The Role of Snai1 during Heart Development

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UNIVERSITY OF MIAMI

THE ROLE OF SNAI1 DURING HEART DEVELOPMENT

By

Ge Tao

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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THE ROLE OF SNAI1 DURING HEART DEVELOPMENT

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Congenital cardiovascular defects (CHD) are the most common causes of infant death from birth defects (Lloyd-Jones, Adams et al. 2010). In the United States, over 25,000 cases with myocardial defects such as hypoplastic left heart syndrome were recorded in 2001. Beyond this astonishing number, an estimated 3 million more people have bicuspid aortic valve (BAV) (Rosamond, Flegal et al. 2008). Despite the clinical significance, there is a lack of effective therapies for treating CHDs, with surgical intervention as the most common treatment. Thus understanding the normal development of these affected cardiac compartments may provide novel insights and therapeutic targets of CHDs. In the primitive heart, the endocardium and epicardium specifically contribute to heart valves and myocardium maturation respectively, by undergoing epithelium/endothelium to mesenchyme transformation (EMT) to generate progenitors cells that give rise to these mature structures. Heart valves are dynamic and delicate structures that maintain unidirectional blood flow throughout life. Insufficient valve function, commonly due to congenital malformations leads to disruptions in hemodynamics and eventual heart failure. During development, the valves are derived from primordial structures called endocardial cushions, formed by EMT in the
atrioventricular endocardium. Endocardial cushions elongate into valve primordium, which will later remodel into valve leaflets. Mature valve leaflets are composed of a heterogeneous population of interstitial cells and stratified extracellular matrix, surrounded by a layer of endothelial cells. This cell-matrix composition provides the valve with all the necessary biomechanical properties required to efficiently function while withstanding constant cyclic shear stress. In addition to development, VECs are also important for maintaining life-long valve integrity and function through the adaptation to the constantly changing hemodynamic environment. Besides VECs, which locate at the lumen-side of the heart chamber, the epicardium, covering the outer surface of myocardium, also gives rise to a group of cardiac progenitors called epicardial-derived cells (EPDCs) through EMT. Following EMT, EPDCs invade the myocardium and differentiate into vital components of the functional myocardium including smooth muscles cells of the coronary vessels, cardiac fibroblasts and a small portion of cardiomyocytes. In the current studies, we showed that Snai1, a zinc-finger transcription factor, is expressed in endocardial cushion cells, VECs in valve primordium, as well as epicardium and EPDCs during normal embryogenesis. Although Snai1 has been widely studied in other EMT model such as cancer metastasis and gastrulation, its direct role during heart development has not been clarified. Our data indicate that Snai1 function is required for endocardial cushion formation, valve remodeling and epicardial development; conditional loss-of-function of Snai1 in endocardial cushion cells and VECs of the remodeling valves leads to abnormal valvular structure. We also identified matrix
metalloproteinase 15 (mmp15) as a novel target gene of Snai1, while knock-down of Snai1 in epicardial cells results in a thinner ventricular walls. Together these studies characterize the specific function of Snai1 during cardiogenesis and improve our understanding of heart valve and epicardium development, which may favor the development of novel treatments for valvular and myocardial disease in the future.
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<td>AdV</td>
<td>adenovirus</td>
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<tr>
<td>AV</td>
<td>atrioventricular</td>
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<td>AVC</td>
<td>atrioventricular canal</td>
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<tr>
<td>αSMA</td>
<td>Smooth Muscle α-Actin</td>
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<tr>
<td>BAV</td>
<td>bicuspid aortic valve</td>
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<td>BMP</td>
<td>bone morphogenetic protein</td>
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<td>caMMP15</td>
<td>constitutively active matrix metalloproteinase 15</td>
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<td>CHD</td>
<td>congenital heart disease</td>
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<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<td>DAB</td>
<td>diaminobenzidine</td>
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<td>dimethyl suloxide</td>
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<td>EC</td>
<td>endocardial cushion</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>cE</td>
<td>chicken embryonic day</td>
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<td>EMT</td>
<td>epithelial/endothelial-to-mesenchymal transformation</td>
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<td>EPDC</td>
<td>epicardial-derived cell</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>HH st.</td>
<td>Hamburger Hamilton stage</td>
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<td>IP</td>
<td>intra-peritoneal</td>
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<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MVP</td>
<td>mitral valve prolapse</td>
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<td>OFT</td>
<td>outflow tract</td>
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<td>PEO</td>
<td>proepicardial organ</td>
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<td>paraformaldehyde</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>VIC</td>
<td>valve interstitial cell</td>
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<td>WT1</td>
<td>Wilm’s tumor 1</td>
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Chapter 1. Introduction

More than 29% of infants who die from a birth defect have congenital heart defects due to abnormal development (Rosamond et al., 2008). Among a series of CHDs, myocardial defects, such as hypoplastic ventricles, normally come in association with severe symptoms including aorta, valves and coronary artery dysfunction (Grossfeld et al., 2009). The treatment option is limited to surgical procedures, which, although improved, can only be considered palliative due to the high postoperative mortality caused by secondary coronary heart disease. Interestingly, this postoperative coronary defect is believed to derive from intrinsic coronary abnormalities such as hypoplasia of the epicardial branches (Abuchaim et al., 2011). During development, the coronary vessel system is largely contributed by EPDCs, which also differentiate into cardiac fibroblasts. These crucial components are required for myocardium maturation. Interrupted epicardium development has been show to severely affect myocardium development in animal models (Martinez-Estrada et al., 2010; Olivey and Svensson, 2010). Worthy of mention, besides congenital diseases, EPDCs recently draw many attentions due to their potential therapeutic role in adult myocardial disease such as myocardial infarction (MI) (Zhou et al., 2011), which caused more than 150,000 deaths each year (Rosamond et al., 2008). In addition to the congenital myocardial defects, congenital valve disease occurs in approximately 2% of live births (Hoffman and Kaplan, 2002a), making it the most common CHD. Despite the clinical significance, there is a lack of effective therapies and surgical intervention is often required, leading to the direct cost of
$1 billion per year in the States (Rajamannan et al., 2003a). Normal heart valve function is achieved by the coordination of valve interstitial cells (VICs), extracellular matrices (ECM) and valve endothelial cells (VECs), providing the leaflets with biomechanical properties to open and close properly during the cardiac cycle while responding to the surrounding hemodynamic signals to establish and maintain valve homeostasis (Balachandran et al., 2011; Hinton and Yutzey, 2011). In contrast to normal valves, valvular disease including prolapse (myxomatous), thickening (sclerosis) and calcification display disorganized ECM, altered VIC distribution and interrupted VEC with attenuated responses to molecular or hemodynamic cues (Fig1D) (Butcher and Nerem, 2007; Ge and Sotiropoulos, 2010; Poggianti et al., 2003). Overall, these alterations have detrimental effects on valve biomechanics leading to impaired leaflet movement and increased resistance to blood flow (Freeman and Otto, 2005; Lincoln et al., 2006b). Due to deficient therapeutic options, the afore-mentioned congenital and adult heart diseases are among the leading causes of end-stage heart failure. Therefore the need to develop alternative and effective therapies is imperative. Due to the worldwide effort in cardiac research, we are gaining insights into the developmental process of the heart. With an improved understanding of heart development, we aim to identify novel potential therapeutic targets for treating valvular and myocardial diseases.

1.1 Heart valve structure and function
Heart valves open and close over 100,000 times a day to maintain unidirectional blood flow through the heart. There are two sets of cardiac valves: the
atrioventricular (AV) valves including the mitral and tricuspid that separate the atria from the ventricles; and the aortic and pulmonic semilunar valves that separate the ventricles from the great arteries (Hinton and Yutzey, 2011). Although the functional demand of each valve set is similar, their anatomies are different. The AV valves are made up of two (mitral) or three (tricuspid) valve leaflets and external supporting chordae tendineae that attach the underside of the valve leaflet to the papillary muscles within the ventricle (Anderson et al., 2000). The three leaflets of the semilunar valves (aortic, pulmonic) are referred to as cusps. Although without external support, a unique supporting structure is apparent within the aortic roots in the form of a fibrous annulus (Fig. 1C) (Anderson, 2000). It is the coordinated movement of these valvular structures that maintain unidirectional blood flow during the cardiac cycle: In diastole, the papillary muscles are relaxed and high pressure in the atrium causes opening of the mitral (left) and tricuspid (right) valve leaflets to promote blood flow into the respective ventricle. Once ventricular pressure increases during diastole, the chordae ‘pull’ the AV valve leaflets closed and maintain coaptation to prevent reversion of the valve into the atria. As the ventricle contracts, blood exits through the now open semilunar valves and then the ventricle relaxes to begin the cycle again (Boignard). So that heart valves are exposed to constantly changing hemodynamic force. To withstand this, the valve leaflets/cusps develop and maintain an intricate and highly organized connective tissue system that provides all the necessary biomechanical properties for efficient function.
The mature valve structures are primarily composed of an outer layer of VECs that surround three stratified layers of specialized ECM, interspersed with differentiated VICs (Fig. 1C) (Garcia-Martinez et al., 1991; Gross and Kugel, 1931; Lincoln et al., 2006b). The tri-stratified ECM layers are arranged according to blood flow and collectively they work together to withstand the continual changes in hemodynamic flow during the cardiac cycle (Fig. 1C) (Balachandran et al., 2011; Lincoln et al., 2006b). The fibrosa layer is located on the ventricular side of the AV valves and atrial side of the semilunar valves, away from blood flow. This layer is predominantly composed of bundles of collagen fibers aligned along the circumferential direction of the leaflets (Icardo and Colvee, 1995; Kunzelman et al., 1993; Lincoln et al., 2006b; Rabkin-Aikawa et al., 2005), providing tensile strength to the valve leaflet during opening, while transmitting forces to promote inosculation of the leaflet free ends when closing (Aldous et al., 2009; Balachandran et al., 2011; Grande-Allen and Liao, 2011). Adjacent to the fibrosa is the spongiosa layer, with a lower abundance of collagens and high prevalence of proteoglycans. This composition provides a more compressible matrix for absorbing high force (Lincoln et al., 2006b; Sacks et al., 2009). Finally, the layer adjacent to blood flow is termed the atrialis (AV) or ventricularis (semilunar) and largely consists of radially orientated elastin fibers that facilitate tissue movement by increasing elasticity (Hinton and Yutzey, 2011; Schoen, 1997; Scott and Vesely, 1995). In addition to the ECM, the VICs and VECs play essential roles in maintaining connective tissue homeostasis in the functional valve leaflet. Although originally thought of as a homogenous population of
fibroblast-like cells, VICs are now considered highly heterogeneous with quiescent, activated and progenitor-like phenotypes reported (Liu et al., 2007). ECM secretion by activated VICs could be different depends on their location within the valve leaflet (Blevins et al., 2008). This feature may be responsible for the dynamic organization of valvular ECM. Meanwhile, VECs are highly responsive to changes in the hemodynamic environment, implying their potential role as “receptor” of external orders, which will be passed down to VICs, functioning as “effectors”. As normal valve function is dependent on the complex arrangement of connective tissue and overall valve morphology, it is not surprising that alterations in localization, and/or contribution of matrix components leads to inefficiencies and valve disease.

Figure 1. Overview of aortic valve maturation and disease. (A) Heart valve development is initiated with formation of endocardial cushions following EMT. These primitive valve structures are composed of cardiac jelly (CJ) containing a population of mesenchymal VICs surrounded by a layer of VECs. (B) As embryogenesis proceeds, endocardial cushions elongate to form aortic valve primorida. At this time, VICs begin to differentiate and secrete specialized ECM to give rise to the trilaminar mature aortic valve structure (C) consisting of the fibrosa (F), spongiosa (S), and ventricularis (V) layers. Arrow indicates blood flow. Throughout this process, the valve endothelium is maintained. (D) In diseased valves, the valve endothelium is disrupted, associated with alterations in VIC and ECM organization. These histopathological often lead to sclerosis and calcification (black lesions) on the fibrosa layer away from blood flow. Myo, myocardium.
1.2 Heart valve development

1.2.1 Endocardial cushion formation

During development, the primitive vertebrate heart tube consists of a myocardial cell layer and an adjacent inner layer of endocardium, connected to myocardium through ECM, referred as cardiac jelly (Fig. 1A) (Person et al., 2005b; Srivastava and Olson, 2000). Soon after rightward looping, myocardial cells localized within the atrioventricular canal (AVC) and outflow tract (OFT) regions increase deposition of ECM components including proteoglycans and hyaluronan and give rise to cardiac jelly ‘swellings’ (Fig. 1A). Concurrently, a sub-population of endocardial cells overlying these ‘swellings’ lose contacts with neighboring cells and undergo EMT (Eisenberg and Markwald, 1995; Person et al., 2005b). As a result, newly transformed mesenchyme cells invade the underlying cardiac jelly and proliferate to populate endocardial cushions (Fig. 1A) (Combs and Yutzey, 2009a; Eisenberg and Markwald, 1995; Person et al., 2005b). This essential role of VECs during early stages of valvulogenesis is initiated in the chick around embryonic day (E) 3, in the mouse at E9.5 and between E31-E35 in human development (Fishman and Chien, 1997; Martinsen, 2005; Moorman et al., 2003).

In the AVC, four endocardial cushions form: the superior, inferior and left and right lateral cushions; while in the OFT, two cushions form in proximal and distal locations (Markwald et al., 2010). These cushion structures serve as physical barriers to prevent backflow of blood through the primitive heart tube (Schroeder et al., 2003), in addition to hosting a population of undifferentiated VICS that serve as precursors to the mature valve structures (de Lange et al., 2004; Lincoln
et al., 2004b). Fused inferior and superior endocardial cushions in the AVC give rise to the septal valve leaflets whereas the lateral cushions form the mural leaflets (de Lange et al., 2004; Lincoln et al., 2004b). However, the precise origins of the right, left and anterior (aortic)/posterior (pulmonic) valve cusps from the proximal and distal endocardial cushions in the OFT following EMT are yet to be defined.

The initiation of endocardial cushion EMT is marked by activation of VECs in response to signals emanating from the adjacent myocardium (Harrelson et al., 2004; Lyons et al., 1990; Ma et al., 2005; Plageman and Yutzey, 2004). Many signaling pathways critical for early embryogenesis have been shown to play roles initiating endocardial cushion EMT, including Transforming Growth Factor (TGF) β, Wnt and Bone Morphogenetic Protein (BMP) (Combs and Yutzey, 2009a). These myocardium-secreted ligands are believed to diffuse through cardiac jelly to reach VECs, where they initiate or promote cell transformation, migration and proliferation through activating downstream target genes like Notch1 (Timmerman et al., 2004) and Snai gene family members, which are all well-known positive regulators of EMT (De Craene et al., 2005).

Standing at the cross roads of the EMT, the Snai family of zinc-finger transcription factors answers to a series of EMT-inducing pathways during development and cancer metastasis (Nieto, 2009; Thiery et al., 2009), including fibroblast growth factor (FGF), WNTs, TGFβ, BMP and Notch (Thiery et al., 2009). The major members, Snai1 (snail) and snai2 (slug), have previously been shown to play major roles in EMT in many developmental systems, although
direct roles for snai1 in the heart have not been examined (Cano et al., 2000b; Niessen et al., 2008b). Among the upstream signaling of Snai1, Notch and TGFβ signaling pathways play vital roles in heart development (Combs and Yutzey, 2009a). Notch is an important activator of EMT, with Notch1 predominantly expressed in VECs (Timmerman et al., 2004). Notch also regulates TGFβ2 production by its activated intracellular domain and TGFβ-mediated activation of the downstream effectors SMAD 2/3 is sufficient to induce expression of Snai1 and Snai2 (Romano and Runyan, 2000). Both of these family members are highly expressed in VECs undergoing EMT, as well as in newly transformed mesenchyme cells within the developing cushion (Niessen et al., 2008a; Oram et al., 2003; Tao et al., 2011). At the downstream, Snai1 and Snai2 promote the loss of epithelial markers and gain of mesenchymal markers through transcription regulation (Thiery et al., 2009). They help breaking down basement membrane though induction of MMPs (Barrallo-Gimeno and Nieto, 2005). To increase cell motility, Snai genes also increase RhoB activity, leading to the changes in cytoskeleton (Thiery et al., 2009). Together, these effects result in the loss of cell-cell adhesion and polarity, changes in cell shape and motility. Studies by Runyan’s group have shown that Snai2 is essential for endocardial cushion EMT (Romano and Runyan, 2000). Mice deficient for Snai2 display hypocellular cushions at E9.5, although this phenotype is compensated by E10.5 due to increased Snai1 (Niessen et al., 2008a). However, Snai2 cannot compensate for Snai1 deficiency and therefore Snai1−/− mice die early during development due to defective gastrulation and mesoderm formation prior to endocardial cushion
formation (Carver et al., 2001a). In other non-cardiac systems, Snai1 is known to regulate EMT by directly repressing expression of cell adhesion molecules including \textit{E-cadherin} (Cano et al., 2000a), \textit{claudins} and \textit{occludins} (Ikenouchi et al., 2003; Martinez-Estrada et al., 2006) and therefore cell-cell contacts are adversely maintained in \textit{snai1} mutant mice.

Collectively, these studies highlight the complexity of the transcriptional networks activated by growth factor signaling during initial stages of endocardial cushion formation. Additionally, studies in several species including chicken, mouse and zebrafish indicate conserved mechanisms underlying EMT and endocardial cushion formation (Hurlstone et al., 2003) (Person et al., 2005b).

1.2.2 Post-EMT and maturation stages of valve development

Once endocardial cushion formation is complete, VECs regain an uninterrupted endothelium (Person et al., 2005b). Meanwhile, mesenchyme cells within the endocardial cushions continue to proliferate, associated with ongoing ECM deposition, under strict regulation (Fig. 1B) (Armstrong and Bischoff, 2004; Hinton et al., 2006). Around this time, the individual endocardial cushions fuse and elongate into mitral and tricuspid valve primordia in the AVC region, and primitive aortic and pulmonic structures in the OFT (Lincoln et al., 2004b). Later, during fetal stages, the valve primordia flatten into valve leaflets with diversified VICs and ECMs located in different compartments (Balachandran et al., 2011; Lincoln et al., 2006b). Compared to the endocardial cushion formation, much less is known about the regulation of valve remodeling. Work by our group and others has shown that during mid-stages of valvulogenesis, VICs lose expression of
mesenchyme genes including *Twist1, Tbx20, Msx1* and *Msx2*, and begin to express differentiated lineage markers including those characteristic of chondrocyte and tendon cells (Chakraborty et al., 2010; Hurle et al., 1994; Lincoln et al., 2006a; Lincoln et al., 2006b; Montero et al., 2002). In the mature chicken heart, Sox9-expressing and Scleraxis-expressing cells are compartmentalized in valve leaflets and supporting structures respectively, indicating the separation of the cartilaginous and tendon-like lineages (Lincoln et al., 2006a). However, in mouse valves, such compartmentalization is not so well-defined due to the small size (Levay et al., 2008; Lincoln et al., 2007). The differentiation potential of embryonic VICs decreases as development progresses (Lincoln et al., 2006a). Along with VIC differentiation, the valve ECM undergoes extensive changes, associated with degradation of the primitive cardiac jelly by matrix metalloproteinases, and deposition of collagens and proteoglycans by differentiating VICs that will later form the fibrosa and spongiosa layers (Dreger et al., 2002; Rabkin et al., 2001). Elastin fibers are laid down later in the atrialis/ventricularis layers of neonatal heart valve, this makes the valve tri-stratified (Hinton et al., 2006). A significant reduction in cell proliferation is another feature of late embryonic valve development (Aikawa et al., 2006; Hinton et al., 2006). In addition to the regulation of cellular processes by complex transcriptional networks during valve maturation, the influence of mechanical flow also plays an important role in mediating valve cell function.
1.3 Heart valve disease
In contrast to healthy valves, diseased valves are characterized by disruptions in ECM layer stratification, VIC disarray and VEC denudation (Fig. 1D) (Hinton et al., 2006). These histopathological changes have detrimental effects on the overall structure and biomechanical properties of the valve, leading to primary insufficiency and secondary ventricular compensation (Hinton and Yutzey, 2011). Clinically, valve disease is most commonly diagnosed as regurgitation, caused by inefficient closing of the valve leaflets leading to backward flow, or stenosis, resulting from outflow obstruction. Both scenarios are caused by disruptions in the structure-function relationship as a result of valve malformations. Clinical phenotypes resulting in structural valve defects are often diagnosed at birth or become apparent later in life. Bicuspid aortic valve (BAV) is the most common congenital valve disease with a reported incidence of 1-2% of the adult population (Hoffman and Kaplan, 2002a) and is characterized by the presence of two, rather than the normal three cusps, leading to stenosis or narrowing of the aortic valve opening (Ward, 2000). The bicuspid cusps are susceptible to calcification: a pathological process mediated by an “osteoblast-like” VIC phenotype and matrix mineralization (Rajamannan et al., 2003b). This pathological disruption to the valve’s normal connective tissue homeostasis leads to valve thickening (sclerosis) and mechanical stiffness, resulting in stenosis or regurgitation that can ultimately require surgical valve replacement (Freeman and Otto, 2005; Garg, 2006). In contrast to BAV and associated calcification, mitral valve prolapse (MVP) affects more than 2% of the US population and is characterized by increased deposition
of collagens and proteoglycans. These changes in ECM contribution and
distribution weaken valve biomechanics leading to “floppy” and regurgitant
leaflets that ‘bulge’ or prolapse into the atrium, preventing valve closure (Grande-
Allen et al., 2003; Gupta et al., 2009). Although end-stage valve insufficiency
could also be the result of predisposing genotypes and structural malformations
that increase susceptibility to degenerative ‘wear and tear’ caused by
hemodynamic exposure over time. While the underlying genotypes of congenital
and latent valve disease remain largely unknown, there is increasing evidence to
suggest that structural abnormalities are the result of defects in the
developmental pathways required for valve formation in the embryo.

1.4. Development and function of epicardium.

1.4.1 Formation of proepicardial organ

The majority of the heart is derived from primary and secondary heart fields,

\[\text{Figure 2. Origin and fate of the proepicardial organ.} \]

(A) Diagram illustrating the location of proepicardial organ (PEO) at the sinus venosus (SV). DM, dorsal mesenchyme; Atr, atrium; AVC, atroventricular canal; Vent, ventricle; OFT, outflow track. (B) Epidermal cells undergo EMT and migrate into myocardium where they differentiate into smooth muscle cells (SMC), endothelial cells (EC), fibroblasts (F) and cardiomyocytes (CM). Epi, epicardium; myo, myocardium; endo, endocardium. *Panel (A) was
modified from (Wessels and Perez-Pomares, 2004). Panel (B) was modified from (Zhou et al., 2008).
however, non-cardiac-derived progenitors from neural crest and the epicardium are indispensable for correct heart formation (Lie-Venema et al., 2007). The epicardium is derived from a proepicardial organ (PEO) which arises from the splanchnopleural mesoderm of the posterior heart field, a structure located dorsal to the developing heart tube and adjacent to the AV canal (Fig. 2A) (Dettman et al., 1998; Mikawa and Gourdie, 1996). PEO formation, common to a range of vertebrate species, shows bilaterally symmetric and asymmetric patterns (Schlueter and Brand, 2009) in mouse and chick respectively. PEO is formed initially pairwise as bilateral structures on the transverse septum and sinus venosus, respectively; Later, the left PEO degenerates while the right PEO persists and forms a grape-like structure (Manner et al., 2001; Schulte et al., 2007), on the other hand, in mouse, both PEOs deliver progenitor cells to the heart (Schulte et al., 2007; Serluca, 2008). Factors required for PEO formation include Fgf8 and Snai1 (Schlueter and Brand, 2009), due to their roles in determination of the left-right asymmetry in both mouse and chick. In chicken, disruption in Fgf8 and/or Snai1 inhibits PEO formation (Murray and Gridley, 2006), however the mechanisms of Snai1 function in this process is not understood.

1.4.2 Epicardium formation.

The PEO is a mixture of superficial methothelial epithelium and mesenchymal cells (Wessels and Perez-Pomares, 2004). The migration of proepicardial cells from the PEO to the myocardial surface during epicardium formation is induced by BMP and FGF signaling through a paracrine interaction (Ishii et al.; Kruithof et
an ECM bridge, as observed in amphibian, avian and rodent embryos (Ishii et al.; Lie-Venema et al., 2007), or detachment and ‘diving freely’ through the pericardial cavity before attaching to the myocardium, as shown in fish (Gallego et al., 1997; Komiyama et al., 1987; Kuhn and Liebherr, 1988; Manner et al., 2001). PEO cells start to spread laterally after attaching to the myocardium to shield the whole heart. In chicken, this process occurs between HH17 (E3) and HH26 (E5.5). The spatiotemporally migratory pattern of the proepicardial cells is shared by all the vertebrates studied thus far (Van den Eijnde et al., 1995). There are increasing evidences indicate the contact point of PEO on myocardium possesses regional signaling that does not exist within surrounding ventricular tissue (Ishii et al.; Lie-Venema et al., 2007). Factors critical for the migration of PEO and the adhesion of epicardium to the underlying myocardium include vascular cell adhesion molecule 1 (VCAM-1), a4 integrin and Tbx5 (Dettman et al., 2003; Hatcher et al., 2004; Kwee et al., 1995; Sengbusch et al., 2002; Yang et al., 1995). Mikawa’s group also described a paracrine interaction between the PEO and heart tube, in which BMP2 and BMP4 signaling directs the formation of the matrix bridge that mediates the contact of PEO and myocardium and their eventual fusion (Ishii et al.). This is the first identified signaling that secreted by PEO-targeted myocardium to directs the protrusion of PEO, where BMP receptors are expressed (Ishii et al.). Lateral spreading of the epicardial sheet is driven by both migration and proliferation, indicating the development from PEO to epicardium is a collaborative process that involves multiple steps.
1.4.3 Epicardium-derived cell development.

Once the heart is covered by the epicardial cell sheet at E4.5 in chicken and E12 in mouse, EMT occurs in a proportion of epicardium cells to generate the epicardium-derived cell (EPDC) population (Perez-Pomares et al., 1997; Vrancken Peeters et al., 1999), which temporarily reside in subepicardium to form subepicardial mesenchyme (Perez-Pomares et al., 1997). The subepicardium serves as a temporary port for EPDCs and is filled with a complex arrangement of ECM components including collagens, fibronectin, laminin and proteoglycans, generated by both myocardial and epicardial cells (Bouchey et al., 1996). From the subepicardial space, EPDCs will invade the myocardium in a spatiotemporally regulated pattern, in which factors expressed by the underlying myocardium define the permissiveness for EPDCs (Fig. 2B) (Lie-Venema et al., 2005). In chicken, EPDC invasion starts from the ventricular sulcus at HH19 (E3.5), followed by other regions of the heart at HH23 (E4), and finally the OFT tract from HH30 (E6.5) (Lie-Venema et al., 2005). The same pattern of EPDC invasion also exists in mouse at equivalent stages, during which EPDC precursors contribute, at least partially, to coronary endothelial cells, besides their major contribution to smooth muscle cell and interstitial fibroblasts (Fig. 2B) (Cai et al., 2008; Dettman et al., 1998; Wilm et al., 2005). The role of cardiac fibroblasts in myocardial homeostasis has been accentuated due to the fact that they are capable of promoting the proliferation of cardiomyocytes besides contributing to the fibrous matrix in the myocardium (Ieda et al., 2009). Besides, the development of EPDCs into coronary vessels has been extensively studied
due to the high mortality caused by CHD and the potential clinical significance of coronary vasculature. Coronary vasculogenesis, which occurs right beneath epicardium, is guided by epicardium-secreted proangiogenic stimuli including VEGFa, FGF9/16/20, Sonic hedgehog and WNT9b (Lavine et al., 2006; Lavine et al., 2005; Merki et al., 2005; Wu et al., 1999; Zamora et al., 2007), among which, FGF is induced by autocrine signaling including erythropoietin and retinoic acid with in the immature epicardium (Merki et al., 2005). In addition, a Shh-VEGF-Ang2 pathway is important for the generation of vascular endothelial cells, whereas PDGF, Wnt-b-catenin, and TGFb are important for driving EPDCs into coronary vascular smooth muscle (Olivey and Svensson). Despite the identification of secreted cytokine signaling during coronary vessel development, understanding how the pathways are knit together by downstream effectors is an area that requires substantially more investigation. However, knowing that Snai family members mediate FGF, VEGF and WNT signaling in the development of other tissue types, a tantalizing possibility is raised that these transcription factors may play a role during EPDC formation and development. Although Snai1 expression has been used as readout of epicardial EMT in previous reports (del Monte et al., 2011; Wilm et al., 2005) due to the fact that epicardial EMT shares several major inductive pathways with previously reported EMT in other tissue, including Notch, VEGF and FGF (Morabito et al., 2001), its function in the epicardium is not fully understood.
1.5 Hypothesis.

In the current studies, we hypothesize that Snai1 function is required for EMT during endocardial cushion formation and epicardium development based on existing knowledge in other systems and its expression pattern in the embryonic heart. The direct roles of Snai1 are determined using mouse models with conditional reduced *snai1* function in endothelial-derived or epicardial-derived cells, and established in vitro systems. Phenotypes in embryonic and fetal hearts with reduced Snai1 in endothelium and epicardium respectively are examined. The possible causative mechanisms underlying the phenotypes are further investigated by molecular and cellular experiments, including identification of possible novel target of Snai1 transcriptional regulation. Besides its function during EMT, we also propose a novel function of snai1 during valve remodeling due to its expression profile at post-EMT stages. For this proposal, mouse model with spatiotemporal loss of *snai1* function in VECs during valve remodeling is employed to elucidate the previously unrecognized role of Snai1. Together these studies improve our understanding of heart valve and epicardial development and characterize the specific functions of Snai1 during cardiogenesis.
Chapter 2. Methods

2.1 Histological Analysis

Whole mouse and chicken embryos staged at embryonic day (E) 9.5, 10.5, 13.5, 16.5, postnatal (PN) day 1 (mouse) and Hamburger Hamilton stage (HH St.) 14 (E2.5-3.0), 18, 26, 35 were collected in 1× Phosphate Buffered Saline (PBS) and either fixed in 4% paraformaldehyde (PFA) overnight at 4°C or left unfixed. Fixed tissues were subsequently processed for paraffin embedding and 6μm tissue sections were cut as previously described (Lincoln et al., 2006a). Alternatively, unfixed tissue was immediately processed for frozen block cryoembedding (Lincoln et al., 2006a) and 12μm tissue sections were cut. For colorimetric and fluorescent immunohistochemistry, fixed tissue sections were processed (Lincoln et al., 2007) and incubated overnight at 4°C with primary antibodies against Snai1, WT1, Smooth Muscle α-Actin (αSMA), and Phospho-histone H3 (Table 1). In contrast, unfixed frozen sections were post fixed in ice-cold acetone at −20°C for 15 minutes, blocked in 5% bovine serum albumin/1xPBS for 30 minutes and incubated with anti-matrix metalloproteinase 15 (Mmp15) for 2 hours at room temperature. Antigen retrieval was used for the detection of Snai1, WT1, Mmp15 and Phospho-histone H3 by boiling tissue sections in unmasking solution (Vector Labs) for 10 minutes and allowing to cool to room temperature prior to blocking. Detection using diaminobenzidine (DAB) was performed according to the manufacturer’s instructions (ABC staining system, Santa Cruz Biotechnology) and visualized on an Olympus BX51 microscope. Immunofluorescent staining was performed using appropriate secondary antibodies (Alexa-Fluor) and
captured using Olympus Fluoview F-1000 confocal microscope. Alcian blue staining was performed on paraffin tissue sections from E10.5 and E13.5 Tie2cre;Snai1fl/+ embryos, E18.5 Tie2ER\textsuperscript{T2}cre;Snai1fl/+ hearts and counter-stained with nuclear fast red solution as previously reported (Lincoln et al., 2007). Trichrome was performed on E18.5 Tie2ER\textsuperscript{T2}cre;Snai1fl/+ hearts and E18.5 WT1ER\textsuperscript{T2}cre;Snai1fl/+ hearts along with controls (Levay et al., 2008).

Table 1. Antibodies used for immunostaining.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Section type</th>
<th>Dilution</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snai1</td>
<td>Abcam</td>
<td>Paraffin</td>
<td>1:500</td>
<td>ABC Colorimetric (Santa Cruz)</td>
</tr>
<tr>
<td>WT1</td>
<td>Santa Cruz</td>
<td>Paraffin</td>
<td>1:200</td>
<td>Alexa Donkey anti-Rabbit 488</td>
</tr>
<tr>
<td>αSMA</td>
<td>Sigma</td>
<td>Paraffin</td>
<td>1:500</td>
<td>Alexa Donkey anti-mouse 488</td>
</tr>
<tr>
<td>Phospho-Histone H3</td>
<td>Millipore</td>
<td>Paraffin</td>
<td>1:200</td>
<td>Alexa Goat anti-Rabbit 488</td>
</tr>
<tr>
<td>MMP15</td>
<td>Abcam</td>
<td>OCT</td>
<td>1:200</td>
<td>Alexa Goat anti-Rabbit 488</td>
</tr>
</tbody>
</table>

2.2 Generation of Adenoviruses

Full-length FLAG-tagged mouse snai1 cDNA was generated by PCR amplification from E14.5 mouse limb template and ligated into the pShuttle-IRES-hrGFP-1 vector (Stratagene). In parallel, a control adenoviral construct was generated with no cDNA insert (AdV-GFP). Adenovirus generation and tittering was performed according to the manufacturer’s instructions using the AdEasy XL and AdEasy Viral Titer kits respectively (Stratagene). For AdV-Snai1 and AdV-GFP, infection efficiency was determined and optimized by counting the number of GFP-positive cells over the number of DAPI-positive nuclei in at least 5 microscopic fields in each culture type used in this study (n=3). The AdV-cre was generated as previously described (Peacock et al., 2010) and snai1 knockdown
in infected AVC explants from $Snai1^{fl/fl}$ mice was determined by qPCR using Taqman probes against mouse Snai1 (Applied Biosystems).

2.3 Primary Cell and Explant Culture

1) 2D-endocardial cushion monolayer culture: AVC ECs were dissected from E4.5 white leghorn chicken eggs (Charles River Laboratories) and E11.5 $Snai1^{fl/fl}$ mouse embryos, trypsinized and cultured for 48 hours as a monolayer collagen-coated 2-well chamber slides (NUNC) prior to treatment as described (Lincoln et al., 2006a).

2) 3D-collagen gel endocardial cushion explant culture: AVC explants were collected from HH St. 14, and E5.0 chicks, and E11.5 $Snai1^{fl/fl}$ mouse embryos and placed lumen-side down on 3D-collagen I gels for 2 hours prior to treatment as previously described (Inai et al., 2008).

3) 2D-Proepicardial organ culture: PEO explants were collected from HH St. 17 chicks and placed in uncoated 2-well chamber slides before treatments.

4) 2D-epicardium monolayer culture: E4.5 chicken hearts were collected and placed in collagen-coated 2-well chamber slides with culture medium (Lincoln et al., 2006a) for 24 hours, the hearts were removed prior treatments.

Following initial culturing, adenovirus expressing Snai1 (AdV-Snai1), Cre (AdV-Cre) (Peacock et al., 2010) or GFP (AdV-GFP) were diluted in serum-free culture media (Lincoln et al., 2006a) and used to infect monolayer cells ($5\times10^6$ PFU) or explants ($5\times10^7$ PFU) for 2 hours. Following infection, fresh serum-free media was added and cells/explants were cultured for a further 24 hours ($iii$), 48 hours ($ii$) or 72 hours ($i$ and $iv$). Alternatively, culture $i$, $ii$, $iii$ were treated with
recombinant protein of the catalytic domain of MMP15 (caMMP15) (Chemicon/Millipore) at a final concentration of 1µg/mL in serum-free media for the same period as adenovirus infection. For controls, explants were treated with serum-free media alone. For MMP inhibition studies, E5.0 chicken endocardial cushion explants were subject to 2µg/mL, 10µg/mL and 20µg/mL GM6001 (Millipore) or dimethyl sulfoxide (DMSO) in the presence of either 5×10⁷ PFU AdV-GFP or AdV-Sna1 for 36 hours. Following treatment, cells/explants were fixed in 4% PFA and subject to IHC or collected for mRNA extraction (2-D monolayer culture). Gene expression level was determined by qPCR. To determine the area covered by migrating cells, 2D images were taken with an Olympus SZX7 and the total pixel counts of each cultured AVC and PEO explant was measured using ImagePro Plus software (n=5-8). To quantify the number of invading cells, immunostained 3-D collagen I gels were captured using an Olympus Fluoview F-1000 confocal microscope and Z-stack images were generated from multiple focal planes sections captured at 5µm intervals down to the furthest distance of invasion using Image J software. The fold change in cell invasion and maximal invading depth was calculated using ImagePro Plus software by counting the total pixel number and distance in Z-stacked images. Statistical significance over respective controls was determined using Student’s t-test (p<0.05) (n=5).

2.4 Chicken Embryonic Whole Heart Culture
Embryonic hearts were collected from chick HH St., 25 in 1X PBS. Explants were placed on a Millipore filter (0.1µm) and immediately infected with 1×10⁸ PFU
adenovirus expressing Snai1 (AdV-Snai1) or GFP (AdV-GFP), diluted in 100 µl of serum-free culture media (Lincoln et al., 2006a) for 2 hours. Then explants were washed in fresh serum-free medium and then placed back to filters for another 46 hours culture time. After 48 hours infection time, explants were fixed in 4% PFA and processed for cryo-section. Tissue sections were counter-stained with DAPI and the proportion of GFP-positive cells inside the myocardium was counted and analyzed using Photoshop CS5 (Adobe) and Microsoft Excel. Statistical significance over controls was determined using Student’s t-test (p<0.05) (n=4).

2.5 mRNA isolation, cDNA generation and quantitative PCR
Total mRNA was isolated from cultured monolayer EC cells as well as from E10.5 AVC regions from Tie2cre;Snai1^{fl/+} and Snai1^{fl/+} mice using TRIzol (Lincoln et al., 2007). 200-400ng of mRNA was used to generate cDNA using the high-capacity cDNA kit (Applied Biosystems) (Peacock et al., 2008). cDNA was subject to quantitative PCR amplification (StepOne Plus, Applied Biosystems) using specific primers (Table 2) targeting chicken vimentin, αSMA, VE-cadherin, claudin1, fibronectin1, mmp15 and GAPDH, or mouse fibronectin1, αSMA, VE-cadherin, E-cadherin, snai1, mmp15 and L7. Also, Taqman probes (Applied Biosystems) were used to targeting mouse snai1. Alternatively, E10.5 mouse AVC cDNA was subject to SuperArray PCR analysis (PAMM-013C-12, SABiosciences) according to the manufacturer’s instructions (n=5). Following PCR analyses, the cycle count (Ct) was normalized to at least one housekeeping gene (GAPDH chicken, L7 mouse or the mean Ct of GAPDH, Gusb, Hsp90ab1)
and the $\Delta$Ct and fold changes in experimental samples over controls were determined as described (Peacock et al., 2008). In addition, the mesenchymal to endothelial gene expression ratio in E10.5 mouse AVC cDNA was determined by subtracting the average $\Delta$Ct of endothelial ($\text{VE-cadherin, E-cadherin}$) from mesenchymal gene expression ($\text{fibronectin1, } \alpha\text{SMA}$) ($n=4$). The ratio was further calculated based upon $\text{Power}^{2,-\Delta\Delta\text{Ct}}$. For absolute transcript calculation, the formula $\text{POWER}^{2, (40-\Delta\text{Ct}+\text{Ct}_{L7})}$ was used, with $\text{Ct}_{L7}$ denoting the average cycle count of $L7$ expression levels. Statistically significant differences in transcript levels were determined using student’s t-test on at least 3 independent experiments with p<0.05 considered significant after Student t-test analysis.

### Table 2 Primer sequences for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chicken</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vimentin</td>
<td>CCGACAGGATTTGACAATG</td>
<td>GGATGTGCTGTCCTGGAGT</td>
</tr>
<tr>
<td>$\alpha\text{SMA}$</td>
<td>CACCAACTCTGCTGACTGA</td>
<td>ACACCATCCCCAGAGTCAAG</td>
</tr>
<tr>
<td>$\text{VE-cadherin}$</td>
<td>ATCTCAGACAACGGCAATCC</td>
<td>GAAAATTGCCACCAGTGTCTT</td>
</tr>
<tr>
<td>claudin1</td>
<td>GGAGGATGACCAAGGTGAAGA</td>
<td>TCTGGTGTTAAGGGTGTGA</td>
</tr>
<tr>
<td>fibronectin1</td>
<td>CGTTCTCTCAGTGGCTACA</td>
<td>ATTAATCCACACACAGAC</td>
</tr>
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<td>mmp15</td>
<td>TGTCGCGGAAACACTCTTTC</td>
<td>TTCTCCGTGTCATCCACTG</td>
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<td>TCCAGCTACATCGCACACCT</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>GGTTCTTATGACACCAGTCC</td>
<td>GTAAGCTTCCCATTCAGC</td>
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<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fibronectin1</td>
<td>GGTTGCTTTCAGCACATGATA</td>
<td>TGAGCTGAACTGGGTGCT</td>
</tr>
<tr>
<td>$\alpha\text{SMA}$</td>
<td>CTGACAGGACCCACTGAA</td>
<td>CATCTCAGAGCTCACACA</td>
</tr>
<tr>
<td>$\text{VE-cadherin}$</td>
<td>ACCGAGAGAAACAGCTGA</td>
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<tr>
<td>$\text{E-cadherin}$</td>
<td>CAAGGACAGCCCTTTCTTTC</td>
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<td>snai1</td>
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<td>GCCAGACTTGGTTGTCTTGT</td>
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<td>mmp15</td>
<td>GACCAGTATGCGCCCAACAT</td>
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<tr>
<td>$L7$</td>
<td>GAAGCTCAGTCTATGAGAAGC</td>
<td>AAGACGAAGGAGCTCGAC</td>
</tr>
</tbody>
</table>

### 2.6 Generation of mice

For the studies in chapter 3, $\text{Snai1}^{fl/fl}$ female mice (Murray et al., 2006) were bred with $\text{Tie2cre}$ males (Kisanuki et al., 2001) to generate heterozygous offspring ($\text{Tie2cre;Snai1}^{fl/+}$) and $\text{Cre}$ negative $\text{Snai1}^{fl/+}$ littermate controls at expected
Mendelian ratios. Genotyping was performed by PCR as previously described (Carver et al., 2001b; Lincoln et al., 2007; Murray et al., 2006). Timed embryonic staged litters of mice were collected at E10.5 and E13.5 from Snai1fl/fl female mice, counting day E0.5 by evidence of a copulation plug. Embryos were either fixed or AVC tissue (E10.5) was collected for mRNA extraction. Quantitation of the number of cells within the endocardial cushions from E10.5 and E13.5 Snai1fl/+ and Tie2cre;Snai1fl/+ mice was determined by counting the number of DAPI-positive nuclei in at least 3 tissue sections collected from 3-4 mutant and control mice.

For studies in chapter 4, Snai1fl/fl females were impregnated by Tie2ERT2cre;Snai1fl/fl male and intra-peritoneal (IP) injected tamoxifen (12.5mg/ml in corn oil, 2mg/mouse/day) at E12, 13 and 14. Tie2ERT2cre;Snai1fl/fl and Snai1fl/fl (Cre negative littermate control) fetal hearts were collected at E18.5, fixed in 4% PFA and subjected to tissue section and histological analysis (Immunostaining, alcian blue staining and trichrome staining) as described above.

For studies in chapter 5, Snai1fl/fl females impregnated by WT1ERT2cre male (Zhou et al., 2008) were IP injected tamoxifen or corn oil as vehicle control at E12, 13 and 14. Heterozygous offsprings (WT1ERT2cre;Snai1fl/+ , with or without tamoxifen treatment) were harvested at E18.5, fixed in 4% PFA and subjected to tissue section and histological analysis (Immunostaining and trichrome staining) as described above.
2.7 Chromatin immunoprecipitation (ChIP)

Six canonical E-box consensus sites were identified within promoter region of the murine *mmp15* gene (NC_000074.5; Chromosome: 8; Location: 8 D1; 8 45.5cM) and conservation between mouse, rat and human was determined using the basic local alignment search tool (NCBI blast). Snai1 binding to *mmp15* was evaluated in whole E11.5 mouse embryonic hearts (8-10 hearts per sample, n=3). Protein/DNA complexes were cross-linked for 10 minutes in formaldehyde (Sigma) at a final concentration of 0.5%. Fixed tissue was lysed and sonicated three times for 10 seconds at 1 minute intervals (Ultrasonic cell disruptor; Microson). For ChIP, cell lysates were incubated with an antibody against Snai1 (6µg; Abcam) and incubated overnight at 4°C with gentle rocking. Immunoprecipitation with normal rabbit IgG was used as a negative control. ChIPs were performed according to the manufacturer’s instructions (EZ ChIP, MilliPore). Immunoprecipitated and input DNA were subjected to qPCR using the following primers to amplify three E-box-rich regions within *mmp15*: region B (Forward: GCCACCACACCTGAATCTACTG; Reverse: GTGACTTGGGAAGCTAGGTTG), region C (Forward: GAAGCAGGTGGATCTCTGTGAA; Reverse: TTGCCGGTTTCTAGCTGTAG) and region D (Forward: CCCCCAGATAGGAGCTAGCA; Reverse: CCTAACCAGGGCTTCCTCAGTA). Primers for region A were also included as a negative control as canonical E-box sites were not identified within this region: Forward: CCAGGAGTCCTAATCCCACACT; Reverse: TGCCCTCTACTGGTGA TTTCTG). Three independent ChIPs were performed and significant enrichment
of E-box regions using the Snai1 antibody over IgG control as determined by qPCR, was determined by student’s t-test (p<0.05).

2.8 Dual Luciferase Assays
Luciferase constructs were generated using PCR to amplify three regions (B, C, D) of the mmp15 enhancer region (NCBI accession number NC_000074) from mouse tail genomic DNA template, using the following primers: pGL3-\textit{mmp15}B, forward TAGGTACC\textit{C}CCCCCTACGCAAGAAAAT and reverse TGAG\textit{C}CTCCACTTGAGGACACACT (970bp). pGL3-\textit{mmp15}C, forward TAGGTACCT\textit{C}CATGCCCTTTCATC and reverse TGAG\textit{C}CTCCACTGTGGTGGACCTCTCTGT (1006bp). pGL3-\textit{mmp15}D forward TAGGTACC\textit{G}CGTGCGGTGTGTAGGGTCTA and reverse TGAG\textit{C}CTCC\textit{C}ACTGCCGAACCTGTAGGTCTA (973bp). Amplified regions were gel purified and ligated into pGL3-basic using introduced Sac1 and Kpn1 (promega) sites. pcDNA-\textit{E47} and pcDNA-\textit{E12} plasmids were kindly provided by Dr. Nakamura (Funato et al., 2001).

Luciferase assays were performed in COS7 cells (ATCC) plated at $2\times10^5$ per well of a 24-well plate 24 hours prior to transfection with Lipofectamine reagent (Invitrogen) according to manufacturer’s instructions. 200ng of pGL3-\textit{mmp15}B, pGL3-\textit{mmp15}C, pGL3-\textit{mmp15}D or empty pGL3, and 200ng of pcDNA-\textit{E47}, pcDNA-\textit{E12} or empty pcDNA, were co-transfected into each well, along with 20ng of pGL4 (\textit{Renilla} luciferase, Promega). All transfection were performed in 400µl OptiMem for 6 hours before the infection of either AdV-Snai1 or AdV-GFP. Cell lysates were collected 48 hours following treatment according to the
manufacturer’s instructions for dual luciferase assays (Promega). Data is represented as an average percent of luciferase activity of all combinations of transfections normalized to pGL4 Renilla signal (n=4) and compared to respective empty vector controls.
Development of heart valve structures is a complex process strictly regulated by multiple molecular pathways that modulate cardiac morphogenesis (Combs and Yutzey, 2009a; Srivastava, 2000). Alterations in these genetic networks during embryogenesis frequently lead to structural defects, malfunction and congenital heart disease (CHD) (Hoffman and Kaplan, 2002b). Understanding the structure-function relationship of genes important during embryonic valvulogenesis will provide insights into genetic causes of CHD. Heart valve formation is initiated in the AVC and OFT regions following formation of endocardial cushions by EnMT (Combs and Yutzey, 2009a; Person et al., 2005a). Targeted loss-of-function of previously shown EMT-regulating pathways, including including Bmp and Tgfβ, as well as more localized signaling from Wnt- and Notch-related pathways (Armstrong and Bischoff, 2004; Combs and Yutzey, 2009a; Person et al., 2005a) has detrimental effects on EC development and embryo survivability (Combs and Yutzey, 2009a; Person et al., 2005a), emphasizing the importance of the EMT process for normal heart valve formation and cardiac function. One critical downstream mediator of these pathways is Snai1, which plays major roles in EMT in many developmental systems during embryogenesis, although direct roles for snai1 in the heart have not been examined (Cano et al., 2000b; Niessen et al., 2008b).

In this study we aim to determine the direct role of Snai1 during EC formation using a mouse model with conditional reduced snai1 function in endothelial-derived cell (Tie2cre;Snai1fl/+)) and established in vitro systems. We
demonstrate that Snai1 is highly expressed in developing ECs, although its expression decreases as development progresses. *Tie2cre;Snai1*/*fl/+* mice display fewer cells within the developing ECs at E10.5, associated with reduced expression of mesenchymal gene markers and matrix metalloprotease (mmp) 15 in the AVC regions. Using the established collagen I gel explant assays (Bernanke and Markwald, 1982; Lincoln et al., 2006a; Runyan and Markwald, 1983), we show that treatment of AVC explants with adenovirus-targeting Snai1 (AdV-Snai1) promotes mmp15 expression and several EMT processes including cell transformation, invasion and migration. In contrast, treatment with a catalytically active MMP15 protein (caMMP15) promotes only cell motility. Further, pharmacological inhibition of MMP activity prevents Snai1-mediated mesenchyme cell migration, while caMMP15 treatment is sufficient to rescue attenuated migration phenotypes observed in AVC explants with targeted *snai1* knockdown. Additional co-immunoprecipitation and luciferase assays indicate that Snai1 binds, and transactivates E-box-rich regions within *mmp15*. Together, these studies support a direct role for Snai1 in EC development, and identify previously unappreciated mechanisms of mmp15 activity, regulated by snai1, for cell motility during EC EMT.
3.1 Reduced Snai1 function in vivo leads to hypocellular endocardial cushions at E10.5.

The expression pattern of Snai1 during endocardial cushion (EC) formation in the atrioventricular canal (AVC) was examined using immunohistochemistry (IHC) and quantitative PCR (qPCR). At E10.5, Snai1 expression is observed in endothelial cells overlaying the ECs (arrowhead, Fig. 3A), in addition to newly transformed mesenchyme cells within the cushions (arrow, Fig. 3A). Similar expression was observed in ECs within the outflow tract regions (data not shown). Nuclear expression is also detectable within the myocardium (M, Fig.3A).
At the mRNA level, snai1 is most highly expressed in AVC regions at E10.5 when EMT is active, while expression is significantly reduced during post-EMT stages from E12.5 through post natal (Fig. 3B).

Although the role of Snai1 during EMT in many systems has been established, its role during EC formation has not been directly examined. To determine this, we used the Tie2cre transgene to target loss of snai1 function in the majority of endothelial, and derived mesenchymal cells of the developing ECs in vivo (de Lange et al., 2004; Lincoln et al., 2004a). Homozygous Tie2cre;Snai1<sup>fl/fl</sup> mice were never recovered after E9.5 and likely die due to vasculature defects as described following early epiblast-specific snai1 deletion (Lomeli et al., 2009). However, heterozygous (Tie2cre;Snai1<sup>fl/+</sup>) mice are viable

![Image](image-url)
through adulthood and show a 50%±17% decrease in snai1 expression in AVC regions at E10.5 (data not shown). Upon histological examination, Tie2cre;Snai1$^{fl/+}$ mice display significantly less cells within the AVC (Fig. 3C-E) and OFT (data not shown) ECs compared to littermate controls (Snai1$^{fl/+}$) at E10.5. Insignificant differences in Phospho-histone H3 and cleaved caspase 3 expression suggest that hypocellular ECs observed in Tie2cre;Snai1$^{fl/+}$ mice at E10.5 was not the result of changes in cell proliferation or apoptosis, respectively (data not shown, Fig. 4F). To determine changes in endothelial cell transformation in E10.5 Tie2cre;Snai1$^{fl/+}$ mice, qPCR was used to quantitatively examine expression levels of mesenchymal (αSMA, fibronectin1) and endothelial (VE-cadherin, E-cadherin) cell markers in AVC regions isolated from Tie2cre;Snai1$^{fl/+}$ and control mice. Expression of mesenchymal cell markers are significantly reduced in Tie2cre;Snai1$^{fl/+}$ mice (SMA, -7.6-fold, fibronectin1, -7.4-fold), although insignificant changes were observed in endothelial markers (Fig. 3F). A consistent decrease in the ratio of mesenchymal to endothelial gene transcript levels confirms that observed decreases in mesenchymal cell marker expression was not a result of decreased cell number within ECs of Tie2cre;Snai1$^{fl/+}$ mice (Fig. 3G).

Despite a significant decrease in the number of mesenchyme cells within ECs from Tie2cre;Snai1$^{fl/+}$ mice at E10.5, by E13.5, cell number is comparable to controls (Fig. 4A-C). This is associated with increased Phospho-histone H3 immunoreactivity, indicative of cell proliferation in valve primordia from E13.5 Tie2cre;Snai1$^{fl/+}$ mice compared to controls (Fig. 4D-F). Collectively, these
findings demonstrate that reduced snai1 function leads to compromised EMT during early stages of EC development, however in association with increased proliferation, this phenotype recovers by later stages.

**Table 3. Superarray analysis to show differential gene expression in E10.5 atrioventricular canal regions from Tie2cre;Snai1<sup>fl/+</sup> mice.** Following analysis, gene expression was normalized to the mean cycle count of GAPDH, Gusb, Hsp90ab1. Fold changes are shown compared to snai1<sup>fl/+</sup>.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emilin1 (elastin microfibril interfacer 1)</td>
<td>0.16 ± 0.05</td>
<td>0.002</td>
</tr>
<tr>
<td>Itgβ3 (Integrin-βeta 3)</td>
<td>0.37 ± 0.1</td>
<td>0.039</td>
</tr>
<tr>
<td>Lami1 (laminin-gamma 1)</td>
<td>0.39 ± 0.09</td>
<td>0.030</td>
</tr>
<tr>
<td>Mmp15</td>
<td>0.27 ± 0.12</td>
<td>0.028</td>
</tr>
<tr>
<td>Sparc (secreted protein, acidic, cysteine-rich)</td>
<td>0.11 ± 0.1</td>
<td>0.006</td>
</tr>
<tr>
<td>Spp1 (secreted phosphoprotein 1)</td>
<td>0.14 ± 0.13</td>
<td>0.023</td>
</tr>
<tr>
<td>Thbs1 (thrombospondin 1)</td>
<td>0.08 ± 0.1</td>
<td>0.022</td>
</tr>
<tr>
<td>Timp2 (tissue inhibitor of metalloproteinase 2)</td>
<td>0.18 ± 0.08</td>
<td>0.016</td>
</tr>
</tbody>
</table>

3.2 **Mmp15 expression is reduced in atrioventricular canal regions from E10.5 Tie2cre;Snai1<sup>fl/+</sup> mice.**

To identify downstream genes that may be affected by reduced snai1 function in developing ECs at E10.5, a gene expression screening approach was used. This high-throughput assay (Superarray) detects quantitative changes in expression of over 84 genes associated with the extracellular matrix (ECM) in AVC regions from E10.5 Tie2cre;Snai1<sup>fl/+</sup> compared to Snai1<sup>fl/+</sup> embryos. Table 3 indicates all the genes that were differentially expressed in the Superarray analysis. Among the list of changed genes, we further examined mmp15 (MT2-MMP) due to the fact that MMPs are widely involved in ECM remodeling and cell migration during various EMT processes. This membrane bound matrix metalloproteinase family member decreased 4-fold in
Tie2cre;Snai1fl/+ embryos (Fig. 5A). Interestingly, expression of other family members including mmps1, 2, 3, 7-14 were not affected (data not shown).

Further IHC analysis shows reduced mmp15 protein expression in both endothelial (arrowheads, Fig. 5B, C) and mesenchymal cells (arrows, Fig. 5B,C) within ECs, highlighted by chondroitin sulfate proteoglycan immunoreactivity (red immunoreactivity, Fig. 5B, C). To examine snai1 expression relative to mmp15 during valve development, qPCR was performed using cDNA from AVC regions at E10.5, E12.5, E14.5 and post natal stages (Fig. 5D). Approximate transcript
levels of *snai1* and *mmp15* showed similar temporal expression patterns throughout valve development with transcript levels being highest at E10.5 and gradually declining to post natal stages. Consistently, co-localization studies show that *snai1* and *mmp15* are both expressed in endothelial (arrowhead, Fig. 5F) and mesenchymal (arrow, Fig. 5F) cells of the valves at E13.5 (Fig. 5E, F). Although due to the expected membrane localization of *mmp15* and largely nuclear distribution of *snai1*, complete overlap is not always observed. Together, these studies suggest that *snai1* and *mmp15* are similarly expressed in developing valve structures and Snai1 function is required for *mmp15* expression during EMT stages.

### 3.3 Snai1, but not MMP15 is sufficient to initiate EMT and promote cell transformation in avian atrioventricular canal explants.

Understanding the mechanisms of EC EMT have been greatly enhanced by studies in the chick using collagen I gel explant systems (Bernanke and Markwald, 1982; Person et al., 2005a). In the avian model, EC formation is initiated in the looped heart at HH St. 14, and by HH St.18, EMT is active in all cushion sets (Person et al., 2005a). Worthy of mention, the EMT inductive signals in the chick appear conserved with the mouse system and include Bmp and Tgfβ signaling (Armstrong and Bischoff, 2004; Combs and Yutzey, 2009a; Person et al., 2005a). To investigate if Snai1 and MMP15 are sufficient to promote EMT in AVC endothelial cells, we employed a published in vitro collagen I gel explant assay (Bernanke and Markwald, 1982; Mjaatvedt et al., 1987; Runyan and Markwald, 1983). In this assay, HH St.14 AV canals are placed on a
3-dimensional (3D) rat-tail collagen I gel with the endothelial cell layer facing down, allowing cells to migrate onto the surface of the gel (Runyan and Markwald, 1983). With the myocardium intact, subsets of endothelial cells then...
invade the underlying gel as newly transformed mesenchymal cells (Inai et al., 2008; Mjaatvedt et al., 1987; Runyan and Markwald, 1983). To validate the use of the avian system in our study, IHC was performed to determine localization of Snai1 and Mmp15 in avian ECs at HHSt.14. As shown in Figures 6A and B, Snai1 and Mmp15 are similarly expressed in endothelial (Fig. 6A, B arrowheads) and mesenchyme (arrows) within the developing cushion. To determine if Snai1 or MMP15 promote cell transformation, HH St.14 AVC explants were subjected to Snai1 gain of function by infecting with a GFP-tagged adenovirus containing full-length FLAG-labeled mouse Snai1 (AdV-Snai1) (Fig. 6E), or a control virus with no insert (AdV-GFP) (Fig. 6D). Exogenous levels of murine Snai1 following adenoviral infection were confirmed by western blot to detect the FLAG epitope (data not shown). For overexpression experiments, the infection efficiency of the AdV-GFP and AdV-Snai1 adenoviruses was comparable with an average of 55.8%±2.29% cells being infected using AdV-GFP and 53.0%±6.45% with AdV-Snai1. To investigate the function of MMP15 during EC EMT, explants were alternatively treated with an exogenous human recombinant MMP15 catalytic domain that functions as a constitutively active form of MMP15 (caMMP15) (Rebustini et al., 2009). Following 48-hour treatment, explants were subjected to IHC to detect transformed αSMA positive mesenchyme cells that migrate over the surface of the collagen gel away from the original explant site as (indicated by dotted white line) (Fig. 6D, E, G, H). Compared to AdV-GFP controls (Fig. 6D), AdV-Snai1 treatment (Fig. 6E) significantly increases the number of αSMA positive cells by over 2-fold (Fig. 6F), whereas compared to respective controls,
caMMP15 does not promote EMT in HH ST.14 AV canal explants (Fig. 6G-H). In addition, increased cell migration in response to AdV-Snai1 treatment was associated with increased mmp15 expression (11.83±1.82-fold), as determined by qPCR (Fig. 6J).

To support these studies, and determine if snai1 or MMP15 can promote cell transformation at later stages of EC development, ECs from HH St. 25-26 (cE4.5) embryos were removed from the adjacent myocardium, trypsinized and cultured as a 2-dimensional (2D) monolayer. This system allows for minimal cell-cell contact and therefore changes in transformation can be examined in a neutral environment (Lincoln et al., 2006a). Dissociated cells were similarly treated with AdV-Snail, AdV-GFP, caMMP15 or vehicle and following 72 hours, changes in expression of mesenchymal (vimentin, fibronectin, SMA) and endothelial (VE-cadherin, claudin1) cell markers were examined by qPCR (Fig. 6J, K). AdV-Snai1 treatment leads to increased expression of mesenchymal cell markers vimentin (+12.6-fold) and αSMA (+2.67-fold), and associated decreased expression of endothelial cell markers VE-cadherin (-4.0-fold) and claudin1 (-15.68-fold), compared to AdV-GFP controls (Fig. 6J). However, gene expression indicative of cell transformation was not affected with caMMP15 treatment compared to respective vehicle (water) controls (Fig. 6K). These data support a role of Snai1, but not MMP15, in promoting cell transformation during EC development.
3.4 Snai1 and MMP15 are sufficient to promote cell invasion and migration in developing endocardial cushions.

Figure 7. AdV-Snai1 and caMMP15 treatments promote cell migration in HH St. 14 AVC explants. (A, B, E, F) HH St.14 AVC explants were placed on 3D collagen I gels and treated with AdV-GFP (A), AdV-Snai1 (B), vehicle (water) (E) or caMMP15 (F). Scale bars in A, B = 200 µm, E, F = 100 µm. Confocal microscopy and Z-stack reconstruction (A, B, E, F) were used to determine the fold change in the number of cells invading the 3D collagen I gel (C, G), and the average maximum distance of invasion (D, H) in AdV-Snai1 (B) and caMMP15 (F) treated cultures compared to respective controls (A, E). Immunohistochemistry indicates α-SMA positive cells (red) and DAPI indicates nuclei (blue). Note increased invasion with AdV-Snai1 and caMMP15 treatments. n=5, *p<0.05, statistical significance compared to respective controls.

For EC formation to occur, transformed cells from the overlying endothelial cell layer must invade the cardiac jelly and migrate to their destination within the expanding cushion (Person et al., 2005a). To mimic EC cell invasion in vitro and examine if Snai1 and MMP15 play a role in this process, HH St. 14 AVC explants with minimal intact myocardium were placed on 3D collagen I gels and subjected
to AdV-Snai1, AdV-GFP, caMMP15 or vehicle treatment. By reconstructing 2D stacks of Z series captured by confocal imaging, we observe that both AdV-Snai1 and caMMP15 treatments increase cell invasion (Fig. 7A-C, E-G), and the distance travelled by invading cells compared to respective controls (Fig. 7A, B, D, E, F, H). In addition, and consistent with observations in Figures 6J and K, the number of αSMA positive cells appears higher with AdV-Snai1, but not caMMP15 treatment. These findings suggest that in ECs, Snai1 and MMP15 play roles in promoting cell invasion during stages of EMT initiation.

Figure 8. Loss of MMP activity attenuates snai1-mediated mesenchyme cell migration in developing endocardial cushions in vitro. (A–D) cE5.0 AVC collagen I explants treated with vehicle (water) (A), caMMP15 (B), AdV-GFP (C) AdV-Snai1 (D), or AdV-GFP (E) or AdV-Snai1 (F) in the presence of the MMP inhibitor (MMP Inhib) GM600. (G) Quantitation to show fold change in cell migration area on the surface of the collagen I gel (dotted line) following each treatment *pb0.05, statistical significance compared to respective controls, #pb0.05, and significance compared to AdV-Snai1. (H) Dose–response effects of GM600 on cell migration in the presence of AdV-Snai1. n=5–8, *p<0.05 compared to 0.2× treatment.
The sufficiency of Snai1 and MMP15 to promote migration of transformed mesenchyme cells within the cardiac jelly of developing ECs was examined using cE5.0 AVC explants on 3D collagen I gels. Similar to cell invasion assays, both caMMP15 and AdV-Snai1 treatments increase the area covered by migrating cells on the surface of the collagen I gel, over respective controls (Fig. 8A-D, G). To further investigate the relationship between Snai1 and Mmps during cell migration, explants were treated with the pan-MMP inhibitor GM6001 (1x) (Fig. 8E-F) in the presence of AdV-GFP (Fig. 8E) or AdV-Snai1 (Fig. 8F). Compared to AdV-GFP controls (Fig. 8C), inhibition of MMP activity (AdV-GFP:MMP Inhib, Fig. 8E) significantly reduces the area covered by migrating cells on the gel surface. Further, MMP inhibition in the presence of AdV-Snai1 (Fig. 8F) attenuates the ability of AdV-Snai1 (Fig. 8D) to promote cell migration in a dose dependent manner (0.2x, 1x, 2x) (Fig. 8D compared to 6F, 6H). These observations suggest that Snai1-mediated cell migration in maturing ECs requires MMP activity.

3.5 caMMP15 treatment rescues attenuated mesenchyme cell migration in murine endocardial cushions with reduced snai1 function.

Tie2cre;Snai1fl/+ mice display defects in cell transformation (Fig. 3F). To determine if reduced snai1 function affects cell migration, AVC regions isolated from E11.5 Snai1fl/fl mice were cultured on 3D collagen I gels and infected with AdV-Cre resulting in a 2.88-fold decrease in snai1 expression. Compared to AdV-GFP controls (Fig. 9A), AdV-Cre treatment significantly reduces cell migration (Fig. 9A, B, E), associated with a 4-fold decrease in mmp15. However,
cell migration defects are no longer observed when explants were co-treated with caMMP15 (Fig. 9B compared to 9D). Therefore suggesting that caMMP15 treatment is sufficient to rescue attenuated cell migration defects observed in ECs following reduced *snai1* function.

3.6 *Snai1* binds and regulates E-box-rich sequences within the promoter region of *mmp15*.

There are increasing data to suggest that *Snai1* may regulate MMP function in several cell types to promote motility (Joseph et al., 2009; Ota et al., 2009; Rowe et al., 2009), however molecular interactions have not been reported. Snail family members regulate transcription via interactions with
E-box binding sites (CANNTG) on target gene DNA. Within 6000bp upstream of the ATG transcription start site of the murine *mmp15* gene, a total of six canonical E-box sites have been identified denoted within three regions (B-D),
each containing two E-boxes (Fig. 10A). Of note, five of the six E-box sites are
conserved with rat or human \textit{mmp15} promoter regions, and two of these are
conserved across all three species (indicated in Fig. 10A). Snai1 binding to
regions A, B, C and/or D (Fig. 10A) in \textit{mmp15} was assessed using chromatin
immunoprecipitation (ChIP) in whole E11.5 mouse hearts. Cross-linked
DNA/protein complexes immunoprecipitated with Snai1 antisera demonstrate
significant enrichment of binding within B, C and D regions of \textit{mmp15} compared
to IgG controls. In contrast, enrichment was not observed in region A that does
not contain canonical E-box sites (Fig. 10B). To further assess the functionality of
E-boxes within regions B, C and D, luciferase assays were performed in COS-7
cells using pGL3 constructs containing \textasciitilde 1kb sequences representative of each
region (pGL3-mmp15B (970bp), pGL3-mmp15C (1006bp), pGL3-mmp15D
(973bp)). Following transfection with respective pGL3-mmp15 constructs, cells
were further infected with AdV-GFP or AdV-Snai1 (as Fig. 6-8). Compared to
AdV-GFP:pGL3mmp15B, AdV-Snai1:pGL3-mmp15B had no significant effect on
transactivation of Region B. However, consistent with ChIP findings, AdV-Snai1
infection significantly increased transactivation of mmp15 region C (pGL3-
mmp15C) (38.49\% ± 8.73\%) over AdV-GFP, with trends of activation also
observed with Region D (pGL3-mmp15D) (29.0\% ± 10.79\%, \(p = 0.3\)).

Previous studies have shown that Snail and E2A (E47/E12) family
members regulate activity of common target genes through interaction with
specific E-box sites within the proximal promoter (Bolos et al., 2003; Cano et al.,
2000b; Peinado et al., 2004). To determine if E47 or E12 function effects Snai1-
mediated transactivation of \textit{mmp15}, luciferase assays were repeated in the presence of pcDNA-E47 or pcDNA-E12, and compared to pcDNA-empty vector controls. Although E2A family members alone have been shown to act as transcriptional activators (Takahashi et al., 2004) and repressors (Batlle et al., 2000; Cano et al., 2000b; Perez-Moreno et al., 2001), co-transfection of pGL3-mmp15B, pGL3-mmp15C or pGL3-mmp15D with pcDNA-E47 or pcDNA-E12 in the presence of AdV-GFP, had no effect on mmp15 activity (data not shown). However, co-transfection of pcDNA-E47 in the presence of AdV-Sna1 significantly repressed pGL3-mmp15B (−70.82%±19.77%), pGL3-mmp15C (−39.09%±0.46%) and pGL3-mmp15D (−48.40%±17.07%). Co-transfection with pcDNA-E12 had no significant effect on AdV-Sna1-mediated mmp15 activity. Collectively these studies reveal that Sna1 physically interacts and activates specific E-box-rich promoter regions within mmp15. Further, we provide evidence to suggest that E47/E12 function may play an important role in Sna1-mediated regulation of \textit{mmp15}.

\textbf{3.7 Summary}

Here we show a direct role for Sna1 in EC formation, and identify \textit{mmp15} as a novel target of Sna1. MMP15 is involved in Sna1-regulated cell motility, but not transformation, during EC EnMT. Our findings are consistent with the previous reports about the indispensible role of Sna1 during EMT, which occurs in a series of pathological and physiological processes (Nieto, 2009). Besides emphasizing the central role of Sna1, once again, in promoting morphological changes and gain of motility in cells, we raise the possibility that there are more
details in the underlying mechanisms that deserved to be dissected. Considering Snai1 functions are crucial for embryonic development, but could be fatal in adult disease, understanding the specific role of each downstream target of Snai1 could provide potential treatment targeting “single-function” molecule while cause minimal side effects. Meanwhile, the function of Snai1 as progenitor-generator during embryogenesis is shut down during adulthood, leading to the question that whether the artificial reactivation of this function could provide fresh progenitors in adult disease where normal somatic cell population is lost.
Chapter 4. Role of Snai1 during valve remodeling and adult

The tri-stratified connective tissue layers of mature valves (Fig. 1C) collectively provide the leaflets with stiffness, compressibility and elasticity, all the necessary biomechanics for efficient function throughout life (Balachandran et al., 2011; Hinton et al., 2006; Lincoln et al., 2006b). Meanwhile, diseased valves are characterized by alterations in connective tissue organization that attenuate the ability of the valve leaflet to open and close during the cardiac cycle (Fig. 1D) (Hinton et al., 2006). Although most of the latent valvular defects were considered as a degenerative process associated with aging, there is increasing evidence implying the underlying molecular aberrations during embryonic development. The highly organized structure within the mature valve leaflet is established during embryonic development at a time when EMT has ceased. This stage is associated with differentiation of mesenchyme precursor cells and ECM secretion and remodeling. It is not clear how the connective tissue layers become organized and highly diversified but it is emerging that VICs are a homogenous population of fibroblast-like cells that differentiate based on their location within the valve leaflet (Liu et al., 2007)(Combs and Yutzey, 2009a). This process is likely mediated by the adjacent VECs, which directly ‘sense’ the hemodynamic environment and exchange signals with VICs.

In contrast to endocardial cushion formation, little is known about the molecular pathways underlying valve remodeling. However in recent years, progress has been made to understand this sophisticated process by several groups, featuring the identification of VEGF-NFATc1 (nuclear factor of activated
T cells cytoplasmic 1) signaling in EMT inhibition and proliferation of VECs (Combs and Yutzey, 2009b), also the identification of Sox9 signaling that regulates the VICs into cartilage-like cell lineage which produces proteoglycan and contributes to the construction of spongiosa layer (Lincoln et al., 2007; Peacock et al., 2010). Interruption of these signaling may lead to either embryonic death or increased vulnerability to adult valve disease (Peacock et al., 2010).

It is well known that VICs largely contribute to the ECM production and maintenance of valvular connective tissue homeostasis, thus the roles of VICs in valve disease has been an active area of research; on the other hand, the VECs, although highly responsive to changes in the hemodynamic environment, are less well understood during valve pathology. As the “mechanosensor” of the valve, VECs show altered and disruptive morphology during early stages of degenerative valve disease (Butcher and Nerem, 2006b), associated with neo-vascularization, infiltration of inflammatory cells and lipid deposition in the case of sclerotic valves (Mohler, 2000; Otto et al., 1994). In addition, VEC dysfunction correlates with VIC activation, proliferation and deposition of ECM in calcified valves, leading to further valve thickening (Hinton et al., 2006). Together, these studies underline the importance of communication between VECs and VICs during valve pathogenesis. However, it remains unclear if alterations in VEC responses directly induce pathogenic processes or are the result of an alternative primary insult. Nonetheless, it is clear that disruption to the valve endothelium is an important initial step in the pathogenesis of heart valve disease.
Intriguingly, it has been shown recently that adult diseased valve tissue has high expression of mesenchyme cell markers, implicating the existence of VEC transformation that could replenish the damaged VIC population in response to pathological stimuli (Paranya et al., 2001; Paruchuri et al., 2006). However, this potential maintained in VECs after developmental stages has not been extensively examined, and the key factors that provoke this potential compensatory response are still elusive.

Our preliminary data show that following EMT, Snai1 expression is maintained in VECs during remodeling stages as well as re-induced in VECs of diseased valves. Therefore the goal of this aim was to determine if Snai1 is important for establishing and maintaining the mature valve structure.

4.1 Snai1 is expressed in remodeling murine valves.

As previously discussed in Chapter 3, consistent with its functions, Snai1 is highly expressed in VECs and VICs formed de novo (Fig. 3). In contrast, during valve remodeling, when EMT is no longer required, Snai1 expression is downregulated in VICs but maintained in VECs of valve primordia (Fig. 11A) and neonatal valves (Fig. 11B). With onward development, the absolute transcript levels of snai1 decrease and become undetectable in healthy adult valves (data not shown). Inspired by

![Figure 11. Snai1 expression in remodeling murine valve.](image-url)
previous report that Snai1 plays a role in cartilage cells by respressing chondrocyte ECM genes including type II collagen (col2a1) (Seki et al., 2003). We proposed that the presence of Snai1 during remodeling stage suggests a potentially novel role of this EMT regulator that has not been reported.

4.2 Snai1 regulates ECM composition in remodeling valves.

Concerning the early onset of Snai1 and the abnormal endocardial cushion formation observed in Tie2cre;Snai1fl/+ embryos, investigating of Snai1 function during valve remodeling with the same mouse model is not feasible. To bypass this early phenotype, we bred a tamoxifen-inducible Tie2creERT2 mouse model (European Mouse Mutant Archives) (Forde et al., 2002) with Snai1fl/fl mouse. This spatio-temporally inducible cre system allows us to target Snai1 knockdown in endothelial cells at post-EMT time point when endocardial cushion formation is complete. In preliminary studies, pregnant Rosa-GFP reporter females bred with Tie2creERT2 males were administrated with daily intraperitoneal (IP) injections of 2 mg tamoxifen from E13 to E15, therefore bypassing the early endocardial cushion formation stages. These conditions are sufficient to induce Tie2creERT2 recombination only in endothelium as green fluorescence was observed specifically in VECs and endothelial cells of great vessels by E18.5 (Fig. 12A, B).
After impregnating Snai1\(^{fl/fl}\) females with Tie2\(^{creER}^{T2}\); Snai1\(^{fl/fl}\) males, histological analysis shows that heart valves from tamoxifen-treated Tie2-\(^{creERT2}\);Snai1\(^{fl/fl}\) embryos at E18.5 have increased ECM deposition of proteoglycans and collagen within the mitral (Fig. 12C-F) and aortic (data not shown) valve core and annulus when compared to littermate controls (Snai1\(^{fl/fl}\)). Further investigation with immunofluorescence staining suggests chondroitin sulfate proteoglycan (CSPG) is, at least partially, responsible for the observed proteoglycan accumulation (Fig. 12G, H). Thus indicating that Snai1 is important in VECs for post-EMT patterning of connective tissue.
4.3 Snai1 is expressed in human pediatric diseased valve and it’s sufficient to reactivate EMT in mature murine valves.

Recent studies highlighting the expression of αSMA in diseased valve suggest the presence of newly transformed mesenchyme cells, indicative of reactivation of signaling pathways involved in EMT, required only in early valve development (Fig. 13A, B). Therefore it suggests the mechanisms of in vivo compensatory response of VECs to pathological conditions. Therapeutically, the identification of effective pathways that could induce EMT in adult VECs to replenish the damaged VIC population would be an innovative tactic in therapies targeting human valvular diseases. Therefore, we started with the examination of αSMA and Snai1 expression in pediatric aortic valve disease patients and observed increased expression of both EMT markers, notably in VECs and the VICs reside within the sub-endothelial region, compared to non-diseased age-matched controls (Fig. 13C, D). This increase in Snai1 expression in diseased valves suggests that it may play an
important role in valvular pathogenesis by contributing to the healing process, in contrast to its normal malignant role in cancer.

4.4 Snai1 induces EMT in post-EMT mouse valve tissue.
To assess the sufficiency of Snai1 during EMT ‘reactivation’ in post-EMT valvular tissue, we overexpressed Snai1 in postnatal murine aortic and mitral valve explants using a published adenovirus (AdVSnai1). As shown in Fig. 14, compared to AdVGFP controls, AdV-Snai1 treatment significantly increased expression of mesenchymal cell genes including fibronectin1 (FN1), and downregulated the endothelial cell adhesion gene, E-cadherin (E-cad). This suggests that VECs from mature valves still possess the potential to undergo EMT, which could be sufficiently induced by Snai1.

4.5 Summary
In the long term, we aim to uncover the unappreciated pathways that response to pathological conditions and these studies provided more insights into the mechanisms that regulate connective tissue remodeling and homeostasis in healthy valves. Collectively, the fact that Snai1 is required for valvular ECM organization and increased Snai1 expression in diseased valves suggest novel functions of Snai1, beyond EMT, in valve remodeling and maintenance. It is highly promising that several key valvular ECM components are involved in Snai1 downstream signaling and Snai1–reactivated EMT serves as a mechanism to maintain and renovate the VIC population during normal and pathogenic scenarios. However, the detailed molecular pathways are waiting to be dissected. On one hand, it is obscure why the specific knockdown of Snai1 in
VECs could affect the ECM profile, normally determined by VICs; on the other hand, the fate and characteristics of Snai1-expressing VECs and VICs in diseased valves remains unclear. Fortunately, these questions will be answered by our group in the near future based on the foundation created by my preliminary work.
Chapter 5 Role of Snai1 during epicardium development

Although considered a minority cardiac cell population, epicardial cells are the major contributors of the coronary vessels, fibroblasts and the collagen-rich fibrous matrix within the myocardium (Wessels and Perez-Pomares, 2004). As the origin of epicardium, proepicardial organ (PEO), a cluster of progenitors located dorsal to the developing heart tube, arises from the splanchnopleural mesoderm of the posterior heart field (Fig. 2A) (Dettman et al., 1998; Mikawa and Gourdie, 1996). Induced by BMP and FGF signaling, PEO cells form the epicardium by attaching and spreading laterally on the outer surface the myocardium to shield the whole heart (Ishii et al.; Kruithof et al., 2006; Schlueter et al., 2006). Later, EMT occurs in a subpopulation of epicardial cells to form epicardial-derived cells (EPDC) (Lie-Venema et al., 2007). Harboring multiple potentials, EPDCs migrate and invade into myocardium and differentiate into abovementioned lineages to promote the myocardial maturation.

The epicardial cells phenocopy mesothelial cell characteristics by expressing Wilms’ tumor 1 (WT1) and Tbx18 (Olivey and Svensson), besides the expression of both epithelial markers such as E-cadherin, and mesenchymal markers including Vimentin. Although after the invasion into myocardium, the mesothelial phenotype is gradually replaced as EPDCs differentiate into coronary smooth muscle, endothelium and interstitial fibroblasts (Wessels and Perez-Pomares, 2004). Transcription factors including Snai2, WT-1, Ets-1 and 2 have all been implied to be involved in the EMT during EPDC formation (Carmona et al., 2000; Lie-Venema et al., 2003; Perez-Pomares et al., 2002), while Ets
signaling is also involved in EPDC migration (Lie-Venema et al., 2003). Our preliminary data shows Snai1 expression in the PEO and epicardium of both mouse and chicken (Fig. 15). Although it has been shown in chicken that snai1 promote PEO formation by mediating FGF signaling (Schlueter and Brand, 2009), its roles in PEO and later epicardial development are not yet defined. Thus we aim to examine the roles of snai1 during epicardium development and myocardium maturation using in vitro explant culture systems and a mouse model with conditional knockdown of snai1 in epicardial and EPDC cells.

5.1 The expression of Snai1 during epicardium development

In order to define the role of Snai1 during epicardial development, we first investigated its expression pattern in chicken (Fig. 15A-C) and mouse (Fig. 15D-F) at comparable stages of epicardial development. Snai1 expression is clearly
observed in the superficial compartments of both chicken (c) and mouse (m) PEOs at chick embryonic (E) 2.5 (Fig. 15A) and mE9.5 (Fig. 15D) respectively, however expression is lower in the interstitial cells in both species (Fig. 15A, D). Later at cE7 and mE16.5, after the formation of the intact epicardium in both mouse (E11.5) and chicken (E4.5), Snai1 expression is maintained in a large portion of epicardial cells, and is also found in EPDCs within subepicardial space (Fig. 15B, E). Meanwhile, Snai1-positive cells are also located within the myocardium (Fig. 15B, E), suggesting that Snai1 could mark the myocardial-invading EPDCs. After epicardial EMT and EPDC invasion, Snai1 is maintained in a small portion of epicardial and EPDC cells in chicken (Fig. 15C), but at a lower levels compared to earlier stages; conversely in mouse, we still can observe evident Snai1 immunoreactivity in the epicardium and subepicardium at postnatal stages, although its expression in the myocardium dramatically decreased. Interestingly, Snai1 is also expressed in endothelial cells of mouse coronary vessels (Fig. 15F). Together, the profile of Snai1 implies its involvement in multiple stages of epicardial development.

5.2 Snai1 promotes pro-epicardial cell migration in vitro

Knowing Snai1 is required for early PEO formation (Schlueter and Brand, 2009), its persistence in the PEO indicates previously unrecognized functions during later developmental stages. Here we attempted to define the role of Snai1 during formation of the epicardium, initiated by PEO spreading over the myocardium. To mimic the outgrowth of PEO, we employed an in vitro explant culture system in which PEOs are harvested from HH17 (cE2.5) chicken embryos and placed on
uncoated chamber slide (Ishii et al.), allowing the cell cluster to attach, flatten out and grow over the slide surface. To determine if Snai1 promotes proepicardial cell outgrowth, PEO explants were subjected to Snai1 gain-of-function by infecting with AdV-Snai1 or AdV-GFP as control. During the first 24 hours of post-infection culture, PEO cells retain the pro-epicardial phenotype as validated by fluorescence activity of WT1 (Fig. 16D) and over 60% of infection efficiency was achieved using both viruses (data not shown). Following 24-hour infection, stereoscopic analysis shows that Snai1 overexpression (Fig. 16B) significantly increased the area (Fig. 16C) covered by the outgrowing cells by 50%, compared to the AdV-GFP control virus-treated group (Fig. 16A). Thus these results imply a novel function of Snai1 during proepicardial cell migration.

5.3 Snai1 facilitates epicardial EMT and EPDC invasion in vitro
Snai1 has been used as an indicator and readout of EMT in many systems including epicardial studies (Martinez-Estrada et al., 2010), however, its direct role during epicardial EMT and EPDC migration hasn’t been recognized. To
determine if Snai1 can promote epicardial EMT, we took the advantage of an in vitro epicardial primary culture protocol, in which cE4.5 hearts are placed on collagen-coated chamber slides to allow the newly formed epicardial monolayer to spread out of the myocardial surface onto the culture slide while maintaining cell-cell contact and key molecular marker, validated by immunostaining of WT1 (Fig. 17A). This 2D monolayer was similarly infected with AdV-Snai1 or AdV-GFP and following 72 hours, qPCR was performed to examine epicardial (TCF21 (or epicardin)) and mesenchymal markers (N-cadherin, fibronectin1, aSMA). AdV-Snai1 infected cultures show significant reduced expression of the epicardial marker Tcf21 (-3-fold) and increased mesenchymal cell marker, N-cadherin (+1.5-fold), aSMA (+1.75-fold) and Fibronectin1 (+1.5-fold), compared to AdV-GFP controls (Fig. 17B). Gene expression changes indicative of epicardial cell transformation was further confirmed by immunofluorescence of aSMA in the same culture system. As shown in Figure 17, compared to AdV-GFP treated cells (Fig. 17C), increased Snai1 expression results in spindle-like cell morphology (Fig. 17D). These data suggest Snai1 is sufficient to promote epicardial cell transformation. To further support the previous study, we performed a whole heart culture assay in which cE5.0 (EPDC invasion stage) heart explants were cultured on 0.1µm filters (millipore) floating on culture media. The usage of the filter largely prevented the epicardial cell from outgrowth, thus we expected the EPDCs to invade into the myocardium (Nesbitt et al., 2009). The epicardium of the explants was labeled by a 2 hours infection of either AdV-Snai1 or AdV-GFP as a control. To avoid the potential effects of cell proliferation on the read out of
this assay, serum-free media was used in the explants. Following 48 hours of culture, explants were subjected to fixation and histological analysis. As shown in Figure 3, following AdVSnai1 infection (Fig3F), the ratio of GFP-positive cells

Figure 17. Snai1 promotes epicardial cell transformation and increases EPDC population in vitro. (A) chickenE4.5 hearts were placed on collagen-coated chamber-slides to let the epicardium spread out as an intact cell layer (A), with the expression of epicardial marker WT1 (arrows in A) confirmed by immunofluorescence. (B) qPCR indicates increased expression of mesenchymal markers (N-cadherin, αSMA and fibronectin1) and decreased epicardial marker (TCF21) in the culture described in A, after treatment with AdVSnai1, compared to AdVGFP. N=3, p<0.05. (C, D) immunofluorescence of SMA in AdVSnai1 (D) and AdVGFP (C) infected group. Note the increased SMA fluorescent activity in Snai1-overexpressing cells (arrows in D), compared to the controls (arrowheads in C). (E-F) whole hearts from cE5 embryos were cultured on 0.1µm filter for 48 hours after labeling the epicardium with AdVSnai1 (F) or AdVGFP (E). (G) Bar graph to show increased GFP-positive EPDC population in the myocardium after AdVSnai1 treatment, compared to AdVGFP control group. N=4, p<0.05.
within the myocardium over the total number of GFP-positive cell within the epicardium and myocardium reached 18% (Fig. 17G), compared to 8% AdV-GFP-infected group (Fig. 17E). This data shows an increased EPDC population promoted by overexpression of Snai1, and further support the role of Snai1 in furthering the epicardial EMT.

5.4 Conditional knockdown of Snai1 in mouse epicardium during development impedes myocardium maturation.

To directly examine the role of Snai1 during epicardial development and myocardium maturation, we utilized the WT1creER^{T2} (Zhou et al., 2008) to target loss of snai1 function in epicardial and EPDCs upon tamoxifen-induced cre recombination. Homozygous WT1creER^{T2};Snai1^{fl/fl} mice could not be generated as both the Cre and Snai1 genes are located on chromosome 2 (Zhou et al., 2008). However, heterozygous (WT1creER^{T2};Snai1^{fl/+}) mice were available and viable at E18.5 following tamoxifen administration in the pregnant female from E12.5 to E14.5. In order to assess the phenotype of the mice with targeted decrease of Snai1 in epicardium lineage, we performed Trichrome staining to examine the gross morphology of E18.5 hearts on tissue sections. As shown in Figure 4, tamoxifen-treated WT1creER^{T2};Snai1^{fl/+} mice have thinner left ventricular walls at E18.5, compared to vehicle treated controls (WT1creER^{T2};Snai1^{fl/+}, corn oil injected only, no tamoxifen) (Fig. 18A). In order to dissect the mechanism underlying this phenotype, we have been examining the coronary vessel system formation in the myocardium of the tamoxifen-treated and control mice, since the circulation established by coronary vasculature is
required for myocardial maturation during fetal stages (Sedmera et al., 2000). Immunofluorescence using antibody against SMA was performed to detect vascular smooth muscle cell distribution in E18.5 myocardium. As shown in figure 4, preliminary findings imply that mice with snai1 knockdown in Wt-1 derived cells (epicardium and EPDCs) have lower density of SMA positive cells (Fig. 18D), compared to the controls (Fig. 18C). Further immunohistological analysis to examine the integrity of epicardium using antibody against WT1 shows

Figure 18. WT1creER<sup>T2</sup>;Snai1<sup>fl/fl</sup> mice have decreased thickness in the ventricular walls, reduced WT1 level in the epicardium and decreased SMA immunoactivity at E18.5 after tamoxifen administration to knockdown Snai1 expression. (A, B) Trichrome staining to show the structure of E18.5 hearts of WT1creER<sup>T2</sup>;Snai1<sup>fl/fl</sup> mice after tamoxifen treatment (B) compared to vehicle control (A). (C, D) Immunostaining to show the expression and localization of WT1 in E18.5 epicardium after tamoxifen (D) and vehicle (C) treatment. Note the decreased WT1 fluorescent activity (arrowheads in D) in Snai1 knockdown group (D). (E, F) Immunostaining of SMA in right ventricles after tamoxifen (F) and vehicle (E) treatments respectively. Arrows in E and F indicate coronary vessels. Asterisk, major coronary vessels. Nuclei were counterstained with DAPI. LV, left ventricle; RV, right ventricle.
a decreased level of WT1 in the epicardium of heterozygous mice (Fig.4E, F). These findings suggest a haploinsufficient function of Snai1 during epicardium development and reduced Snai1 function could lead to abnormal myocardium during development.

5.5 Summary
Here we show that Snai1 plays an additional role during cardiogenesis by regulating epicardium development. Snai1 expression persists during pro-epicardial cell migration, EPDC formation and invasion. Interestingly, in both mouse and chicken PEO, Snai1 expression is intense in the majority of the cells at the outer surface, while it is detectable only in a portion of the interstitial cells at lower level. This may be explained by the heterogeneity of the PEO, which harbors different groups of progenitors that express epithelial, mesenchymal or endothelial cell markers (Lie-Venema et al., 2007). It is still a hypothesis that if Snai1 could mark a certain subpopulation of these precursors, fated to transform and invade after reaching their position in the epicardium.

Although previously shown that Snai1 positively regulate several key steps of EMT, including cell transformation and migration in the endocardial cushions, it seems that the Snai1-promoted migration is not transformation-dependent. As overexpression of Snai1 in pro-epicardial cell promotes cell migration but does not induce cell transformation during the first 24 hours in our in vitro culture. More importantly, Snai1-promoted transformation occurs in different cell types including epithelial cells and more specific endothelial and mesothelial cells, as shown in Figure 3. This implies conserved mechanisms underlying the formation
of progenitor cell populations during heart formation. The lack of these progenitors, which could be due to decreased Snai1 function, may lead to severe consequence in both valve and myocardium development. These facts, along with our studies, prove the versatility of Snai1 during embryogenesis, also raise more questions about the potential novel functions of an widely-studied molecule.

Current studies include further characterizing the developmental phenotypes of WT1creER^{T2};Snai1^{fl/+} mice at E18.5 and 14.5 and the mechanisms underlying them. The ventricular wall thickness of the fetal hearts will be quantified, and we will assess the cardiac fibroblast population by immunostaining with Thy1 antibody; cardiomyocytes morphology, apoptosis and proliferation ratio will also be assessed by double-immunofluorescence of cleaved-Caspase-3 or phosphor-Histone H3 respectively, along with ventricular myosin heavy chain. In vitro studies with loss and gain of function of Snai1 are also being performed in order to examine if the role of Snai1 is sufficient and required for epicardial cell transformation.
Chapter 6 Discussion

Over the past several decades, the fields of heart development and pathogenesis have made significant advancements in understanding the structure-function relationship of different cardiac compartments in both health and disease. These findings established the foundation for us to further investigate the ins and outs of cardiac development and pathology, in order to catch the strategic points of heart disease for novel therapeutic approaches. As a long-lasting hot topic, transcription factors play vital roles during many physiological and pathological processes by answering one or multiple upstream signals from distant (endocrine) and adjacent (paracrine) loci, even directives within the same cells (autocrine), and performing different roles by targeting the transcription of various downstream targets (Barrallo-Gimeno and Nieto, 2005; Combs and Yutzey, 2009a; Lie-Venema et al., 2007). The complexity of signaling transductions in living cells is well recognized, thus identifying the crucial regulators among these communication webs is essential for promoting our understanding the overall situation (Thiery et al., 2009). The above-mentioned projects performed by our group featured Snai1, one of the master regulators of embryonic development, in cardiac development and diseases. The insights we gained from these studies provide us new perspectives of the build-up, maintenance and maladaptive steps of cardiac function.
6.1 Endocardial cushion formation
In developing ECs, the EMT program converts endothelial cells into migratory mesenchymal cells through sequential steps that require many phenotypic changes (Barrallo-Gimeno and Nieto, 2005; Thiery et al., 2009). The transcription factor Snai1 has previously been shown to be indispensible for EMT in many tissues during embryonic development and pathological processes (Nieto, 2009; Thiery et al., 2009). Despite \textit{snai1} expression correlating with EMT activity in developing valve structures (Liebner et al., 2004; Luna-Zurita et al.; Meadows et al., 2009; Nath et al., 2008; Wang et al., 2005), a direct role in EC formation had not previously been reported. This is likely due to the premature lethality of \textit{Snai1}^{-/-} embryos prior to cardiogenesis and heart defects have not been reported in \textit{Snai1}^{+/+} mice (Carver et al., 2001b). To address this limitation, we performed in vivo studies using viable \textit{Tie2cre;Snai1}^{fl/+} mice, in parallel with gain and loss of \textit{Snai1} function in AVC explants in vitro. The goal of this study was to use these previously established systems to reveal new insights into the role of \textit{Snai1} for cell transformation, invasion and migration in developing ECs in the embryonic heart.

6.1.1 The role of \textit{Snai1} during endocardial cushion formation
Prior to EMT, \textit{Snai1} is highly expressed in endothelial cells of the AV canal and expression is maintained in this cell layer throughout endocardial cushion formation (Fig. 3A, B, 6A). During EMT, \textit{Snai1} expression is also observed in transformed mesenchyme cells, however expression is downregulated as these cells differentiate during remodeling stages (Fig. 3A, 11A, B) (Lincoln et al.,...
In this study we show that Snai1 is important in many sequential steps during EMT beginning with the early transformation of a subset of cardiac endothelial cells into mesenchyme cells, and proceeding mesenchyme cell invasion and migration. Further, we show that MMP activity is required for Snai1-mediated mesenchyme cell migration and more specifically, MMP15 activity is sufficient to rescue migratory phenotypes observed in ECs with targeted snai1 knockdown. Together, findings from this study suggest that Snai1 is an important regulator of EC formation and directly binds and regulates mmp15 to promote cell motility during this process.

6.1.2 The role of Snai1 during EMT

Snai1-mediated signaling pathways in EMT have been extensively studied and therefore parallel mechanisms in the developing heart are considered. Many known upstream regulators of Snai1 are expressed during cardiogenesis including Notch1 in endothelial cells overlying the ECs, and Tgfβs and Bmps expressed in the adjacent AV canal and OFT myocardium (Luna-Zurita et al., 2010; Molin et al., 2003; Niessen et al., 2008b; Thiery et al., 2009). In addition, manipulation studies of these growth factors, their receptors or intermediate signaling kinases affect snai1 expression in developing cushions, and therefore Snai1-induced EC EMT is likely initiated by these established signaling pathways in vivo (Luna-Zurita et al., 2010; Meadows et al., 2009; Wang et al., 2005). Downstream, we can speculate that Snai1 promotes EMT in developing cushions through mechanisms previously described in other non-cardiac tissues including repression of cell adhesion genes (Cano et al., 2000b; Liebner et al., 2004;
Meadows et al., 2009; Nath et al., 2008) and activation of mesenchymal markers (Batlle et al., 2000; Cano et al., 2000b; Guaita et al., 2002; Ikenouchi et al., 2003; Person et al., 2005a). In Tie2cre;Snai1^{fl/+} mice, it is likely that these signaling pathways are abrogated as a result of reduced snai1 function, leading to hypocellular ECs. In addition to attenuated transformation, it is plausible that deficient mesenchyme cell motility and/or imbalanced cell survival also contribute to this phenotype. Indeed, our data supports a positive role for Snai1 in cell invasion and migration in developing ECs, however changes in cell proliferation or apoptosis were not observed in Tie2cre;Snai1^{fl/+} mice at E10.5. In contrast, cell proliferation was enhanced at E13.5 and levels were sufficient to increase mesenchyme cell number to levels comparable with controls. Compensation of EC cellularity was similarly reported in Snai2^{-/} mice that exhibit hypocellular cushions at E9.5 but not E10.5, and attributed to increased snai1 expression (Niessen et al., 2008b). However, increased expression of Snail family members were not detected in Tie2cre;Snai1^{fl/+} mice (data not shown), consistent with observations reported in snai1^{-/} mice (Carver et al., 2001b). Therefore it is likely that alternative mechanisms promote the compensatory proliferative response in ECs from mice with reduced snai1 function.

6.1.3 Novel transcription activity of Snai1 in EMT

In addition to developmental processes, Snai1-mediated EMT is active in many cancer cell lines and high expression levels correlative with increased cell migration and metastasis (Thiery et al., 2009; Wu and Zhou). A recent study suggested that this pathologic function of snai1 is mediated through induction of
MT-MMPs, that when activated, recapitulate snai1’s ability to degrade basement membranes and promote cancer cell migration (Ota et al., 2009). In this study, we present several lines of evidence to suggest that in addition to disease, activated MT-MMPs also promote cell motility, but not transformation, in the embryonic heart during EC development. Further, we identify mmp15 (or MT2-MMP) as a potential new target gene of Snai1 to promote mesenchyme cell invasion and migration. This finding extends the current repertoire of known target genes regulated by Snai1 during EMT beyond cell transformation processes (Batlle et al., 2000; Cano et al., 2000b; Guaita et al., 2002; Ikenouchi et al., 2003; Person et al., 2005a).

In ECs from Tie2cre;Snai1^{fl/+} mice, mmp15 was the only MMP family member whose expression was affected by reduced snai1 function, however, decreased trends of the MMP15 target gene mmp9 were also observed (data not shown) and snai1 knockdown in mouse embryonic EC explants attenuated mesenchyme cell migration even with the presence of caMMP15 (Figure 9 C-E), indicating other migration-promoting factors could be affected by decreased snai1 expression. The widespread protein localization of mmp15 throughout the ECs suggests that this mmp family member may target ECM substrates in both endothelial and mesenchymal cells. Type IV collagen is a known substrate of MMP15 in basement membranes (Hotary et al., 2000; Rebustini et al., 2009) and its degradation in endothelial cells lining the AV canal and OFT regions is important for EMT and EC formation (Song et al., 2000). Therefore leading to the conclusion that mmp15 may facilitate endothelial cell delamination, independent
of transformation in developing ECs. Mmp15’s function in mesenchymal cells is less speculative and its activity correlates with increased mesenchyme cell invasion and migration in AV canal explants. Like mmp15, other family members including mmp2 and 9 are also highly expressed in mesenchyme cells during EC EMT, where they facilitate ECM degradation to promote mesenchymal cell motility through the hyaluronan-rich cardiac jelly (Rupp et al., 2008; Shelton and Yutzey, 2007). The observation that Snai1 directly binds and activates mmp15 is consistent with a recent study showing that Snai2 (slug) binds and regulates activity of another family member, membrane-type 4 MMP (Huang et al., 2009). Findings from this current study have revealed a new role for snai1 as a transcriptional activator of mmp15 to promote cell migration during EC EMT.

The observation that Snai1 acts as a transcriptional activator of mmp15 is somewhat surprising as despite emerging evidence to support this function (Batlle et al., 2000; Cano et al., 2000b; Guaita et al., 2002; Hwang et al., 2011; Ikenouchi et al., 2003; Person et al., 2005a), Snai1 is more commonly known as a repressor (Nieto, 2009). In our system, Snai1 increases mmp15 transactivity by 40%, which could be considered a subtle, albeit significant, difference. As this study is one of the first to show direct transactivation of target genes by Snai1, it is not clear if recruitment of co-activators (or removal of co-repressors) could be sufficient to increase mmp15 activity above this level. In contrast to AdV-Snai1 treatment, E47 alone has no effect on mmp15, yet E47 in the presence of AdV-Snai1, leads to repression. As previous studies have shown that Snai1 and E47 regulate common target genes through interaction with specific E-boxes (Bolos et
al., 2003) (Peinado et al., 2004; Perez-Moreno et al., 2001), we speculate that exogenous E47 binds with no functional effect, to prevent Snai1 interaction and transactivation. This mechanism may also in part explain the subtle increase in mmp15 activity with AdV-Snai1 treatment: as endogenous E47 competes with Snai1 for E-box binding. This competition may also be dependent on differential binding affinities of Snail1 and E2A family members on the same target genes (Bolos et al., 2003). Although this might explain the lost activation of mmp15 by Snai1, the mechanism in which Snai1 and E47 cooperate to repress mmp15 is not clear, but likely requires recruitment of co-repressor machinery at the putative E-box sites. Our studies using the in vivo mouse and in vitro chick models, strongly suggest that Snai1 positively regulates mmp15 in these systems, therefore suggesting that endogenous E2A expression levels are low at this time. However, it remains unknown how Snai1-E2A cooperate to regulate mmp15 during post-EMT stages when cell motility is less active.

6.2 Snai1 function in heart valve maturation and maintenance
After EMT, endocardial cushions elongate as the result of VIC and VEC proliferation, meanwhile the connective tissue starts to undergo remodeling (Combs and Yutzey, 2009b). In addition, VECs reform an uninterrupted endothelium and gradually gain the canonical endothelial cell morphology (Person et al., 2005b). During this stage, VICs lose expression of mesenchyme genes, and begin to express chondrocyte and tendon cell markers (Chakraborty et al., 2010; Hurle et al., 1994; Lincoln et al., 2006a; Lincoln et al., 2006b; Montero et al., 2002), signifying VIC diversification. Earlier studies have
extensively characterized the role of VECs in establishing the pool of valve precursor cells that later give rise to the mature valve structures. However, less is known about the pathways involved in valve remodeling.

6.2.1 The role of Snai1 in heart valve remodeling and maturation

The maintenance of Snai1 in VECs during post-EMT stages (Fig. 11A, B) give rise to many questions as to its function and the role of VECs in valve remodeling. Although Snai1 expression pattern in VECs seems to be consistent during early and late valve development (Fig. 3A and Fig. 11), the cobblestone-like VECs in endocardial cushions serve as a source of progenitors formed by EMT, while VECs of remodeling valve form a “canonical” endothelium that lose the capacity to transform in vivo. This switch of phenotype in VECs may demand Snai1 to adapt to different VEC-derived signaling and functions, at the same time, it provides a new candidate regulator that may fit into the largely-unknown valve remodeling pathways. To test these possibilities, loss of function studies were performed using an inducible-conditional knockdown system (Tie2creER\textsuperscript{T2}) that targets snai1 in VECs during remodeling stages (Fig. 12 A, B). Interestingly, E18.5 atrioventricular valve leaflets show increased total proteoglycan, including Chondroitin-sulfate proteoglycan (CSPG), and collagen deposition following Snai1 knockdown (Fig. 12 C-H). These results imply a novel role of Snai1 in a non-EMT process, the VIC diversification and ECM remodeling stages of valvulogenesis. Although function of Snai1 other than an EMT regulator has been reported in chondrocytes (Seki et al., 2003), the switch from an EMT promoter in endocardial cushion VECs to a potential regulator of ECM remodeling suggests
the involvement of different molecular environments. As mentioned previously, Snai1 may have co-repressor during transcription regulation of EMT (Fig. 10), thus it is highly possible that novel co-factor is present in VECs of remodeling valve that assist Snai1 to target other genes.

It is well known that VICs are the major producer of valvular ECM, and combine with the observed phenotypes, which involved the whole leaflet, we speculate the existence of molecular communication between VECs and VICs, established by either secreted factors or juxtacrine signaling during valve remodeling stages. As the “mechanosensory” of valves, VECs have been indicated to correlate with the VICs in valvular pathogenesis (Hinton et al., 2006) and also regulate VIC phenotype and matrix synthesis responding to shear stress in vitro (Butcher and Nerem, 2006a). However, in order to ascertain the precise roles of each cell type, proper animal models are required. As a further matter, instead of showing a myxomatous phenotype, the valve leaflets possess grossly normal morphology after Snai1 knockdown, this could be explained by the requirement of aging as a pathological stimuli. Thus the long-term effects on heart valve structure and function in viable Tie2-creERT2;Snai1$^{fl/fl}$ mice, as well as the possible requirement of Snai1 in VECs after birth for maintenance of valve connective tissue, will be characterized. The most challenging goal is to elucidate the mechanism(s) in which VEC-Snai1 molecularly communicates with VICs to regulate ECM deposition within the valve leaflet and identify the unrecognized roles of Snai1 in a non-EMT context.
6.2.2 The role of Snai1 in heart valve maintenance

Developmental abnormalities in valves could lead to birth defects or increase susceptibility to adulthood disease (Lincoln and Yutzey, 2011; Peacock et al., 2010). Current treatments including replacement surgeries are inefficient and often temporary (Mann and West, 2001) due to improper procedure and immature bioprosthetic valve tissue (Schoen, 2008). Therefore, addressing currently unanswered questions about the establishment of the valve structure during development may provide us insights into valve pathogenesis and clues of therapeutic targeting of VECs and VICs, allowing for intervention prior to end-stage heart failure. As previously discussed, transformation of a subset of VECs in endocardial cushions is essential for establishing the pool of mesenchymal VICs that later give rise to the mature heart valve structures (Lincoln et al., 2004a). With the emerging concept that valve disease processes recapitulate developmental gene programs, there are undergoing investigations about the potential of adult VECs to undergo EMT for replacing damaged endothelia and replenishing VICs in response to pathological stimuli (Paranya et al., 2001; Paruchuri et al., 2006). Therefore, we examined the expression of Snai1, normally undetectable in healthy mature valves (Tao et al., unpublished), and mesenchymal marker αSMA in pediatric diseased valve sample, compared to non-valvular disease controls (Fig. 13A, C). Intriguingly, consistent with the expression of αSMA (Fig. 13B), a shown diseased valve marker, in endothelium and sub-endothelial area of the pathological leaflet, Snai1 is highly expressed in the similar area (Fig. 13B), suggesting ongoing EMT. Bearing the question that if
Snai1 is sufficient to reactivate EMT in post-EMT VECs, we overexpressed it in mouse postnatal mitral valve leaflets, where Snai1 indeed induced the alteration of EMT marker genes (Fig. 14). Thus, it is worthy to focus future studies on the potential of VECs to re-activate the EMT program to repair or replace damaged lesions in diseased valves. However, based upon the limited regeneration capacity of mature endothelial cells compared to embryonic endothelial cells (Dimmeler and Zeiher, 2004), it is considered that VEC “plasticity” may be greater in the embryo than in adult valve, and the question about how to boost the healing capacity of adult VEC during disease may be answered by identification of the major EMT regulators during development.

6.3 Snai1 function in epicardial development
It is known that Snai family members mediate FGF, VEGF and WNT signaling (Olivey and Svensson), all of which are essential for coronary vasculogenesis. Thus a tantalizing possibility is raised that Snai1, found to express in developing epicardium (Fig. 15), may play a role during EPDC formation and development. Knowing that epicardium gives rise to a group of multipotent progenitors during embryogenesis by undergoing EMT, we aim to detail the underlying mechanisms of the development of epicardial and epicardium-derived lineages, also find out if Snai1 function as EMT-regulator is conserved in developing heart, between endocardial cushion and epicardium. Interestingly, we located Snai1 expression during almost every stage of epicardium development in both mouse and chicken (Fig. 15), suggesting its involvement in different steps. The formation of the single layer epicardium from PEO requires pro-epicardial cell outgrowth, mainly
achieved by cell migration (Wessels and Perez-Pomares, 2004). To examine Snai1 function during this process, we overexpress Snai1 in PEO explant culture (Ishii et al.). As shown in Figure 16, Snai1 overexpression significantly increased the motility of PEO cells. Worthy to mention, the PEO is a mixture of superficial methothelial epithelium and mesenchymal interstitial cells (Wessels and Perez-Pomares, 2004), each of them shows different level of endogenous Snai1 expression (Fig. 15). Different from Snai1-promoted migration in endocardial cushion, here the increased motility is EMT-independent, shown by the preexisting mesenchymal phenotype in PEO and well-maintained epicardial morphology and gene marker in the culture (Fig. 16B). This could be due to a different migratory mechanism that Snai1 is involved, and it is also possible that Snai1-promotes cell migration and transformation in parallel, and specific co-factors and regulators may be required to let them occur simultaneously.

The major event through which epicardium contributes to the myocardium maturation occurs after the hearts are wrapped by the epicardial cell sheet. During this time, EMT occurs to generate the epicardium-derived cell (EPDC) population (Perez-Pomares et al., 1997; Vrancken Peeters et al., 1999), which temporarily reside in subepicardium (Perez-Pomares et al., 1997), from which they invade the myocardium in a spatiotemporally regulated pattern (Lie-Venema et al., 2005). To test the EMT-promoting role of Snai1 in epicardium, we overexpress it in epicardial primary monolayer culture. Although showing either significant changes or at least a clear trend, none of the mesenchymal markers we examined dramatically increased at the transcript level (Fig. 17B) as what we
observed in Snai1-promoted endocardial cushion EMT, which could be interpreted again by the mesothelium phenotype of epicardial cells that hampered further promotion of mesenchymal genes. In contrast, tcf21, an epicardial marker, did show a dramatic decrease. To support the qPCR study, we performed Immunostaining in the identical culture system and observed increased αSMA immunofluorescent activity in Snai1-overexpressing cultures (Fig. 17C, D). To further study Snai1 function in EPDC, we labeled the epicardium layer of cE5.0 heart using AdVSnai1, or AdV-GFP as controls. Analysis after 48 hours showed accumulation of Snai1-overpressing cells inside the myocardium (Fig. 17F), compared to AdV-GFP controls (Fig. 17E), this could be the result of increased invading capacity rather than more transformation, consistent with our previously data. So far, these results show a similar but not identical role of Snai1 during epicardium development compared to endocardial cushion EMT, once again prove the versatility of Snai1 in different molecular environments.

Further in vivo studies are impeded due to the lack of a proper model. However, after testing the available mouse model, in which heterozygous knockdown of Snai1 in epicardium and EPDCs was directed by the transgene WT1creER<sup>T2</sup> following tamoxifen administration, we found that conditional loss of Snai1 resulted in defective ventricle (Fig. 18B), decreased epicardium marker WT1 (Fig. 18C, D) at E18.5, coincident with reduced coronary vasculature (Fig. 5-18E-G). As signs of defective maturation of myocardium, these data suggest that targeting Snai1 in epicardium during early embryonic stages could
compromise the role of EPDC during myocardium development. Together with our previous studies, the Snai1 function during heart development shows a haploinsufficient pattern, and its role varies based on tissue type and developmental stages. This raises the possibility that a transcription factor could show versatile capacity based on the cell, tissue and molecular context.

6.4 Therapeutics and perspectives

The prevalence of valve disease is increasing, yet effective treatments remain limited. The long-term goal is to generate a therapeutic alternative that will replace damaged VECs, VICs, and ECM, as well as adapt to the changing hemodynamic environment in the absence of secondary pathologies, throughout life. As initiation of valve disease is concomitant with damaged endothelia, therapeutic strategies have recently focused on stimulating autologous endothelial cells to replenish injured lesions, and replacing diseased tissue with bioprosthetic valves seeded with healthy endothelial cells. With the emerging concept that valve disease processes recapitulate developmental gene programs, there are opportunities to focus future studies on the potential of VECs to reactivate the EMT program to repair or replace damaged lesions in diseased valves. However, the limited regeneration capacity of mature VECs needs to be considered and solved.

The molecular regulation of endothelial cell function during the EMT process is not limited to the valvulogenesis, and therefore findings have proven applicable to other development systems, such as epicardium. Although there is a lack of reports showing a direct causative or responsive role of the epicardium
in cardiac disease, these specialized mesothelial cells hold great potential as compensatory units during pathological conditions (Zhou et al.). The differentiation of EPDCs into coronary vessels has been extensively studied due to the high mortality caused by CHD and the potential clinical significance of coronary vasculature; meanwhile, the role of cardiac fibroblasts in myocardial homeostasis has been accentuated due to the fact that they are capable of promoting the proliferation of cardiomyocytes besides contributing to the fibrous matrix in the myocardium (Ieda et al., 2009). As a major contributor of both coronary vessels and interstitial fibroblasts, the role of epicardium during development and diseases certainly deserves more attention.
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