A ROLE FOR TIE1 IN LATE-GESTATIONAL SEMILUNAR VALVE DEVELOPMENT

By

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LIST OF ABBREVIATIONS

β-gal	Beta-galactosidase
AFM	Atomic force microscopy
Ang	Angiopoietins
ao	Aorta
av	Aortic valve
AVC	Atrio-ventricular canal
BMP	Bone morphogenetic protein
CHD	Congenital heart disease
DAPI	4',6-diamidino-2-phenylindole
E_	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Endothelial-mesenchymal transformation
EPC	Endothelial progenitor cell
f/f	flox/flox
Flk1	Fetal Liver Kinase1
GAG	Glycosaminoglycans
GSK3β	Glycogen-synthase kinase 3
H&E	Hematoxylin and Eosin
HUVEC	Human umbilical vein endothelial cell
Lv	left ventricle
Lz	LacZ
Mv	Mitral valve
NFAT	Nuclear factor of activated T-cells
NHR	NFAT homology region
PBS	Phosphate buffered saline
PE	Proepicardium
PECAM	Platlet endothelial cell adhesion molecule
PI3K	Phosphatidyl inositol 3-phosphate
OFT	Outflow tract
Pv	Pulmonary valve
qRT-PCR	Quantitative real-time PCR
RHR	REL homology domain
RTK	Receptor Tyrosine Kinase
Tie	Tyrosine kinase with immunoglobulin-like and EGF-like domains
VEC	Valvular endothelial cells
VIC	Valvular interstitial cells
Ys	Yolk sac

CHAPTER 1

INTRODUCTION

Overview

Cardiovascular defects are the most common congenital abnormality in the human population, affecting approximately one out of every 100 live births worldwide (J. Hoffman, 2002). Defects in valve development account for 25-30% of these malformations, therefore being responsible for the majority of morbidity and mortality that occurs in association with abnormal heart development. It is thought that subtle cardiac developmental anomalies as well as some genetic predispositions can be linked to adult-onset heart disease. Despite advances in surgical techniques such as the Ross procedure (replacing the aortic valve with the patient's pulmonary valve) and implantation of artificial valves, valvular insufficiency remains difficult to treat. Most notably, such procedures are limited to short-term resolution of the overlying valve insufficiency. Synthetic valve replacement may be effective in elderly patients, however, use in infants and children are not practical due to their relatively short lifespan (approximately 10 years) and their inability to grow with the child.

Recently, much work has been put forward into engineering an artificial heart valves using biological scaffolds and a patient's own cells (Dohmen PM, 2009). If successful, this holds the most promise for the future of surgical valvular repair. Viable heart valves, unlike synthetic ones, have the potential to grow, remodel and regenerate, thus leading to longer lifespans of the replacement and less complications to the patient

(Chambers JC, 1997). Understanding the specification of cardiac lineages from progenitors to differentiated cells to how these cells coordinate their activity to create a functional valve leaflet is a crucial first step in the development of viable synthetic heart valves.

Much work has gone into identifying genes and signaling pathways associated with early stages of heart valve development and endothelial-mesenchymal transformation (EMT), though later stages of valve development have been more difficult to study due to early embryonic lethality in knockouts of critical gene pathways such as TGFβ, BMP, Flk and others. Our laboratory has developed a Cre transgene driven by the P2 promoter of NFATc1 which is specific for the pro-valvular endothelial cells of the developing heart valve. This tool will allow us to observe later stages of valve remodeling by bypassing the necessity of aforementioned gene pathways in the vasculature and other regions of the heart. We chose to examine a role for Tie1 in the developing valve leaflet based on its expression pattern during later stages of valve development and the evidence for Tie1 function elsewhere in the developing endocardial endothelium (Puri MC *et al.*, 1995; Sato TN *et al.*, 1995). Using the P2Cre, in conjunction with a ^{flox}ed Tie1 allele that has been generated in our laboratory, we hypothesize that Tie1 plays a role in late-gestational valve remodeling that was until now unable to be studied using the original knockout animals.

The focus of this research has been to identify a role for Tie1 during valve development using an *in vivo* mouse model in which Tie1 is efficiently deleted in the valve endocardium and is normally expressed elsewhere in the mouse. Chapter I will describe heart development and outline stages of valve initiation, EMT and remodeling.

It will go on to discuss the Tie family of RTKs and their contribution in heart and vascular development as well as their role in endothelial cell survival and quiescence. Chapter II will outline the spatial-temporal patterns of both Tie1 and the P2Cre in the developing mouse and provide insight into the origin of adult valvular endothelial cells. Chapter III will detail our experimental model and outline the phenotype of the P2cre Tie1$^{flox/lz}$ mouse. Finally, Chapter IV will conclude by discussing potential Tie1 signaling mechanisms and highlight future experiments to further determine Tie1's role in the developing valve leaflet.

Heart Development

In vertebrate embryos, the heart is the first organ to function and is essential for circulation of oxygen and nutrients throughout the body. Early heart defects commonly lead to embryonic demise and it is thought that up to 30% of embryos/fetuses lost prior to birth likely had some type of cardiac malformation (Bruneau, 2008). Cardiac development in the mouse begins at embryonic day 7.5 (E7.5) in the splanchnic layer of the anterior lateral plate mesoderm between the ectoderm and the anterior visceral endoderm (Fig. 1.1a, b). This collection of cells takes on a crescent shape that is well conserved across species and is collectively referred to as the cardiac crescent (Harvey, 2002; Sirvastava, 2006). Heart progenitors move ventrally to form a linear heart tube, which is characterized by an endothelial cell lining and an overlying myocardial epithelium. This linear tube serves as the structural basis for subsequent cardiac morphogenesis (Fig 1.1c). Around E9.5, the linear heart tube begins a complex process termed cardiac looping in which the chambers become defined and the atria and outflow

tract are positioned above the ventricle. Swellings within the outflow tract and atrioventricular canal (AVC) mark the position of the developing valves and aid in unidirectional blood flow through the embryo. By E10.5, activated endocardial cells invade these swellings of the cardiac jelly to become valvular interstitial cells (VICs) that populate the valve leaflet (Barnett JV, 2003; Armstrong EJ, 2004) (Fig 1.1e, f). Through a distinct process of differentiation, proliferation, apoptosis and ECM remodeling, endocardial cushions transform from regional swellings into thin valve leaflets of endothelial cells overlying VICs and an intricate matrix of elastin, collagen, proteoglycans and glycosoaminoglycans (GAGs) (Schroeder JA *et al.*, 2003). Cardiac looping is completed by E12.5 (Fig 1.1g, h) and from this point the heart undergoes remodeling. The remodeling process persists not until birth, but well into the juvenile period. Trabeculae form along the inner surface of the ventricles, the four chambers are divided via septation, and the heart grows along with the embryo at a rapid pace.

The endocardium, myocardium and extra-cardiac lineage cells are not the only players critical for normal heart development. The Proepicardium (PE) and cardiac neural crest cells are also key to formation of a functional heart. The PE originates at the septum transversum and migrates as a sheet over the myocardium at around E10.5 to contribute to the epicardium (Mikawa T, 1996). These epicardial cells then undergo EMT and delaminate into myocardium where they then form myofibroblasts, smooth muscle cells and endothelial cells. Cardiac neural crest cells migrate down from the hindbrain and contribute to the outflow tract, developing semilunar valves, and parasympathetic innervations of the heart. These neural crest cells are also responsible for populating pharyngeal arches 3, 4 and 6 and remodeling these into the aortic arch and

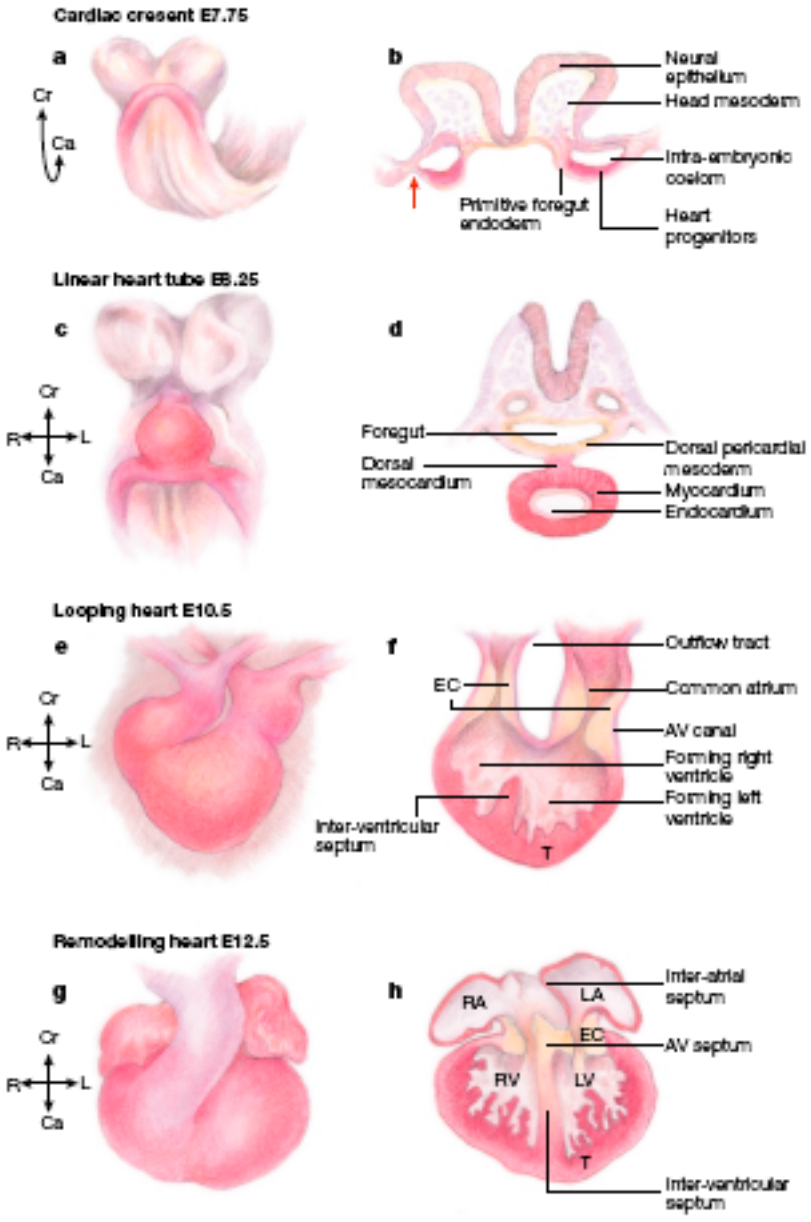


Figure 1.1 ***Developmental events in cardiac formation***. Depiction of the initial formation and remodeling of the murine heart. Adapted from Harvey (2002).

Cranial, Cr; caudal, Ca; endocardial cushion, EC; atrioventricular, AV; right atria, RA; right ventricle, RV; left ventricle, LV; left atria, LA; trabeculae, T.

its branches (Waldo K, 1999). In addition, contributions to the outflow tract aid in septation of the pulmonary and aortic arteries and generate myocardium and smooth muscle in this region (Brown CB, 2006; Red-Horse K, 2010).

Primary and secondary heart fields

Construction of the heart is a complex process which involves the integration of different cell populations at distinct sites at precise points during the developmental process. Two waves of cell migrations are responsible for bringing heart progenitors to the ventral midline where the heart tube will later form. The first wave is associated with gastrulation and is responsible for moving head and heart mesoderm from the node to the cranial region of the embryo where the cardiac crescent then forms (P.P. Tam, 1997). This migration event is regulated by Fgf8, as well as transcription factors *Mesp1* and *Mesp2* (Sun X, 1999; Kitajima S, 2000). The second wave of migration moves cardiac progenitor cells ventrally to form the heart tube (DeHaan, 1963) and relies on an established fibronectin gradient for this movement. Disruptions in fibronectin lead to a complete or partial failure of the cardiac primordial to fuse leading to a condition known as cardiac bifida (George EL, 1997).

During these migrations, heart progenitors cluster in two distinct fields (Fig 1.2), the primary heart field and the secondary heart field. The secondary heart field is positioned dorsally and cranially to the primary heart field. The primary heart field contributes mainly to the left ventricle myocardium as well as parts of the left and right atria. It was previously thought that the endocardium that populates the inner portion of the heart tube originates from the primary heart field (Kirby, 2007). However, more

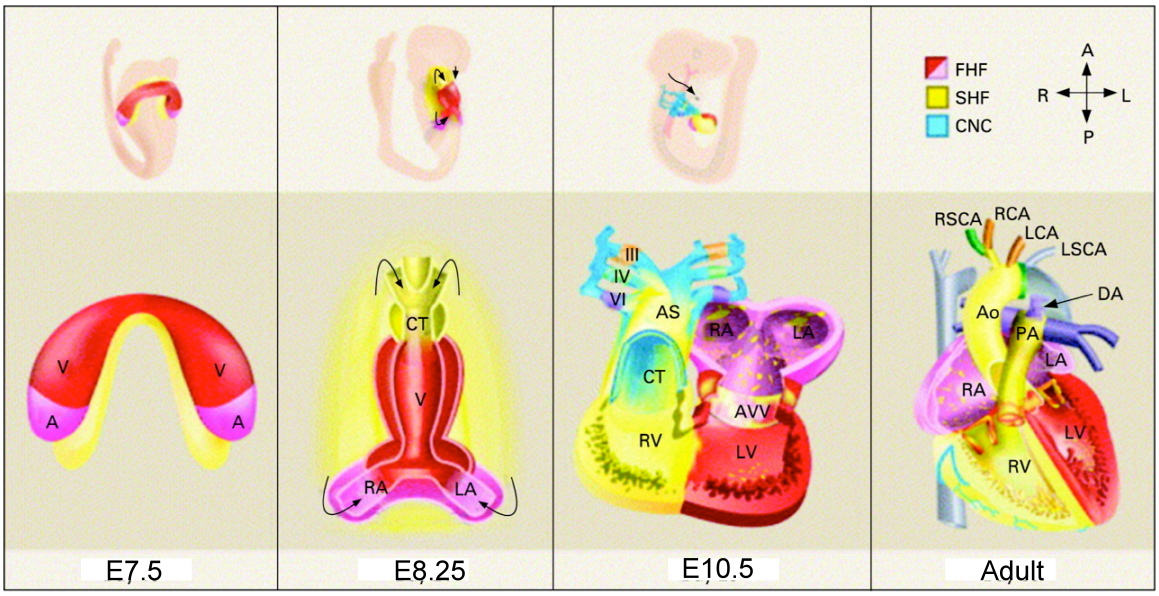


Figure 1. 2 ***Contributions of the primary heart field, secondary heart field and cardiac neural crest to the developing heart***. The first heart field (FHF) (red) contributes to the left ventricle (lv) and parts of the left and right atria (la, ra). The secondary heart field (SHF) (yellow)also contributed to the atria as well as the right ventricle (rv), aorta (ao) and pulmonary artery (pa). Cardiac neural crest (blue) populate regions of the outflow tract and developing valves. Adapted from Sirvastava (2006).

recent work looking at *Nfatc1* as a marker for endocardial endothelium indicate that these cells originate from the secondary heart field (Misfeldt AM, 2009). In addition, the secondary heart field contributes to the myocardium of the right ventricle, pharyngeal arches, and most notably, the outflow tract (Kirby, 2007) (Fig 1.2).

Heart Valve Development

During development of the heart, the linear heart tube consists of an outer layer of myocardium and an inner layer of endocardium that is separated by an acellular layer of ECM commonly referred to as the cardiac jelly (Fig 1.3). By E9.5, swellings of the cardiac jelly can be seen in the outflow tract as well as in the atrio-ventricular canal. Endothelial cells lining these swellings become activated, downregulate endothelial markers such as *Pecam-1,* and *VE-cadherin* and activate mesenchymal markers such as *Snail1* and *Twist*. These cells then migrate into the cardiac jelly in a process known as endothelial-mesenchymal transformation (EMT) to form the primordial for both the septa and the valves. BMPs and TGF-βs are the inductive signals for the onset of EMT, though endocardial Notch signaling must be activated in order for those cells to respond to those signals (Timmerman LA, 2004). It is known that cellular movements during EMT are regulated in part by ECM secretions, though this process is poorly understood. All mesenchymal cells of the mitral and tricuspid valves, as well as the proximal cells of the semilunar valves are derived from endocardial cells that underwent EMT (Kisanuki YY, 2001).

Disruptions or complete failure to undergo EMT can result in a myriad of developmental valve defects. Inactivation of Has-2, versican, BMPs, Nfatc1 and many others results in a complete absence of valve formation (Yamamura, 1997; Ranger AM *et*

al., 1998; Camenisch TD, 2000), whereas mutations in HB-EGF (Jackson LF *et al.*, 2003), Smad-6 (Galvin KM, 2000) and Neurofibromin1 (Brannan CI, 1994) result in hyperproliferative valve leaflets and subsequent hyperplastic valves.

While mechanisms regulating EMT are well understood, post-EMT differentiation and remodeling of the cushions into thin, mature valve leaflets (Fig 1.3) with organized and stratified matrix components has been largely under studied. It is thought that following EMT, a wave of proliferation and apoptosis on the ventricular and arterial side of the semilunar valves, respectively, carve out the valve cups (Fig 1.3) and provide the location where the coronary vessels later invade. Following this stage, the valves begin remodeling from regional swellings into thin leaflets. The ductus arteriosis closes around birth which causes a dramatic increase in hemodynamic pressure across the aorta. The heart and valves themselves undergo a rapid growth period after birth. In humans, the heart triples in size in the first year of life and continues to grow and remodel for several years. Previously mentioned efforts to surgically replace defective valve leaflets in infants and children have been minimally successful due to this rapid growth and expansion of the heart and valve tissue. Synthetic valve replacements must be of a precise size to function properly *in vivo* and any amount of growth in the patient can lead to cardiac regurgitation or outright failure of the implant.

As noted, mutations in many of the genes involved in early cardiovascular development are lethal prior to this stage of valve remodeling. FGF and TGF-β are involved in post-EMT valve remodeling by promoting VIC proliferation (Zhao Z, 2000; Goumans MJ, 2002). VEGF is also proposed to be involved in valve remodeling by inducing endocardial proliferation through NFATc1 (Dor Y, 2001).

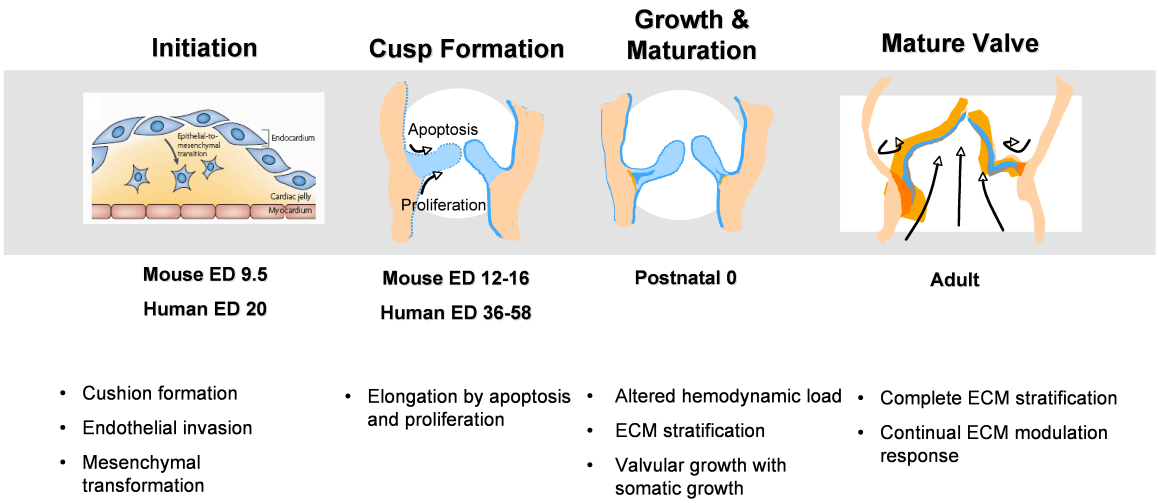


Figure 1.3 ***Schematic of heart valve development***. The three stages of valve development leading up to the mature heart valve; initiation, cusp formation and growth and remodeling and the corresponding developmental day in mouse and human.

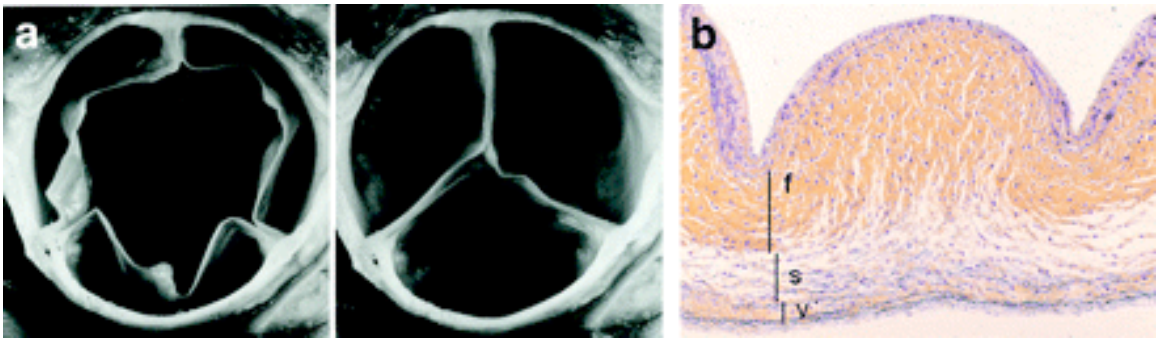


Figure 1.4. (**A**) Semilunar valves during systole (left) and diastole (right). (**B**) Mature valve leaflets segregate into trilaminar structures. The ventricularis (v) is the caudal-most region which contains high amounts of elastin. The spongiosa (s) layer lies in the middle and is composed of proteoglycans and glycosoaminoglycans. The fib (f) layer lies atop the spongiosa and contains largely collagens (yellow). Adapted from Schoen 2008.

The ECM is set up during EMT and plays a critical role in later stages of valve remodeling. ECM provides support for mechanical function, promotes endothelial cell invasion into the mesenchyme, and serves as a scaffold for cell migrations (Camenisch TD, 2000; Schroeder JA *et al.*, 2003). During EMT, the main component of the matrix is hyaluronan. During valve remodeling, however, the matrix becomes stratified into a trilaminar structure composed mainly of collagen, elastin and proteoglycans (Fig 1.4 b). This trilaminar stratification is essential for normal mechanical properties and function of the valve leaflets. Elastin fibers in the ventricularis, the caudal most region of the semilunar valve, provide support and elasticity to the valve to extend during systole and to recoil when the valve closes in diastole (Schoen, 2008) (Fig 1.4 a). The spongiosa layer of the valve sits above the ventricularis and is composed of proteoglycans and glycosoaminoglycans. This layer absorbs the contractile forces of the leaflets and coordinates movements between the ventricularis and the overlying fib layer (Schoen, 2008). Collagen is the major structural component of the fib layer (Fig 1.4 b). Collagens type I, III and V provide the majority of stiffness and strength to the leaflet (W G Cole, 1984). Early in development, proteoglycans and glycosoaminoglycans compose the majority of the ECM in the valve leaflet. During remodeling and throughout adulthood, collagen becomes more abundant while proteoglycan and glycosoaminoglycan content decreases (Aikawa *et al.*, 2006; Chakraborty S, 2008; Peacock JD, 2008).

Altered expression and distribution patterns of valvular ECM proteins are not surprisingly associated with developmental valve abnormalities. Periostin knockouts, for example, lead to truncated heart valves, ectopic cardiomyocytes and smooth muscle cells,

perinatal lethality, and disorganized ECM stratification resulting in functional deficiencies (Norris RA, 2008). A total loss of elastin in the mouse embryo results in embryonic death approximately mid-gestation, but heterozygous mutants survive long enough to display aortic valve insufficiency (Li DY, 1998). Mutations in *fibrilin-1* are associated with Marfan syndrome in humans, which leads to aortic valve abnormalities. Likewise, mice lacking the associated protein fibulin-4 also exhibit aortic valve anomalies including thickened valves and aortic calcification (Dietz HC, 1991; Hanada K, 2007). In addition, mutations in numerous collagens including collagen 1, 2, 3, 5 and 11 all correlate with various human disorders. Deletions of these genes in the mouse all result in embryonic lethality and display varied cardiovascular abnormalities including aortic valve insufficiency (Kuivaniemi H, 1997; Lincoln J, 2006).

The Origin of Valvular Endothelial and Interstitial Cells

While much work has been done examining cardiomyocyte specification and differentiation, much less is known regarding the origin of the endothelial cells that line the inside of the developing heart and valve structures. The endocardial endothelium is known to arise from mesodermal precursors early in development, but markers to distinguish endocardium from other endothelium have, until recently, been lacking (Misfeldt AM, 2009). Our laboratory has identified NFATc1 as the only gene expressed in early endocardial cells and not other vascular populations (Zhou B *et al.*, 2005). Chapter II will discuss NFATc1 in more detail.

In the adult animal, much controversy exists over the origin of VICs and the overlying endothelial cells. Several studies have provided evidence that VICs are

replaced by circulating endothelial or mesenchymal precursors derived from the bone marrow. Visconti et al. inserted a green fluorescent marker driven by a ubiquitous promoter into hematopoietic stem cells and implanted them into lethally irradiated mice. Upon dissection, it was found that green fluorescent cells were present in the valve interstitium and analysis of those cells demonstrated a function similar to that of VICs (Visconti RP, 2006). What is interesting to note is that no study has provided direct evidence of circulating hematopoietic progenitor cells contributing to the endothelial lining of the valve leaflets. Using NFATc1 as a marker of the valve endocardium, we will address the lineage of valvular endothelial cells in Chapter II.

The Tie Family of Receptor Tyrosine Kinases

The Tie family of receptor tyrosine kinases (RTKs) were first described in 1992 (Partanen J *et al.*, 1992) and is comprised of two members; Tie1 and Tie2. Tie1 and Tie1 are selectively localized to endothelial cells and cells of the hematopoietic lineage (Dumont DJ *et al.*, 1992; Partanen J *et al.*, 1992; Shahrara S, 2002; Puri MC, 2003). Tie1 and Tie2 share a high degree of structural and sequence homology, though the angiopoietin (Ang) ligands have been found to associate with Tie2 and not Tie1. Genetic deletions of both Tie1 and Tie2 in the mouse have identified roles in early cardiovascular development (Puri MC *et al.*, 1995; Sato TN *et al.*, 1995; Puri MC *et al.*, 1999). While Ang 1-4 have been shown to regulate Tie2 signaling, Tie1 signaling mechanism, ligands and downstream targets remain unknown.

Deletions of Tie1 in the embryo result in severe edema, hemorrhage, a breakdown of the microvasculature and ultimate demise after E14.5 (Puri MC *et al.*, 1995; Sato TN

et al., 1995; Qu X, 2010) (Fig. 1.5). Tie1-/- embryos develop a normal vascular plexus, suggesting that Tie1 is not essential in setting up the vascular network. After E13.5, however, the integrity of the vascular plexus is lost, indicating that Tie1 is essential for maintenance, survival and quiescence of the endothelium (Puri MC *et al.*, 1995; Sato TN *et al.*, 1995; Qu X, 2010). Tie2 knockout mice die around E10.5 due to a lack of vascular remodeling, absent endothelial cell tight junctions, and an overall lack of vascular integrity (Dumont DJ *et al.*, 1992). Double knockouts of both Tie1 and Tie2 are also embryonic lethal early in development due to severe cardiovascular abnormalities. In addition, endothelial cells lacking both Tie1 and Tie2 do not contribute to the mature vasculature (Puri MC *et al.*, 1999).

Tie1 expression in the mouse embryo is turned on around E8.5 following activation of Flk-1, Flt-1 and Tie2, thus being the last RTK to be activated during vascular development. In the early embryo, Tie1 expression can be visualized in the yolk sac and developing vascular plexus. By E12.5, Tie1 is robustly expressed in the lung, vasculature, heart, liver, brain and kidneys. In the adult animal, low levels of expression remain in the heart and branch points of the aorta (Woo KV, 2011), although expression is lost in other organs.

The Tie1 and Tie2 receptors have remarkably similar structure. The extracellular region of each receptor has two immunoglobulin (Ig) domains, followed by three EGF repeats, an additional Ig domain, and three fibronectin type III repeats. The intracellular portion contains the kinase domain (Partanen J *et al.*, 1992; Macdonald PR, 2006) (Fig 1.6). The sequence homology of the intracellular domain is quite high, approximately 80%, however the extracellular domain is much more diverse. The highest amino acid

conservation is in the EFG repeats (50-55%), an area essential for Ang ligand binding (Fig 1.6 a). The homology rates in the EGF repeat region may explain why Ang 1-4 are selective for Tie2 but not Tie1 (Macdonald PR, 2006).

Endothelial Specific Receptor Tyrosine Kinases in Valve Development

Tie1 and Tie2 are not the only endothelial specific RTKs expressed during cardiovascular development. NFAT has been shown to be a negative regulator of VEGF, thus promoting the onset of EMT in valve development and Flk-1 (VEGFR-2) has been shown to induce translocation of NFATc1 to the nucleus (Johnson EN, 2003). Flk-1 null mice lack mature endothelial cells and die around E8.5-9.5 (Shalaby F, 1995). A lack of endothelial cells limits EMT, which would lead to subsequent valvular defects should these embryos live past E9.5. Flt-1 (VEGFR-1) is highly expressed in the endocardial endothelium prior to EMT and subsides following EMT initiation. While Flt-1 mice do possess mature endothelial cells, blood vessels are poorly organized therefore ultimately leading to embryonic demise at E8 (Fong GH, 1995).

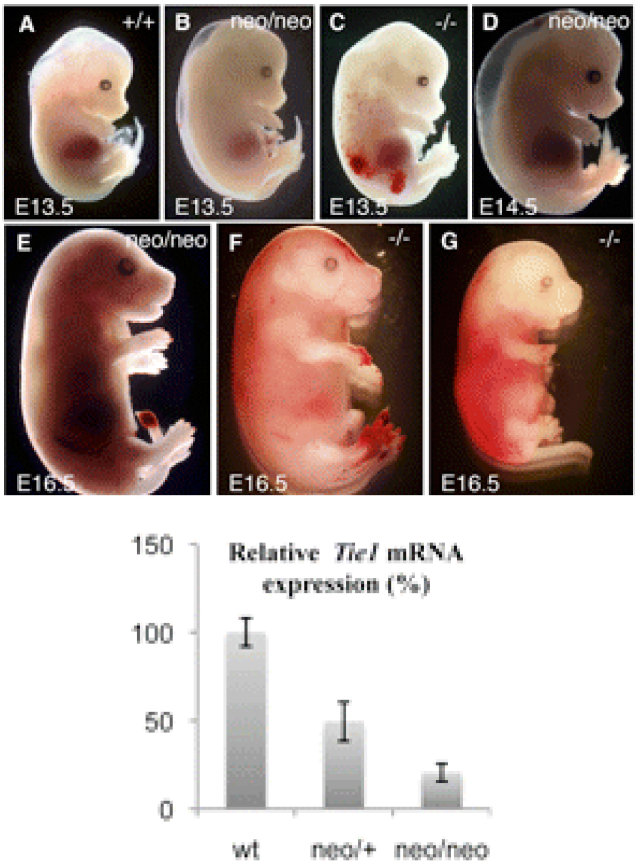


Figure 1.5. ***Loss of Tie1 leads to early embryonic lethality***. Deletion of Tie1 leads to a loss of vascular integrity in the mouse embryo resulting in edema and embryonic death by E16.5 (**A-G)**. (**H**) Insertion of a neomycin cassette into the Tie1 locus significantly reduces Tie1 expression. Figure adapted from Qu et al 2010.

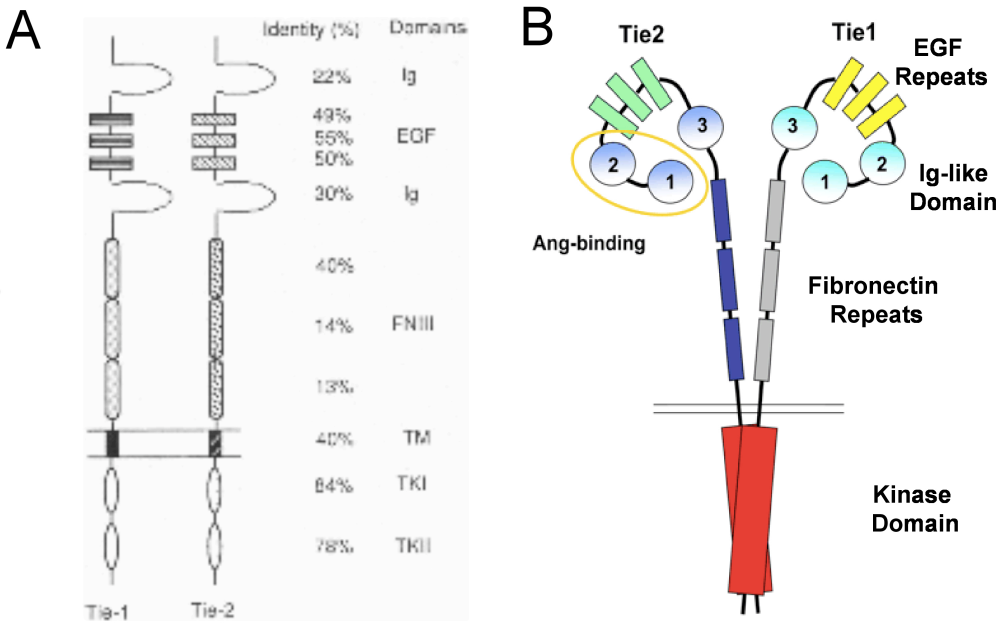


Figure 1.6. ***Tie1 and Tie2 receptor structure***. (**A**) Early model of Tie1 and Tie2 receptor structure with domain homology comparisons. (**B**) Latest model of Tie1 and Tie2 receptor structure. Note the recent identification of a third Ig-like domain. These receptors can homo- or heterodimerize with each other at the intracellular kinase domain. Interactions between the extracellular regions has not been reported. Adapted from Sato et al (1993) and Macdonald et al (2006).

Immunoglobulin-like, Ig; epidermal growth factor, EGF; Angiopoietin, Ang; Transmembrane, TM; Fibronectin Type III, FNIII; Tyrosine kinase, TK.

Mechanisms of Tie1 Signaling

Although there is a high degree of structural and sequence homology between Tie1 and Tie2, a ligand for Tie1 has yet to be identified. This has made efforts to elucidate the signaling mechanisms of Tie1 quite difficult.

Evidence has shown that Ang1 and Ang2 can activate the Tie1 receptor in some instances, although no direct receptor-ligand interaction has been identified *in vivo* (Davis S *et al.*, 1996; Maisonpierre PC *et al.*, 1997; Valenzuela DM *et al.*, 1999) (Fig 1.7). To gain insight into Tie1 signaling, two groups utilized a chimeric receptor approach (Marron MB *et al.*, 2000a; Kontos CD *et al.*, 2002). Marron et al. generated a fusion of the TrkA receptor tyrosine kinase ectodomain to the intracellular and transmembrane domains of Tie1. Activation of TrkA via NGF signaling did not result in Tie1 phosphorylation. While this approach was unsuccessful, the same group identified an association between Tie1 and Shp2 (Marron MB *et al.*, 2000b). Shp2 is a protein tyrosine phosphatase that plays a regulatory role in cell signaling, migration and transcription regulation. Mutations in Shp2 have been implicated in Noonan's Syndrome. Data from this latter paper suggests that the phosphorylated state of Tie1 may be transient, therefore not detected in previous studies.

A later paper by Kontos et al used a similar approach to examine the signaling mechanism and function of Tie1. This group used a cell line that stably expressed csf-Tie1 (colony stimulating factor receptor-Tie1 fusion) to show that Tie1 can auto-phosphorylate in the absence of Tie2 (Kontos CD *et al.*, 2002). This paper also demonstrates that *in vitro* the Tie1 endodomain can associate with the p85 subunit of

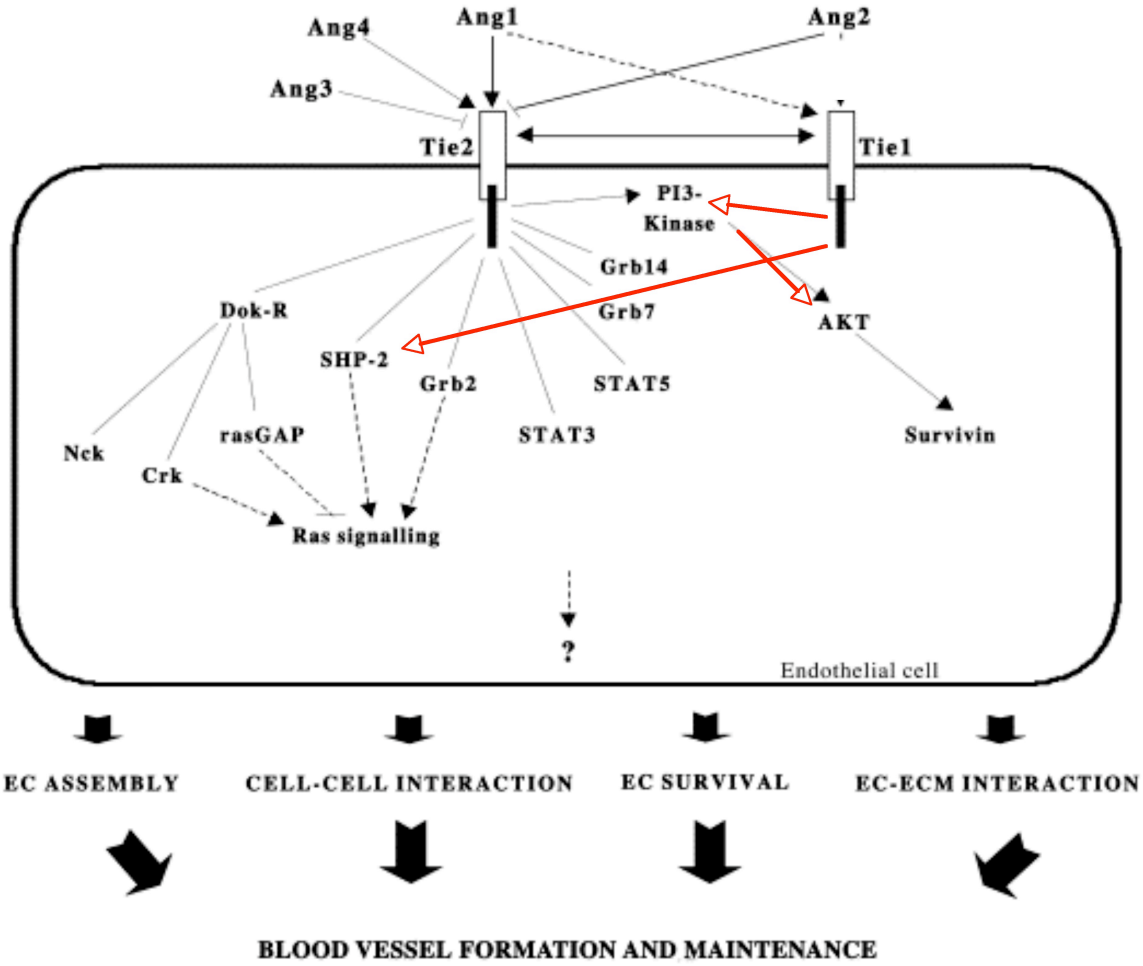


Figure 1.7. ***Tie1 and Tie2 signaling pathways***. Summary of Tie1 and Tie2 signaling. Many more signaling pathways have been identified downstream of Tie2 as compared to Tie1, though more recent work has identified SHP-2 and PI3K/AKT as targets of Tie1 signaling. Adapted from Loughna and Sato (2001).

PI3K subsequently causes phosphorylation of Akt, thereby inhibiting endothelial cell apoptosis (Fig. 1.7).

This study was the first to suggest that Tie1 can homodimerize and may autophosphorylate, therefore being able to signal in the absence of Tie2. Taken together, Tie1 may play a role in signaling independent of Tie2, even though their signaling pathways may overlap.

A more recent paper sought to examine the interactions between Tie1 and Tie2 *in vitro* (Kim KL, 2006). This group studied expression levels of both Tie1 and Tie2 in endothelial progenitor cells (EPCs) and in HUVECs and found similar levels of expression in each cell type. Tie1 was found to associate with Tie2 in the mature cell type (HUVECs) but not in EPCs, suggesting that Tie1s role in development is context-dependent. In addition, Tie1-Tie2 heterocomplexes inhibit Ang2 from binding to and activating Tie2 signaling in HUVECs. More work needs to be done in this area to determine if similar events take place *in vivo*.

Tie1 is not only functional during development. Recent studies have implicated a role for Tie1 in inflammatory diseases such as rheumatoid arthritis, osteoarthritis and atherosclerosis (Shahrara S, 2002; Porat RM, 2004; Woo KV, 2011). Tie2 activation via Ang1 promotes endothelial cell survival and integrity (Kwak HJ, 2000; Papapetropoulos A, 2000), therefore Tie1 may be able to activate endothelial cells by inhibition of Ang1-mediated Tie2 activation. The overexpression of Tie1 in HUVECs increases expression of the inflammatory markers VCAM-1, ICAM-1 and E-selectin (Aikawa *et al.*, 2006; Chan B, 2008). Likewise, a reduction of Tie1 expression in HUVECs results in a

decrease in TLR2, IL-1β, VCAM-1, ICAM-1 and E-selectin, though Tie2, eNOS and TGF-β were unperturbed (Chan B, 2009). Taken together, these data suggest that Tie1 may play a role in promoting inflammation as well as endothelial cell activation during development and disease.

In summary, the Tie family of RTKs is essential for the maintenance of endothelial and vascular integrity in the developing embryo. Global knockouts of either receptor result in embryonic lethality, and double knockouts compound this phenotype suggesting that interactions exist between Tie1 and Tie2, and that these interactions may be context dependant. Although Tie1 and Tie2 share much homology in the intracellular region, their extracellular regions are quite different from each other. Since the extracellular region is critical for ligand binding, the lower homology rates between Tie1 and Tie2 is likely why the Ang ligands have been found to interact with Tie2 but not Tie1. With the exception of the original knockout papers and a few recent manuscripts, the majority of the information on Tie1 has been ascertained from *in vitro* experiments. To effectively determine a role for Tie1 we must be able to make conclusions on what actually happens in an *in vivo* model.

Aims of this Dissertation

Global knockouts of Tie1 result in embryonic demise due to a breakdown in the integrity of the microvasculature. Because of the high expression levels of Tie1 in the valve, we hypothesize that Tie1 may have a role in valve development that was previously undetermined due to the vascular phenotype and early demise. To test this hypothesis we propose to:

Aim 1. *Define the expression pattern of Tie1 in the developing valve leaflet and determine the spatio-temporal pattern of the valvular endothelial specific NFATc1-P2Cre*. To this point, evaluation of endothelial specific genes in late stages of valve remodeling has been unattainable due to the requirement of these genes in the vasculature. The NFATc1-P2Cre is specific for the developing pro-valve endocardium, and provides a tool to bypass the requirement of essential genes in vascular development. Tie1 promoter-LacZ reporter mice were dissected and stained with X-gal to determine the expression pattern of Tie1 in the developing valve leaflets. Likewise, we examined the spatio-temporal patterning of the NFAT-c1 P2Cre using a R26R reporter and real time PCR. We have determined that endothelial cells of the embryonic valve leaflet go on to contribute to the endothelium of the mature adult valve.

Aim 2. *Define the effect of Tie1 deletion in the developing valve endocardium*. Using a conditional Tie1 ^{flox}ed allele, the Tie1 LacZ knockin, and the NFATc1-P2Cre, we will generate mice that are deficient for Tie1 in the valve endocardium (P2Cre Tie1$^{flox/lz}$). Semilunar and atrio-ventricular valves of these animals will be compared to Tie1$^{flox/flox}$ littermates that are equivalent to wild-type animals. We have determined that P2Cre Tie1$^{flox/lz}$ aortic valves are significantly larger than Tie1$^{flox/flox}$ valves.

Aim 3. *Determine if interactions between Tie1 and Tie2 exist in the developing valve leaflet.* The signaling mechanisms and downstream targets of Tie1 are mostly unknown, and no information on Tie1 signaling *in vivo* has been determined. To elucidate Tie1 signaling mechanisms, we have isolate valve leaflets from P0 P2Cre Tie1$^{flox/lz}$ and Tie1$^{flox/flox}$ animals and use Western blotting to determine if a loss of Tie1 has any effect on the phosphorylation of Tie2 in this context. We will also construct a Tie2 ^{flox}ed allele

to determine potential genetic interactions between Tie1 and Tie2 in the developing valve. RT-PCR and RNA sequencing will be used to determine the downstream signaling targets of Tie1.

CHAPTER II

SPATIAL AND TEMPORAL PATTERNING OF TIE1 AND NFATc1 P2CRE DURING HEART DEVELOPMENT

Introduction

Animal models to assess gene function during late gestational heart development have been largely unavailable since most gene knockouts that affect heart development result in embryonic lethality before valve remodeling takes place. Our laboratory has developed a Cre transgenic line using the P2 promoter of NFATc1 that is specific for the pro-valve endocardium (Zhou B *et al.*, 2005). In conjunction with a conditional ^{flox}ed allele, we are able to bypass the requirement for essential genes in the developing embryo to examine their function specifically in the endothelial cells lining the valve. In addition, the Cre marks a specific subset of valvular endothelial cells that do not transform into mesenchyme, therefore lineage tracing via R26R does not yield any Cre positive cells in the valve interstitium.

The Nuclear Factor of Activated T-cells (NFAT) family of transcription factors consists of five members: NFATc1, NFATc2, NFATc3, NFATc4 and NFATc5. NFAT is found not only in T-cells but in various other tissues including the developing heart (Rao A, 1997; Crabtree GR, 2002; Hogan PG, 2003; Macian, 2005). The structure of the NFAT family members is highly conserved (Fig 2.1 a). Each has an NFAT homology domain (NHR), which has numerous serine residues to regulate activity via phosphorylation and a transactivation domain with binding sites for calcineurin, NFAT

kinases and GSK3β. Each NFAT family member also has a REL homology region (RHR), which consists of a DNA binding domain with homology to the REL family of transcription factors (also known as the NF-κB family of transcription factors). Calcium signaling regulates the transcriptional activity of NFATc1-4 (Fig 2.1 b). When calcium waves are released, formation of calmodulin and calcineurin phosphatase complexes are induced that in turn dephosphorylate NFATc. NFATc then translocates into the nucleus where it activates transcription of downstream targets.

During heart development, NFATc1 is expressed in endocardial precursors in the linear heart tube. By E10.5, expression is restricted to the endocardium of the AVC and OFT valves. NFATc1 is no longer expressed in the heart after E12.5. Deletions of either the RHR or NHR result in an absence of valve leaflets. Promoter analysis of NFATc1 in our laboratory identified a 250bp region just upstream of the P2 promoter element that was responsible for autoregulation of *NFATc1* (Zhou B et al., 2005). This element was able to drive lacZ reporter gene expression in the pro-valve endocardium at E9.5 through E13.5. No reporter gene expression was noted in other regions of the endocardium. Therefore, this region was selected to develop a highly region specific and tissue specific mouse line; the NFATc1 P2 Cre.

Tie1 has been shown to be expressed throughout the endothelium of the developing embryo after E9.5 (Partanen J *et al.*, 1992). However, a full detailed timeline of expression in the heart has not yet been described. Tie1 expression is high in the embryo prior to E14.5 then is reduced in most regions of the heart and vasculature. We show that Tie1 is expressed specifically on the arterial side of the semilunar valve leaflet in the adult, as well as branch points along the aorta where turbulent flow is present.

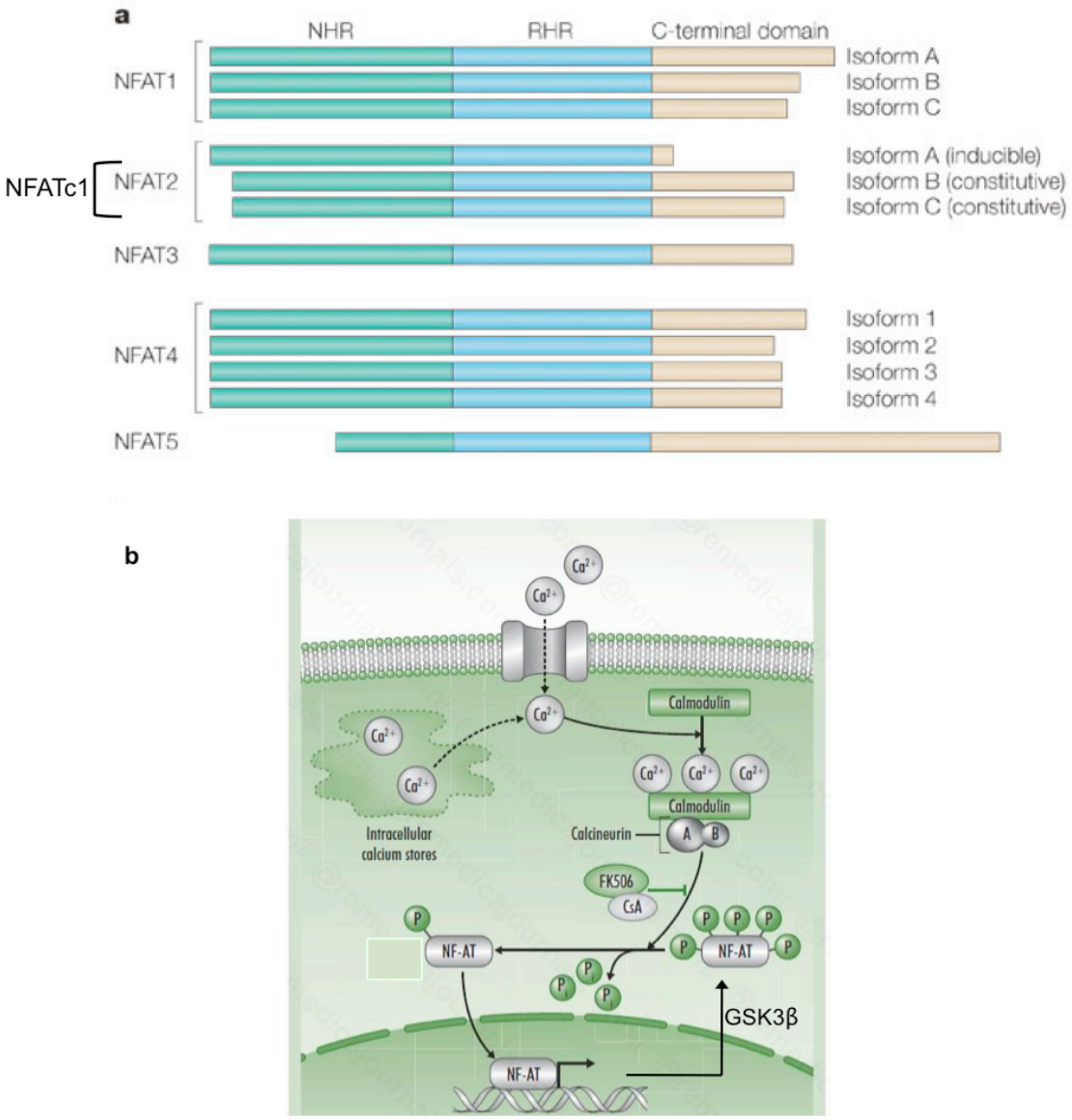


Figure 2.1. ***Structure and signaling of NFAT transcription factors***. NFAT family members (a). Adapted from Macian et al 2005. When intracellular calcium increases, (b) the calcium-calmodulin-calcineurin complex dephosphorylates NFAT. This leads to nuclear localization and activation of downstream transcriptional regulation. Adapted from Steinbach et al 2007.

Regions of the heart and vasculature under laminar sheer stress express little to no Tie1 in the adult mouse.

Experimental Procedures

Genotyping

Tail samples were obtained from three week old mice and digested in a buffer containing 100 mM Tris pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCl and 100 μg/ml Proteinase K overnight at 55°C. Tail DNA was then genotyped by PCR using *Taq* polymerase (Roche) or REDExtract-N-Amp PCR Reaction Mix (Sigma) using the following primers:

Tie1-lz: 5' – TGC CCC CCC TTC CAG AGA CTT CC – 3', 5' – GCA AAG AGG ATC CCC ACC AGA CCA TACT -3' and 5' – GGG GAT GTG CTG CAA GGC GAT TAA G – 3'

Cre: 5' - TCC GGG CTG CCA CGA CCA A -3' and 5' – GGC GCG GCA ACA CCA TTT TT -3'

R26R: 5' – GCG AAG AGT TTG TCC TCA ACC -3', 5' - GGA GCG GGA GAA ATG GAT ATG – 3' and 5' – AAA GTC GCT CTG AGT TGT TAT -3'

Animal Breeding

Tie1 lz/+ mice (a kind gift from Dr. Mira Puri, University of Toronto) were bred to WT ICR mice in our animal facility. Likewise, R26R mice were backcrossed to each other to maintain homozygosity. All animals were maintained in microisolator cages and

were fed normal rodent chow (Purina Mills). All experimental procedures were performed according to Vanderbilt University's Institutional Animal Care and Use Committee.

Detection of β-galactosidase

Tissue was collected in PBS and fixed overnight at 4°C in 4% PFA/PBS. Fixed tissue was then washed in a solution containing 2 mM MgCl, 5 mM EGTA, 0.01% deoxycholate, 0.02% NP-40, and 0.1 M phosphate buffer. The tissue was then stained in 1 mg/ml X-gal, 5 mM potassium ferro/ferricyanate, 2 mM MgCl, 0.01% deoxycholate, 0.02% NP-40, overnight at 30°C. Stained tissue was then post fixed in 4% PFA/PBS for up to eight hours, washed and cleared in a glycerol gradient and photographed with a dissection photomicroscope. Once photographed, samples were again washed, dehydrated in a graded ethanol series, and embedded in paraffin. Paraffin blocks were sectioned at 6-7μm on a microtome. Slides were counterstained with Eosin and mounted with Permount.

qRT-PCR

All qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on the iQ5 Real Time PCR Detection System (BioRad). Assays were performed in triplicate using glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) as the normalization control. Relative expression was calculated as $\Delta^{2\text{-ct}}$ (Schmittgen, 2008). Primers used are as follows:

GAPDH: 5' – CAC TGG CAT GGC CTT CCG TG -3'and 5'- AGG AAA TGA GCT TGA CAA AG -3'

Cre: 5'- TGC AAC GAG TGA TGA GGT TC -3'and 5' –GCA AAC GGA CAG AAG CAT TT -3'

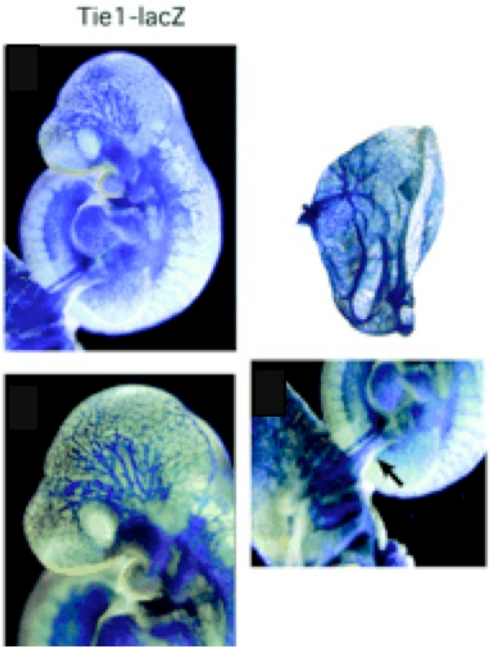
Heart tissues from E9.5, E12.5 and E14.5 were collected from P2Cre mice and yolk sacs were collected as control tissue. The cranial half of the P0 heart was collected and in the absence of P0 yolk sacs, the lung was collected as the negative control. RNA was isolated with a standard Trizol preparation (Invitrogen). For statistical purposes, E9.5 yolk sac was set to a value of 1 and all fold changes shown are set relative to that value.

Results

Tie1 Expression is Localized to the Endothelium of the Developing Embryo

At E8.5, Tie1 is the last RTK to be turned on during vascular development. Tie1 is localized to the developing endothelium and can be seen early in the developing yolk sac vasculature and the great vessels of the embryo. By E9.5, Tie1 is robustly expressed in all the embryonic vasculature (Fig 2.2 A). By E12.5, Tie1 expression is also seen in the developing lung, brain and heart, including the endothelium of the ventricles, atria, and valve leaflets (Fig 2.2 B). High levels of Tie1 expression persist through the postnatal period then begin to taper off in older animals. In the adult mouse, Tie1 expression is localized to regions of the cardiovascular system with high levels of turbulent flow in areas of resistance. These regions include the arterial side of the semilunar valves and branch points of the aorta. Laminar flow has been shown to downregulate Tie1 expression (Chen-Konak L, 2003), which explains why Tie1 is not expressed on the ventricular side of the valve leaflets nor in straight regions of the aorta.

A



B

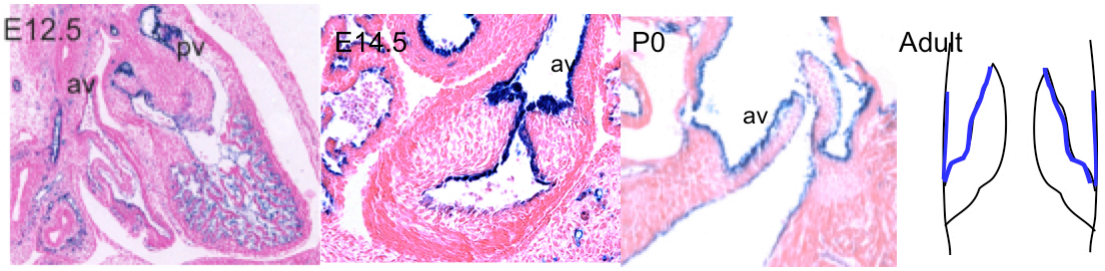


Figure 2.2. ***Tie1 expression throughout development.*** (**A**) Tie1 is expressed throughout the embryonic endothelium at E9.5 including the umbilical vein (arrow) and the yolk sac. Adapted from Uyttendaele et al 2001. (**B**) Using the Tie1 LacZ (Tie1 lz) knockin (Dr. Mira Puri), we are able to visualize Tie1 expression throughout the developing heart and valve leaflets. Tie1 is first expressed around E8.5 in the developing vasculature. By E12.5, high expression levels are seen in the lung, brain and cardiovascular system. Expression after P0 diminishes, but are seen on the arterial side of the valve leaflets and branch points of the aorta in the adult.

Spatio-temporal patterning of the NFATc1 P2Cre

When the P2Cre transgenic line was first generated, it was communicated that the Cre was only expressed for a short window during development; sometime between E9.0 and E12.5. There was no definitive evidence, however, to support this timeline. Here we are the first to document the timeline of the P2Cre. Quantitative Real Time PCR data shows that the P2Cre is expressed by E9.5 in isolated heart tissue. Expression continues through E12.5 and E14.5 when it is then turned off (Fig 2.4). No Cre expression is noted after E14.5. As expected, no Cre expression was noted in yolk sac or lung at any stage examined.

We crossed P2Cre animals with R26R reporter mice to determine the spatial patterning of the P2Cre. R26R stains robustly in the pro-valve endocardium at E12.5 (Fig. 2.3) but can be seen as early as E9.0 in the common AVC and OFT. The R26R reporter also labels valve endocardial cells in P0 and adult animals. This result was unexpected even though we have shown that the Cre is not being expressed at these later time points. This indicates that the cells of the adult valve endocardium originated from early embryonic endocardial cells and are not replaced by circulating endothelial progenitor cells or other methods as previously thought.

Discussion

Few *in vivo* studies have focused on Tie1 other than the original knockout papers (Puri MC *et al.*, 1995; Sato TN *et al.*, 1995), in part because no ligand has been identified for Tie1 but also the early lethality makes studying later developmental events impossible. Using the P2Cre, we now have a tool to study the effects of conditionally

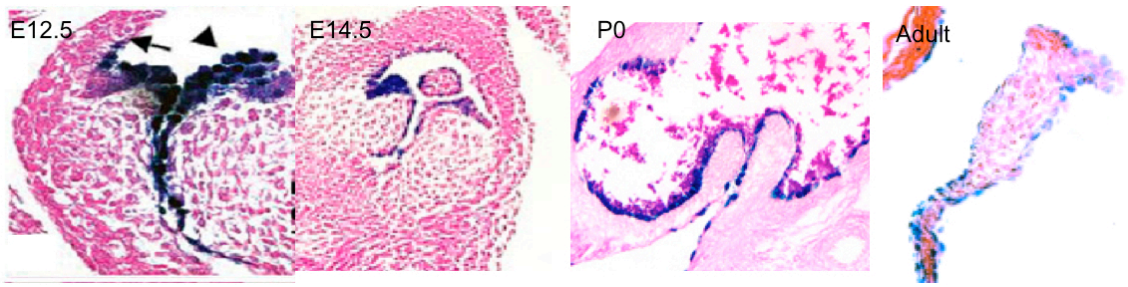


Figure 2.3. ***P2Cre R26R expression.*** The P2Cre is active from E8.5 through E14.5 in the pro-valve endocardium. Expression of the Cre is restricted to the endothelium of the developing valves (arrowhead) and not the endothelium of the OFT (arrow). After the Cre is turned off, R26R expression remains in the majority of the valvular endothelial cells through adulthood.

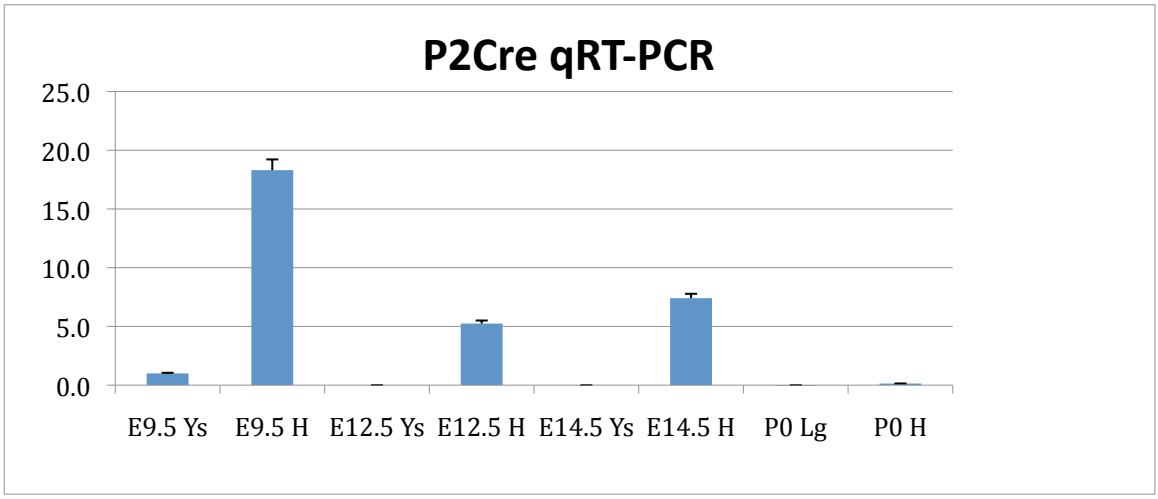


Figure 2. 4 ***P2Cre qRT-PCR.*** P2Cre expression was measured by quantitative Real Time PCR. Expression levels were normalized to E9.5 yolk sac.

Yolk sac, Ys; heart, H; lung, Lg.

deleted genes in the developing heart valves. We can therefore bypass the requirement for Tie1 in the developing vasculature and keep these embryos alive so that we can study the role of Tie1 in late-gestational valve development and remodeling.

We first examined the expression pattern of Tie1 throughout embryonic development to be certain that Tie1 is expressed in the developing valve leaflet at the time that the Cre is active. Tie1 expression turns on at approximately E8.5, within hours of P2Cre activation. We also determined that Tie1 expression in the valve persists into adulthood, indicating that it may play a role in maintenance of the valve endothelium.

We provide the first definitive timeline for the P2Cre during development. The P2Cre is expressed by E9.0 and remains active until E14.5. More importantly, using an R26R reporter we have shown that cells once expressing Cre (or descendents thereof) make up the vast majority of the valve endothelium well into adulthood. This indicates that deletion of Tie1 in between E9.0 and E14.5 likely is sufficient to eliminate all Tie1 expression in the valve leaflet for the life of the animal, as the valvular endothelial cells are not being replaced. We can conclude from these data that the endothelial cells in the valve leaflet at E9.5 are the same cells as those in the P0 animal and the same as those cells in the adult mouse. The cells of the valve endocardium are not replaced by circulating EPCs or by cells from any other source. This settles a long debate of the origin of adult valvular endothelial cells in the field of cardiovascular development.

CHAPTER III

TIE1 IS A REGULATOR OF LATE GESTATIONAL SEMILUNAR VALVE REMODELING

Introduction

Chapter II outlined the spatio-temporal pattern of Tie1 expression in the developing embryo. Expression of Tie1 appears in the cardiac cushions by E9.5 and persists through the neonatal period. Adult Tie1 expression is restricted to the arterial side of the valve leaflet. We hypothesize that this localized expression of Tie1 aids in valve remodeling by sending information to the VICs and the ECM to properly stratify the matrix during late gestational remodeling. Since Tie1 global knockouts die around E14.5, examining a role for Tie1 in later stages of valve development *in vivo* has been impossible. Thus, the majority of literature on Tie1 comes from *in vitro* studies. Tie1 *in vitro* appears to play a role in endothelial cell survival and quiescence (Patan, 1998), though these studies examined only cell lines of the vasculature. It is likely that Tie1 may play a similar role in developing valve, but it is just as likely that the role for Tie1 is context dependant both spatially and temporally.

Using a conditional Tie1 ^{flox}ed allele that was developed in our laboratory (Qu X, 2010) in conjunction with the Tie1 lz knockin and the P2Cre, we generated mice that lack Tie1 in the valve endocardium (further referred to as P2Cre Tie1$^{flox/lz}$). These animals have aortic valves that are much larger than their wild type littermates. We hypothesize that Tie1 does play a critical role in valve development and here we examine in depth the

effects of a loss of Tie1 in the valve leaflet and how this loss contributes to structural changes and functional abnormalities in the mouse.

Experimental Procedures

Genotyping

Tail samples were obtained from three week old mice and digested in a buffer containing 100 mM Tris pH 8.5, 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCl and 100 μg/ml Proteinase K overnight at 55°C. Tail DNA was then genotyped by PCR using *Taq* polymerase (Roche) or REDExtract-N-Amp PCR Reaction Mix (Sigma) using the following primers:

Tie1-lz: 5' – TGC CCC CCC TTC CAG AGA CTT CC – 3', 5' – GCA AAG AGG ATC CCC ACC AGA CCA TACT -3' and 5' – GGG GAT GTG CTG CAA GGC GAT TAA G – 3'

Tie1 ^{flox}: 5' – TCG GGC GCG TTC AGA GTG GTA T -3' and 5' – ATG CCT GTT CTA TTT ATT TTT CCA G -3'

Cre: 5' - TCC GGG CTG CCA CGA CCA A -3' and 5' – GGC GCG GCA ACA CCA TTT TT -3'

Generation of Tie1 mutant alleles

Tie1^{flox} animals were developed in our laboratory. A loxP site and a neomycin resistance cassette were inserted into the first intron of Tie1, and a second loxP site was inserted just upstream of the minimal promoter region. When introduced to Cre recombinase, the Tie1 minimal promoter and exon 1 are excised.

Animal Breeding

Tie1 $^{flox/flox}$ mice were bred to Tie1 $^{lz/+}$ (A lacZ knockin to the Tie1 locus resulting in one null allele) to generate Tie1$^{flox/lz}$ mice. These animals were then crossed to P2Cre Tie1 $^{flox/flox}$ mice. Results of this cross generates 25% of each of the following offspring; Tie1$^{flox/flox}$, Tie1$^{flox/lz}$, P2Cre Tie1 $^{flox/flox}$ and P2Cre Tie1 $^{flox/lz}$. All animals were maintained in microisolator cages and were fed normal rodent chow (Purina Mills). All experimental procedures were performed according to Vanderbilt University's Animal Care and use Committee.

Immunohistochemistry

Adult, P0 and prenatal tissue was extracted in PBS and flash frozen in OCT (Tissue Tek). Tissue was sectioned at 7-10μm onto charged slides using a cryostat (Leica) and stored at -80°C until ready for use. The following primary antibodies were used to examine the valve phenotype of P2Cre Tie1 $^{flox/lz}$ mice;

Rat monoclonal CD31 antibody (GeneTex) 1:100

Mouse monoclonal Chondroitin Sulfate (Abcam) 1:200

Rabbit polyclonal Fibronectin (Abcam) 1:100;

Rat monoclonal Heparan Sulfate Proteoglycan 2 (Abcam) 1:200

Phosphohistone H3 (Cell Signaling) 1:100

Rabbit polyclonal Smooth muscle actin (Abcam) 1:200

Rat monoclonal Tenascin C (Abcam) 1:200

Rabbit polyclonal VE Cadherin (Abcam) 1:50

Rabbit polyclonal ZO-1 (Invitrogen) 1:200

Rabbit monoclonal Periostin (a kind gift from Dr. Stan Hoffman, Medical University of South Carolina) 1:200.

Sections were fixed with 4% PFA or acetone as required. Primary antibodies were incubated overnight at 4°C and immunodetected with the appropriate secondary antibody for 30 minutes at room temperature. Samples were mounted with Vectashield containing DAPI (Vector Labs). Sections were visualized on a Nikon Elipse E800 epifluorescence microscope and imaged using SPOT Imaging software.

Laser Capture Microdissection

P0 and E14.5 hearts were flash-frozen in OCT (Tissue Tek) and sectioned at 7 μm on a cryostat (Leica). Sections were then washed with water, dehydrated and stained briefly with toluene blue for visualization. Pulmonary and aortic valve tissue were immediately collected via laser capture (Leica).

RNA isolation and cDNA preparation

RNA from laser-captured tissue was isolated using the PicoPure RNA Isolation Kit (Arcturus). RNA from microdissected tissue was isolated using a standard Trizol prep (Invitrogen). cDNA was obtained using a standard protocol (Invitrogen).

Quantitative real-time PCR (qRT-PCR)

All qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) on the iQ5 Real Time PCR Detection System (BioRad). Experiments were performed in

triplicate using glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) as the housekeeping control to normalize data. Relative expression was calculated as $\Delta^{2\text{-ct}}$ (Schmittgen, 2008). Primers used are as follows:

Tie1: 5' – GTG CCA CCA TTT TGA CAC TG -3' and 5' – CAG GCA CAG CAG GTT GTA GA -3'

GAPDH: 5' – CAC TGG CAT GGC CTT CCG TG -3'and 5'- AGG AAA TGA GCT TGA CAA AG -3'

Area Measurements

H&E stained sections were measured for overall valve area. 3-5 animals per genotype per age group were selected and valve area in 5-8 consecutive sections of the aortic and pulmonary valves was quantitated. Areas given represent the mean area of two valve leaflets in a field. Measurements were performed using The Image Processer software. Statistical analysis was performed using a Student's T-Test in Excel.

Von Kossa Stain for Calcium

Paraffin sections were deparaffinized and hydrated to water. Slides were placed in 3% silver nitrate and exposed to light for 30 minutes until calcium minerals in the tissue absorbed the stain. Sections were washed in water, placed in sodium thiosulfate for two minutes then counterstained with Nuclear Fast Red. Tissue was then dehydrated in a graded ethanol series, cleared in Xylene and mounted with Permount.

Hyaluronic Acid Staining

Frozen sections were used examine hyaluronic acid content in the adult valve. Sections were washed with PBS and fixed for 10 minutes in 4% PFA. Slides were then rinsed with PBS and half the samples were digested with bovine testicular hyaluronidase 1:20 in 5% PBST for one hour at room temperature. All slides were then rinsed with acetic acid 3% and stained with alcian blue (pH 2.5) for 15 minutes. Slides were counterstained with Nuclear Fast Red for 10 minutes, washed with water, dehydrated through a graded ethanol series and mounted with Permount.

Modified Movat's Pentachrome Stain

Paraffin sections of adult valve leaflets were used to study the effects of a loss of Tie1 on the ECM. Sections were deparaffinized in xylene and hydrated through a graded ethanol series into water. Slides were then stained in alcian blue for two hours, washed in running water then exposed to alkaline alcohol for one hour to convert alcian blue into insoluble monastral fast blue. Slides were then washed with water and stained in Verheoff's hematoxylin to visualize elastin and nuclei. A wash in sodium thiosulfate was followed by five minutes in crocein scarlet-acid fuchsin to stain fibrin and muscle red/pink. Slides were then differentiated in 5% aqueous phosphotungstic acid, dehydrated and stained in Saffron for 15-20 minutes to visualize collagens. Slides were finally washed in 100% ethanol, two changes of xylene, and mounted with Permount. The Movat's Pentachrome kit was purchased from PolyScientific.

Echocardiograms

The mice were sedated with isoflurane then placed on a heated table with isoflurane for continued anesthesia. The mice were restrained and an echocardiogram was performed with a VisualSonics Vevo 2100 using the MS-400 probe (18-38 MHz). 2-dimensional imaging was obtained in the long axis, short axis, apical 4-chamber, and aortic arch views; color flow mapping was used to assess for insufficiency of the semilunar and atrioventricular valves and Pulsed wave Doppler of the semilunar and atrioventricular valves was obtained to evaluate for valvuar stenosis. M-mode measurements were performed in short axis to assess ventricular function and chamber dimensions.

Atomic Force Microscopy

The stiffness of the valves was determined by measuring the modulus using a Catalyst Bioscope atomic force microscope (AFM) (Bruker AXS, Madison WI, USA). Where Ftip is the force on the tip, E is the Young's modulus, R is the tip radius, d is the distance between the sample and tip, and Fadh is the adhesive force between the sample and tip. The tips were borosilicate glass beads attached to silicon nitride-coated cantilevers with a nominal diameter of 5 mm and nominal spring constant of 0.03 N/m (Novascan Technologies, Inc., Ames IA, USA. Tips were calibrated on a PDMS substrate of known stiffness prior to sample measurements. Scan parameters were: scan size between 25 mm x 25 mm and 30 mm x 30 mm, scan rate 0.1 Hz, and peak force setpoint of 1 nN. A total of 3 samples per genotype were used and each sample was scanned three times in adjacent areas on the valves.

ECM Quantitation

Collagen from Tie1$^{flox/flox}$ and P2Cre Tie1$^{flox/lz}$ was measured using the Sircol Soluble Collagen Assay (Biocolor Ltd.). Aortic and pulmonary valves from 4 animals were pooled for each sample. A total of 3 Tie1$^{flox/flox}$ and 3 P2Cre Tie1$^{flox/lz}$ samples were measured with a microplate reader. Glycosoaminoglycans from Tie1$^{flox/flox}$ and P2Cre Tie1$^{flox/lz}$ was measured using the Blyscan Sulfated Glycosaminoglycan Assay (Biocolor Ltd.) Again, a total of 3 Tie1$^{flox/flox}$ and 3 P2Cre Tie1$^{flox/lz}$ samples (each sample contained a pool of leaflets from 4 mice) were used for quantitation.

Results

Loss of Tie1 in the valve endocardium reduces embryonic survival rates

As described above, Tie1$^{flox/lz}$ mice were crossed with P2Cre Tie1$^{flox/lz}$ animals to generate 25% of each of the following offspring: Tie1$^{flox/lz}$, Tie1$^{flox/flox}$, P2Cre Tie1$^{flox/lz}$ and P2Cre Tie1$^{flox/flox}$ (Fig 3.1 A). Deletion of Tie1 in the valve leaflets results in reduced survival of P2Cre Tie1$^{flox/lz}$ offspring. Less than half of P2Cre Tie1$^{flox/lz}$ pups survive past weening (Fig. 3.1 A, green bars). Embryos that die *in utero* typically exhibit edema after E14.5. The majority of P2Cre Tie1$^{flox/lz}$ pups born are smaller and more lethargic than WT littermates, a common phenomenon associated with human patients that have heart defects.

qRT-PCR from laser captured valves shows that we have deleted Tie1 by approximately 5-fold in the embryo and P0 pulmonary and aortic valves (Fig 3.1 B).

Reduction of Tie1 in the adult was less dramatic, likely due to the diminished endogenous expression of Tie1 in the adult valves.

P2Cre Tie1$^{flox/lz}$ mice have larger aortic valves than Tie1$^{flox/flox}$

Since we have deleted Tie1 in the valve endocardium, we sectioned valve leaflets at different stages of development to examine their morphology. We noted that after E14.5 P2Cre Tie1$^{flox/lz}$ aortic valves were larger than Tie1 $^{flox/flox}$ valves (Figure 3.2 A). We quantitated this and discovered that indeed, P2Cre Tie1$^{flox/lz}$ aortic valves are larger than that of their littermates (Fig 3.2 B). Adult aortic valves in P2Cre Tie1$^{flox/lz}$ mice were nearly twice the size as what was measured in Tie1$^{flox/flox}$ animals. No differences in valve area were observed in the pulmonary valve (Fig 3.2 B) or in the AV valves.

Tie1 does not appear to be involved in endothelial cell survival in the developing valve leaflet

Tie1 has been shown to be essential for endothelial survival and quiescence in the vasculature (Puri MC *et al.*, 1995; Sato TN *et al.*, 1995) therefore we first sought to examine P2Cre Tie1$^{flox/lz}$ valves for a loss of endothelial cells or endothelial cell integrity. The endothelial cell marker PECAM-1 did not show any differences in staining patterns or intensity in P2Cre Tie1$^{flox/lz}$ vs. Tie1 $^{flox/flox}$ valves (Fig. 3.3 A-F), suggesting that the endothelium was indeed present in these valves. We also used the tight junction marker ZO1 (Fig. 3.3 I, J) and again concluded that endothelial cells in the valve leaflets were

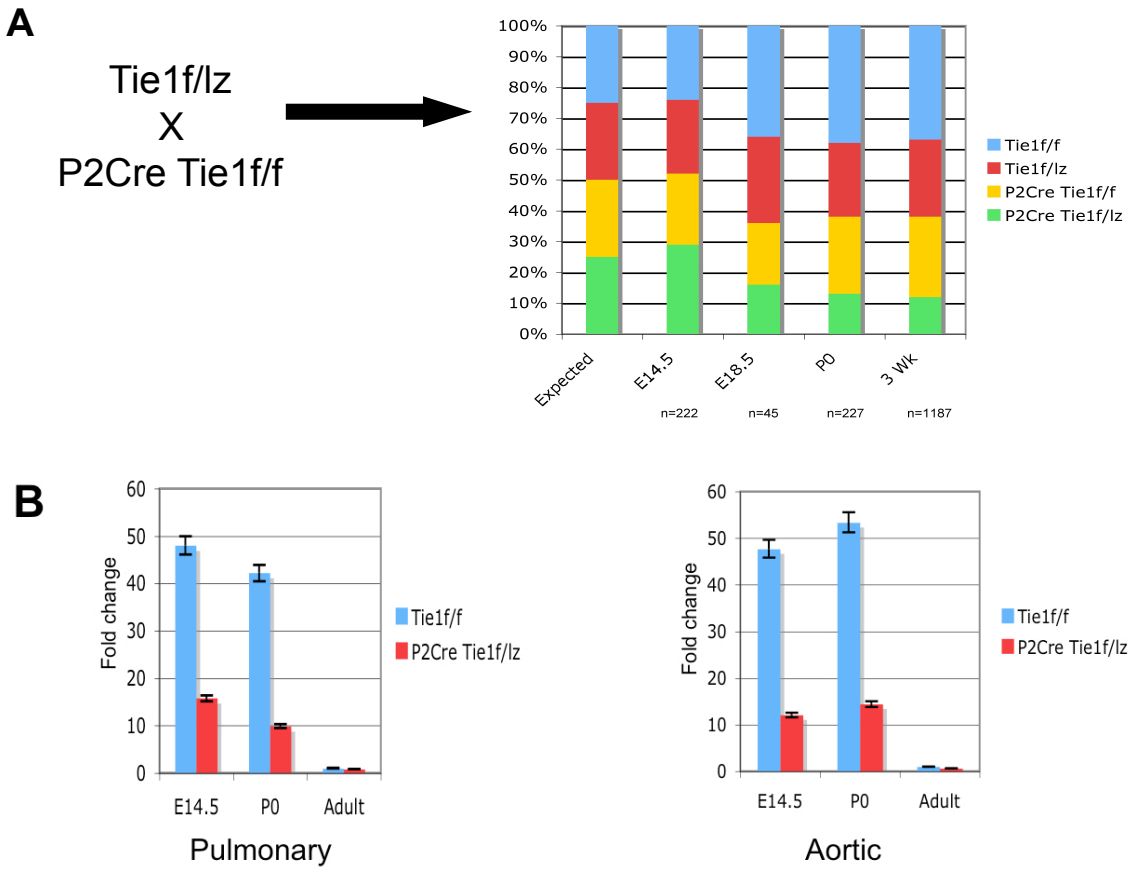


Figure 3.1. ***Tie1 deletion in the valve endocardium results in decreased survival rates***. (**A**) As described, P2Cre Tie1$^{flox/lz}$ mice were generated by crossing Tie1$^{flox/lz}$ mice to P2Cre Tie1$^{flox/flox}$ mice. Rather than having a typical 25% Mendelian cross, we note a decrease in survival of P2Cre Tie1$^{flox/lz}$ offspring (green). (**B**) Tie1 is efficiently deleted in the embryonic valve leaflets, as well as in the adult.

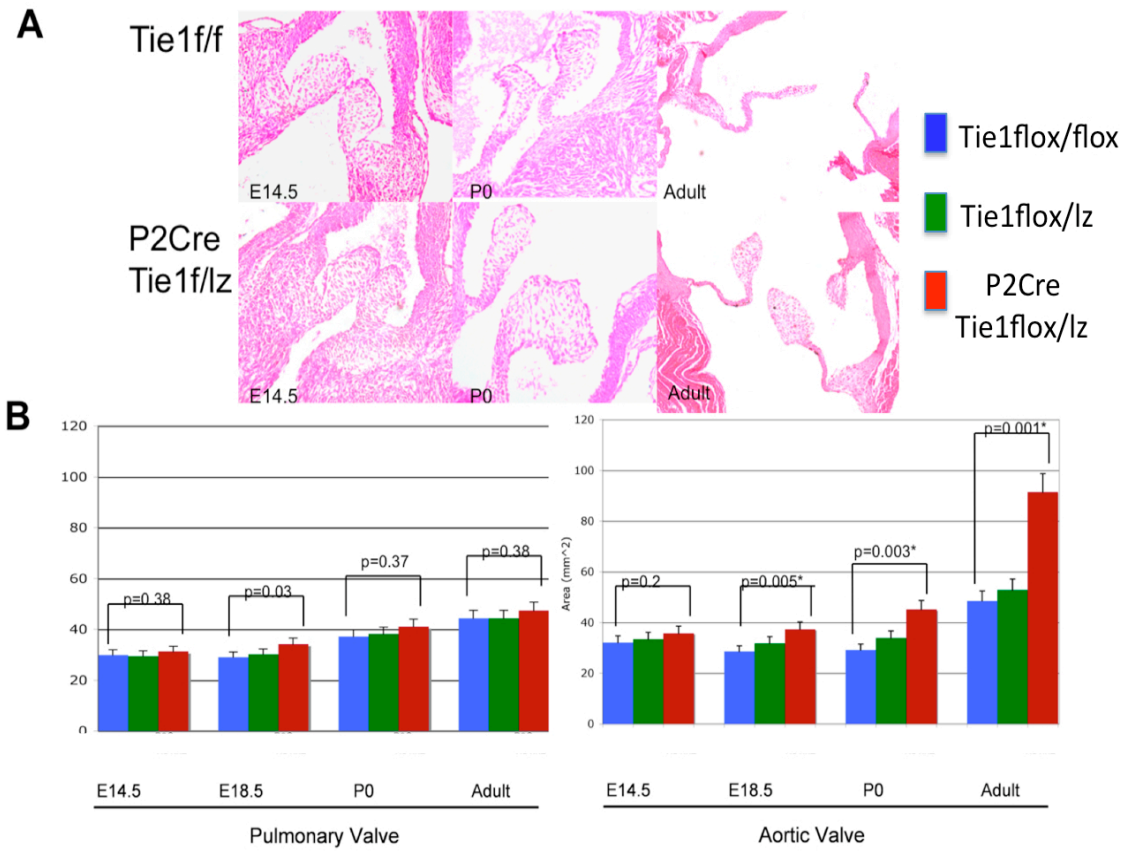


Figure 3.2. ***P2Cre Tie1$^{flox/lz}$ animals have enlarged valve phenotype***. (**A**) At E14.5 Tie1 $^{flox}/^{flox}$ aortic valves are indistinguishable from that of P2Cre Tie1$^{flox/lz}$ aortic valves. Following this stage, a significant increase in size can be noted. This size difference has been quantitated (**B**). Blue bar, Tie1$^{flox}/^{flox}$; Green, Tie1$^{flox/lz}$; Red, P2Cre Tie1$^{flox/lz}$. p-value compares Tie1 $^{flox}/^{flox}$ with P2Cre Tie1$^{flox/lz}$.

present and did form intact epithelial sheets. Furthermore, inspection of the valve leaflets with transmission EM showed no abnormalities in the presence or appearance of valvular endothelial cells (Fig 3.3 G, H). No abnormalities were seen in overall cell number, proliferation or apoptosis rates within the valve leaflets, suggesting that abnormalities in these processes likely did not lead to the hyperplastic valve phenotype of P2Cre Tie1$^{flox/lz}$ mice.

Abnormal calcium deposition is observed in P2Cre Tie1$^{flox/lz}$ adult mice

Von Kossa staining revealed that numerous calcium deposits were present in young (2-3 month) adult P2Cre Tie1$^{flox/lz}$ valve leaflets, whereas no calcium deposits were noted in Tie1$^{flox/flox}$ animals (Fig 3.4). The significance of this finding has not yet been determined, however we speculate that a loss of Tie1 in the valve may alter other signaling pathways responsible for secreting or degrading calcium deposits. I will discuss this observation further in Chapter IV.

Extracellular matrix content and organization is altered in P2Cre Tie1$^{flox/lz}$ semilunar valves

We used Movat's Pentachrome stain to assess the deposition and organization of ECM within the valve leaflet (Fig 3.5 A). Deposition of GAGs (blue) was much greater in P2Cre Tie1$^{flox/lz}$ valves as compared to Tie1$^{flox/flox}$. Furthermore, intricate stratification of ECM components within the leaflet was not observed in P2Cre Tie1$^{flox/lz}$ mice.

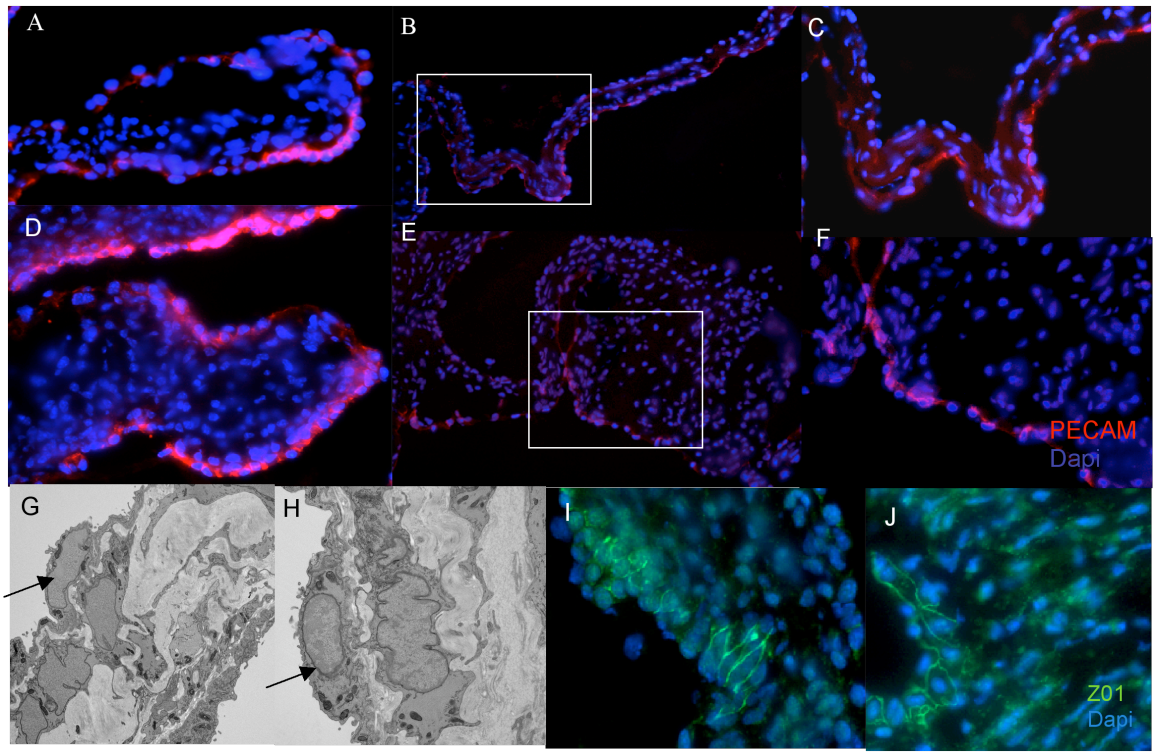


Figure 3.3. ***Valvular endothelial cells lacking Tie1 do survive and do maintain an intact epithelial sheet.*** (**A-F**) PECAM-1 immunofluorescence. $Tie1^{flox/flox}$ endothelial cells are indistinguishable from P2Cre $Tie1^{flox/lz}$ endothelial cells at P0 (**A, D**) and adulthood (**B, C, E, F**). Transmission EM of $Tie1^{flox/flox}$ (**G**) and P2Cre $Tie1^{flox/lz}$ (**H**) endothelial cells also appear normal. Tight junctions and epithelial sheets are also found to be intact in both P2Cre $Tie1^{flox/lz}$ (**J**) and $Tie1^{flox/flox}$ valve leaflets (**I**) as visualized by ZO1 immunohistochemistry.

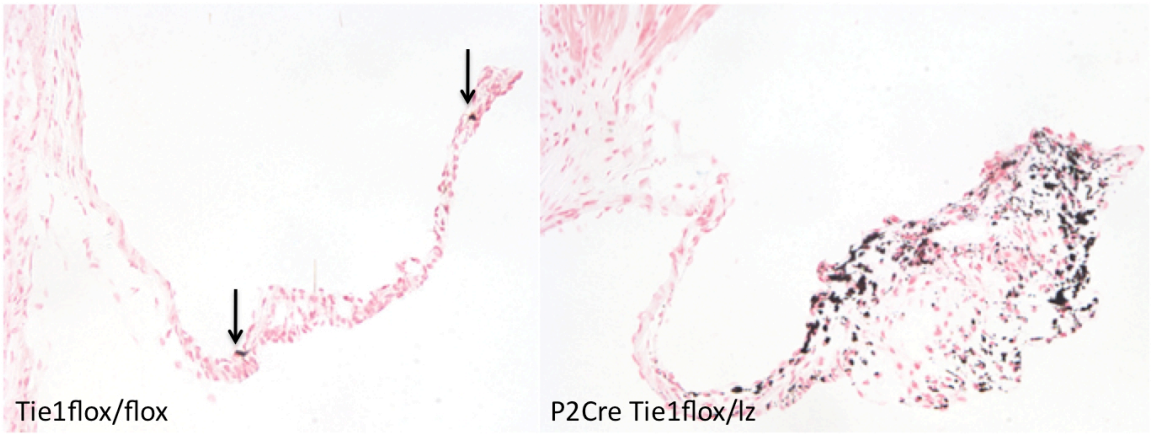


Figure 3.4. ***Abnormal calcium deposits are seen in P2Cre Tie1$^{flox/lz}$ valves.*** Normal Tie1$^{flox/flox}$ aortic valves have minimal calcium deposits (black spots) at 3 months of age (arrows). Age matched P2Cre Tie1$^{flox/lz}$ valves, however have numerous calcium deposits .

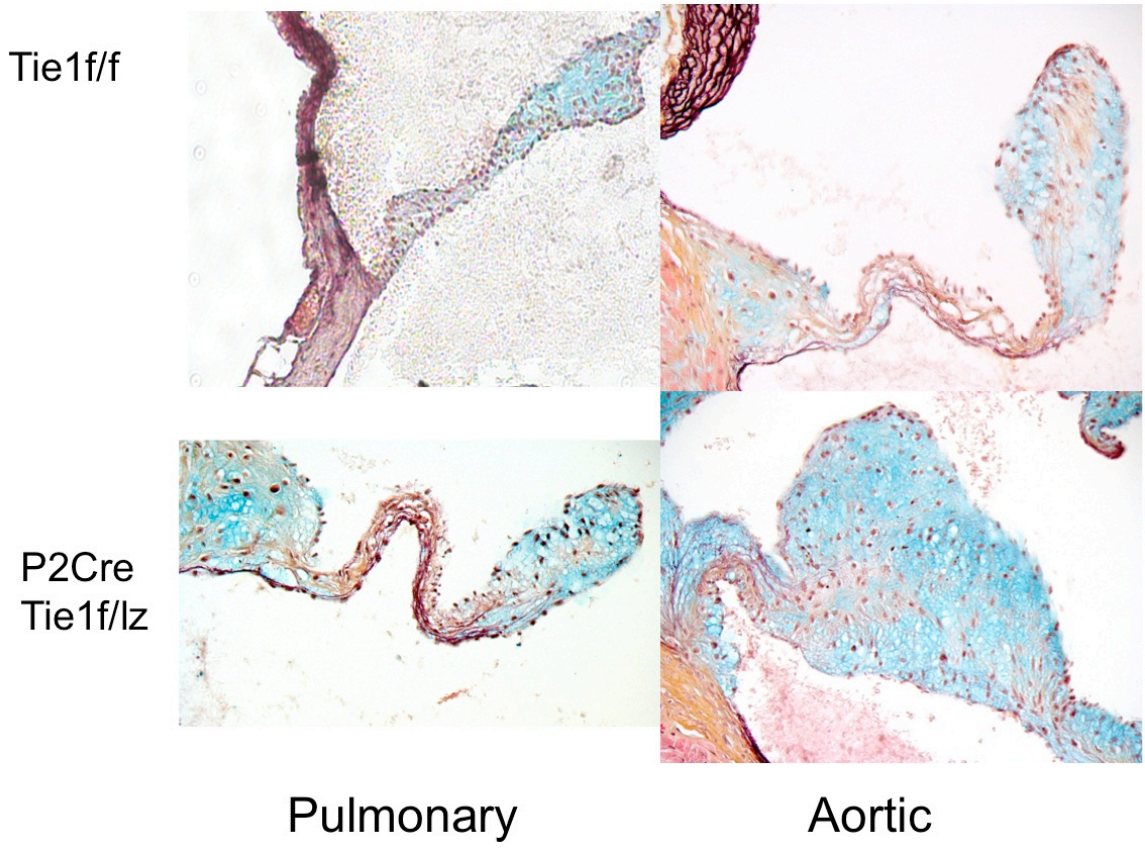
Quantitation of the amount of GAGs in the adult valve leaflets was done using a colormetric assay (Fig 3.5 B). The amount of GAGs was increased in both aortic and pulmonary P2Cre Tie1$^{flox/lz}$ valves over that of both Tie1$^{flox/flox}$ and Tie1$^{flox/lz}$. Likewise, we measured collagen content and a similar trend was noted. The amount of collagen in P2Cre Tie1$^{flox/lz}$ adult valves was decreased in both aortic and pulmonary valves in a dose-dependent manor (Fig 3.5 C).

An antibody stain against chondroitin sulfate, a GAG found in the valve leaflet, also revealed that valves lacking Tie1 are insufficient at effectively organizing the ECM. Rather than being localized in the interstitium on the arterial side of the leaflet, chondroitin sulfate in the P2Cre Tie1$^{flox/lz}$ valve is clustered in random locations throughout the leaflet (Fig. 3.6), including both arterial and ventricular regions.

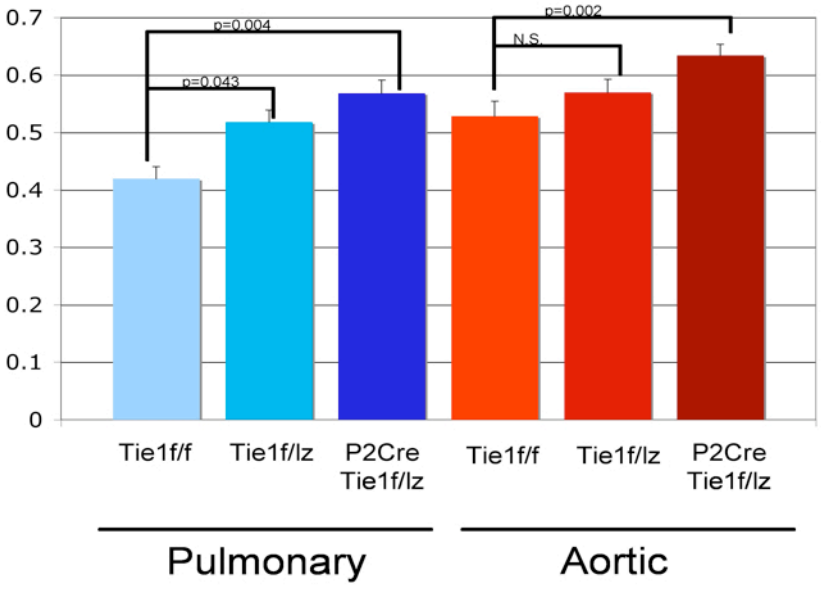
P2Cre Tie1$^{flox/lz}$ valves are structurally and functionally insufficient

Atomic force microscopy (AFM) measures the "stiffness" of tissue or polymers. Information is gathered by projecting a laser onto a cantilever. This causes the tip on the underside of the cantilever to touch the underlying sample. The laser is reflected back to a photodiode which processes the information gained by displacement of the cantilever and tip. Using this feedback, an image is generated and a color scale represent stiffness measured in kPa. In this study, we used AFM to assess the integrity or "stiffness" of the valve leaflet. Stiffness of the adult Tie1$^{flox/flox}$ aortic valve measured at approximately 500 kPa, whereas corresponding regions on P2Cre Tie1$^{flox/lz}$ aortic valve leaflets measured about 5-fold lower, close to 100 kPa (Fig 3.7 C). This supports our GAG and

A


Tie1f/f
P2Cre
Tie1f/lz
Pulmonary
Aortic

B


0.7
0.6
0.5
0.4
0.3
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0
p=0.004
p=0.043
p=0.002
N.S.
Tie1f/f
Tie1f/lz
P2Cre Tie1f/lz
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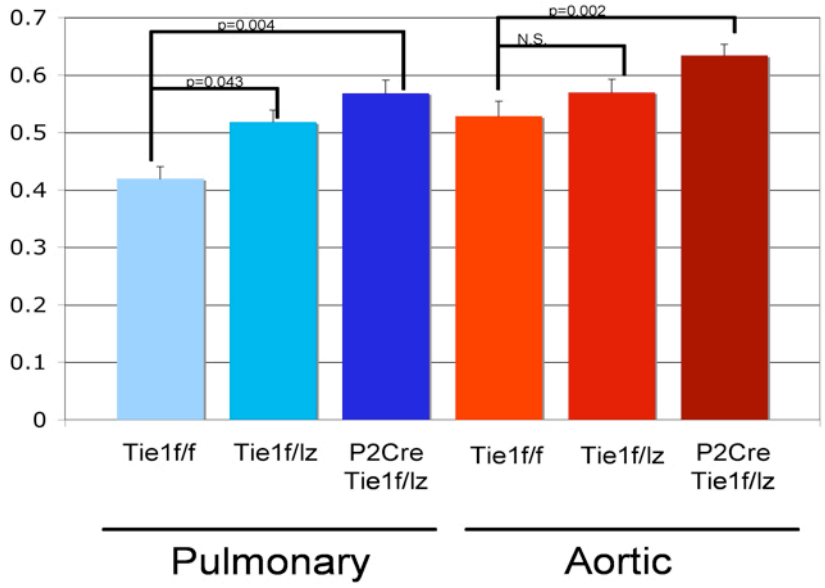

Figure 3.5. ***Proteoglycan and Glycosaminoglycan content in valves lacking Tie1 is increased as collagen levels decrease.*** GAG deposition (blue) appears to be increased in P2Cre Tie1$^{flox/lz}$ valves as shown by Movat's Pentachrome stain (**A**). Sulfated GAGs were measured using a colormetric assay from Biocolor (**B**). A significant increase (p=0.004 and 0.002) in the amount of sulfated GAGs were noted in both P2Cre Tie1$^{flox/lz}$ aortic and pulmonary valves. Collagen was also quantitated using a similar method from Biocolor. (**C**) A significant decrease in collagen content was noted in both pulmonary (p=0.006) and aortic (p=0.002) P2Cre Tie1$^{flox/lz}$ valve leaflets as compared to Tie1$^{flox/flox}$ littermates. 12 valve leaflets were pooled per sample and all samples were performed in triplicate.

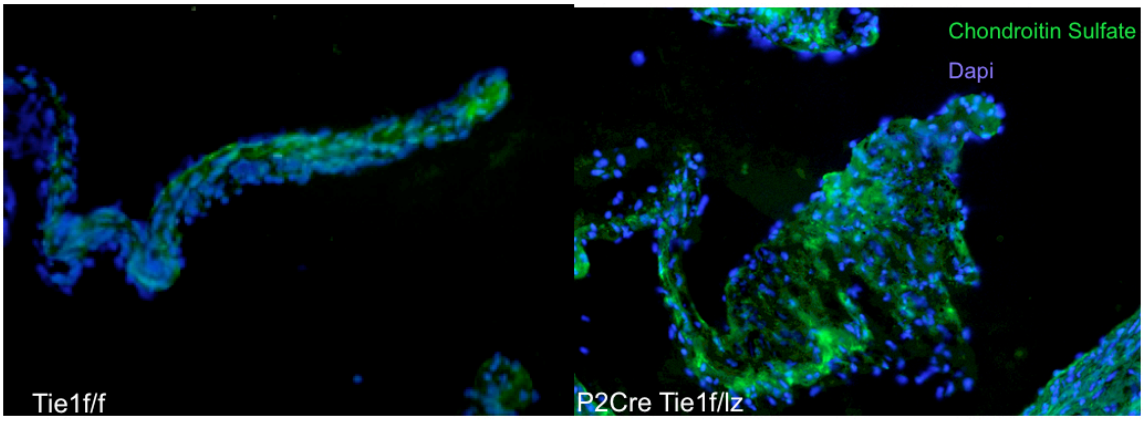

Figure 3.6. ***Chondroitin sulfate organization is abnormal in P2Cre Tie1$^{flox/lz}$ valves***. Chondroitin sulfate deposition is organized to the arterial side of the valve leaflet in the Tie1$^{flox/flox}$ adult animal. Such organization is lost in P2Cre Tie1$^{flox/lz}$ valves.

collagen quantitation data that indicate that P2Cre Tie1$^{flox/lz}$ valves have less collagen and more proteoglycans in the leaflets, thus reducing the rigidity of the valve.

To assess functionality of Tie1$^{flox/flox}$ and P2Cre Tie1$^{flox/lz}$ valve leaflets, echocardiograms were performed on adult animals (n=6 mice per genotype). Though overall heart rate was unchanged, P2Cre Tie1$^{flox/lz}$ mice showed significant changes in systolic volume, ejection fraction, fractional shortening and left ventricle mass (Table 3.1). These values correlate with valvular insufficiencies and ultimate heart failure. In addition, 100% (6/6) of P2Cre Tie1$^{flox/lz}$ aortic valves showed some degree of aortic and pulmonary regurgitation (Fig. 3.8) whilst only 17% of Tie1$^{flox/flox}$ (1/6) displayed minor pulmonary regurgitation.

Discussion

This chapter focused on the effects of Tie1 deletion in the pro-valve endocardium of the developing mouse embryo. We demonstrated that Tie1 is an essential component of valve remodeling as a loss of Tie1 in valve endothelium resulted in embryonic and perinatal demise as well as enlarged valves that persisted through adulthood. Endothelial cell survival and morphology appeared unperturbed in mutant animals, as visualized by PECAM-1, ZO1 and transmission EM. Though Tie1 has a role in endothelial cell survival and quiescence in the developing vasculature, it does not appear that this is the case in the developing valve as endothelial cells are present and form intact epithelial sheets.

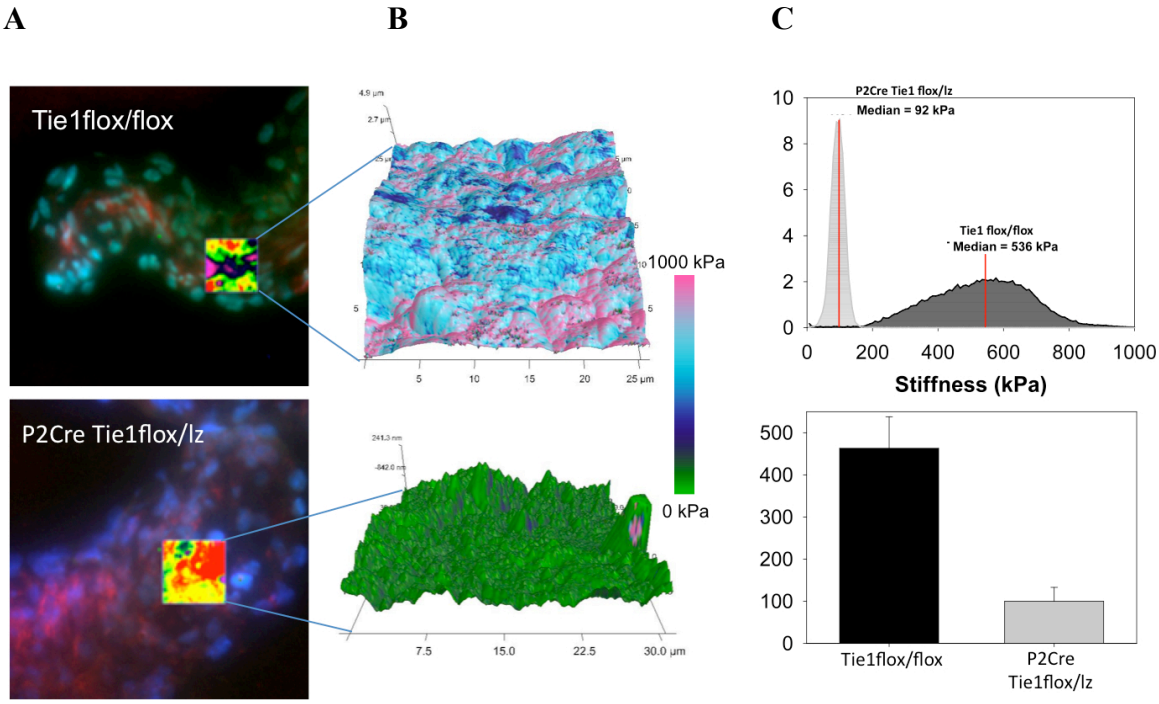

Figure 3.7 ***P2Cre Tie1$^{flox/lz}$ valves lack rigidity as measured by atomic force microscopy***. (**A**) Corresponding regions of aortic valve sections were examined and each region was imaged three times. (**B**) Relative stiffness of P2Cre Tie1$^{flox/lz}$ valves is much lower than what is seen in Tie1 $^{flox}/_{flox}$ valve leaflets. (**C**) Graphical representation of P2Cre Tie1$^{flox/lz}$ and Tie1 $^{flox}/_{flox}$ valve stiffness shown in **B**. Tie1 $^{flox}/_{flox}$ aortic valves are approximately 5-fold stiffer than P2Cre Tie1$^{flox/lz}$ aortic valves.

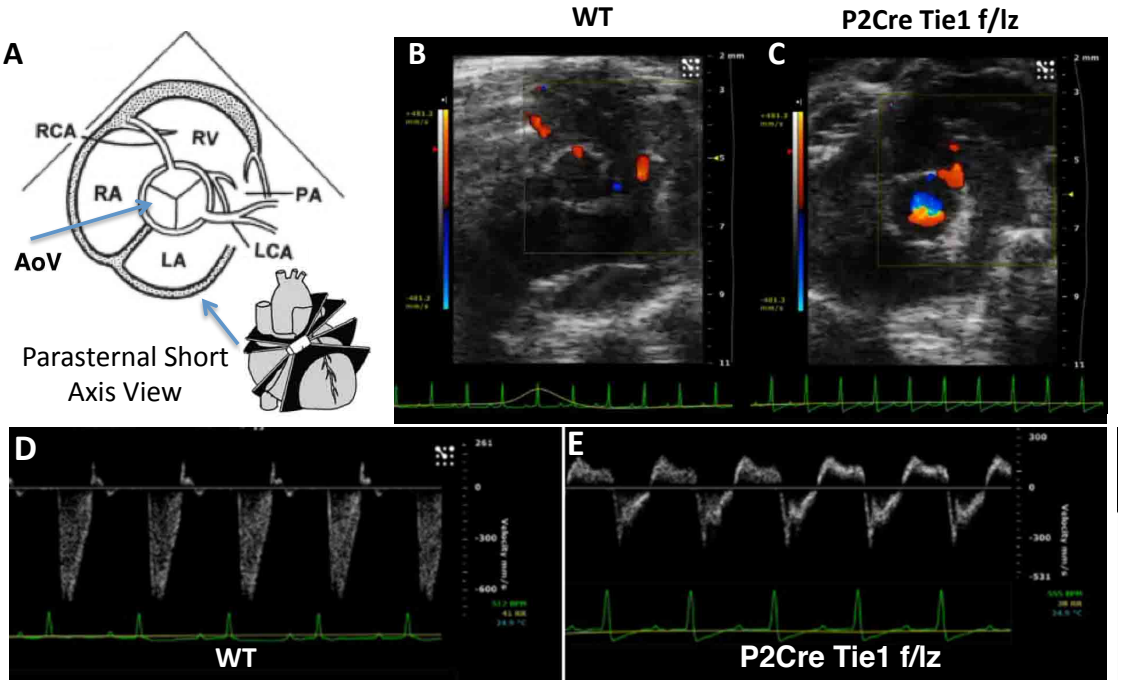

Figure 3.8 ***P2Cre Tie1$^{flox/lz}$ valves are functionally abnormal.*** (**A**) Transverse depiction of the aortic valve. (**B**) WT and (**C**) P2Cre Tie1$^{flox/lz}$ aortic valves. Unidirectional blood flow exists in (**B**) (red) and regurgitation is shown in (**C**) (red and blue). Doppler profile of (**D**) WT and (**E**) P2Cre Tie1$^{flox/lz}$ aortic valves. Regurgitation is seen in (**E**).

	Tie1f/f n=6	P2Cre Tie1f/lz n=6
Heart Rate	501 (+/- 13)	497 (+/- 8)
Vol Syst	17.4 (+/- 4.2)	34.6* (+/- 7.1)
Vol Diast	50.6 (+/- 8.3)	59.9 (+/- 10.9)
Ejection Fraction	67% (+/- 3)	53%* (+/- 2)
Fractional Shortening	36% (+/- 3)	29%* (+/- 1)
LV mass (corrected)	60 (+/- 1)	73* (+/- 6.5)
AoV Valve	1.07 (+/- 0.03)	1.23* (+/- 0.07)
Ao Root	1.48 (+/- 0.03)	1.76* (+/- 0.11)
Ao Velosity Time Integral	34.8 (+/- 3.5)	53.2* (+/- 13.6)

Table 3.1. ***Echocardiogram statistics for Tie1$^{flox/flox}$ and P2Cre Tie1$^{flox/lz}$ adult mice.*** Measurements taken from 3 month old female Tie1 $^{flox/flox}$ and P2Cre Tie1$^{flox/lz}$ mice. Asterisk denotes a p value of < 0.005.

Deletion of Tie1 in the valve endocardium results in abnormally large hyperplastic aortic valves. There are no changes in endothelial or mesenchymal proliferation or apoptosis in P2Cre Tie1$^{flox/lz}$ valves, nor are there significant changes in cell number. However, deposition and organization of numerous ECM components are disregulated in later stages. Changes in ECM are noted in both pulmonary and aortic valves, though area measurements show a significant increase in aortic valve size. We hypothesize that this phenomenon is due to the increased hemodynamic flow across the aortic valve and less physical stress on the pulmonary valve. This change in flow occurs around birth when the ductus arteriosis closes, which coincides with the most dramatic increases in aortic valve size.

A close examination of the ECM revealed that not only the organization of matrix components was abnormal, but the amount of GAG and collagen deposition was abnormal as well. As animals age, the collagen content of the valve typically increases as GAG content decreases. Furthermore, periostin deposition is altered in P2Cre Tie1$^{flox/lz}$ mice. Periostin is made and secreted by the mesenchyme, and because we deleted a gene in the endothelium, it is the endothelium that must be responsible for miscommunication. In P2Cre Tie1$^{flox/lz}$ valves, the matrix never receives the proper message to make the switch from GAG filled valve leaflets to fully remodeled leaflets with a higher collagen content, suggesting that Tie1 plays a role in crosstalk between endothelium and mesenchyme in the embryonic and post-natal remodeling valve leaflet. A lack of Tie1 in the valve endocardium disrupts this crosstalk, therefore disrupting the process of valve remodeling. This may be in part due to the loss of Tie1 expression itself, as in the adult Tie1 is typically localized to the arterial side of the valve leaflet. A loss of this

expression pattern may also cause miscommunication within the leaflet in terms of knowing which "side" of the valve is arterial and which side is ventricular.

Not only do we observe molecularly based anomalies in aortic valve size and ECM organization, P2Cre Tie1$^{flox/lz}$ valves are functionally insufficient as well. AFM data confirm, as we suspected, that P2Cre Tie1$^{flox/lz}$ valves containing high levels of GAGs and lower levels of collagens are physically "softer" and less rigid than that of Tie1$^{flox/flox}$ leaflets. Echocardiograms of adult P2Cre Tie1$^{flox/lz}$ and Tie1$^{flox/flox}$ mice indicate that the less rigid valves as indicated by the AFM correlates with insufficient valve function in mice lacking Tie1 in the valve endocardium, likely leading to eventual heart failure. One hundred percent of the P2Cre Tie1$^{flox/lz}$ mice (n=6) examined by echocardiogram showed some degree of both pulmonary and aortic valve regurgitation whereas only 17% of Tie1$^{flox/flox}$ mice had minor pulmonary regurgitation. The degree of insufficiency varied within P2Cre Tie1$^{flox/lz}$ mice, ranging from mild regurgitation to more severe regurgitation reaching the aortic arch and abdominal aorta. We are the first to show that Tie1 plays a role outside the vasculature of the developing embryo and that cell-cell communication between endothelium and the mesenchyme are critical during valve development.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

The major focus of this work was to identify a role for Tie1 in the developing valve. Most global Tie1 knockouts do live long enough to develop valves that undergo remodeling (Sato TN *et al.*, 1995). Of the few Tie1$^{-/-}$ mice that do survive to E14.5, early in the valve remodeling process, the focus has been on the more obvious vascular phenotype and the valves are not examined in depth. Little information on Tie1 has been documented in the literature, and the majority of those studies have been done *in vitro* under extraordinary conditions that are mostly unrelated to real events *in vivo*. This study was an important step away from the previous *in vitro* studies and a step closer to identifying what Tie1's role is in the developing murine embryo.

The secondary focus of this work has been to describe the role of the NFATc1-P2Cre as a tool that can be used to study later stages of valve remodeling. Using the P2Cre, we are now able to examine the role of vascular endothelial genes in the developing valve. Global deletions of vascular endothelial genes resulting in early embryonic lethality has, until now, made such studies unattainable.

The origin of adult valvular endothelial cells

The origin of cells in the adult heart valve has been a topic of discussion in the field for many years. Cells of the adult valve endocardium must either arise from embryonic endocardium, mesenchyme-endothelial cell transformation from cells of the valve interstitium, or from circulating progenitor cells. Proliferation and apoptosis rates

in VICs and VECs are quite low in the adult valve, so the question has always been whether these cells are replaced by other sources, such as circulating progenitor cells. As discussed in Chapter I, Visconti et al used a green fluorescent marker to label hematopoietic stem cells and implanted them into lethally irradiated mice. Upon dissection, it was found that green fluorescent cells were present in the valve interstitium and analysis of those cells displayed markers and demonstrated a function similar to that of mature VICs (Visconti RP, 2006). It is important to note that no green fluorescent cells were observed contributing to the endothelium of the valve leaflet. So where do the endothelial cells of the adult valve come from? Using the NFATc1-P2Cre, and an R26R reporter we are able to label the valve endothelial cells in the early embryo and observe the progression of these cells through development into adulthood. We have noted that the majority of endothelial cells lining the adult heart valve retain R26R expression even though the P2Cre is turned off after E14.5. In addition, no R26R expressing cells are ever seen in the valve interstitium. From these data, we conclude that the endothelial cells present in the early developing embryo are the same cells that are maintained through adulthood. These endothelial cells do not become replaced by circulating EPCs or cells of mesenchymal or other origins. This finding will without a doubt change the way that we think about the valve endothelium.

Tie1 is essential for late-gestational semilunar valve remodeling

At E8.5, Tie1 is the last RTK to be turned on during vasculature development. Global knockouts of Tie1 result in a breakdown of the microvasculature, edema and eventual embryonic demise around E14.5 (Puri MC *et al.*, 1995; Sato TN *et al.*, 1995).

Tie1 is highly expressed in the heart and developing valve leaflets during embryonic development, however no heart or valve phenotype was noted in the global knockout papers. Using the NFATc1-P2Cre, we were able to delete a conditional Tie1 floxed allele in the pro-valve endocardium of the developing heart, therefore bypassing the requirement for Tie1 in vasculature. P2Cre $Tie1^{flox/lz}$ mice do not survive in expected ratios and closer examination revealed that these animals have larger aortic valves as compared to $Tie1^{flox/flox}$ littermates. No size difference was noted in the pulmonary valves of these mice, which may be attributed to the differences in hemodynamic flow in the left heart vs. right heart after closure of the ductus arteriosis. The mitral and tricuspid valves also appear unaffected.

Endothelial cells lacking Tie1 in the valve leaflet survive and maintain intact epithelial sheets. This is in direct contrast to what is observed in other endothelial cell models in which Tie1 expression is lost. Previous studies of Tie1 in the vasculature or in vasculature cell lines have indicated that Tie1 is essential for endothelial survival (Partanen J *et al.*, 1992; Puri MC *et al.*, 1995; Sato TN *et al.*, 1995). Our data show that the role of Tie1 in the endothelium is context dependent and that Tie1 is essential for functions other than basic endothelial survival in the valve.

A loss of Tie1 in the developing valve appears to have the most severe effect on organization and deposition of ECM within the leaflet (Fig. 4.2). We provide data that suggests this process is dose-dependent, and mice with one functional Tie1 allele ($Tie1^{flox/lz}$) display an intermediate phenotype when examining GAG and collagen content in the valve, even though these valves look and function physically similar to

Tie1$^{flox/flox}$ valves. Aortic valve size is also dose dependent, as Tie1$^{flox/lz}$ aortic valve area measures between that of Tie1$^{flox/flox}$ and P2Cre Tie1$^{flox/lz}$.

Numerous immunohistochemical stains and other assays indicate that the ECM makeup in the valve leaflets of P2Cre Tie1$^{flox/lz}$ mice is abnormal, but does that translate to a functional insufficiency? Using AFM, we determined that the excess GAG content in these valves leads to a lack of stiffness and rigidity. This observation is confirmed by echocardiogram. Functional analysis of valves lacking Tie1 show that the disorganization of valvular ECM and the delay in remodeling leads to aortic and pulmonary regurgitation in 100% of P2Cre Tie1$^{flox/lz}$ animals that were examined. Valvular insufficiency leading to aortic stenosis and cardiac regurgitation is likely the cause of death in P2Cre Tie1$^{flox/lz}$ mice. Again, we speculate that this is based on the amount of Tie1 that is deleted in the valve. The P2Cre is not 100% efficient, and it is possible that some of the variability in the phenotype that we see in P2Cre Tie1$^{flox/lz}$ mice is due to fluctuating amounts of residual Tie1 expression that was not fully deleted by the P2Cre. This may be one explanation as to why only half of the P2Cre Tie1$^{flox/lz}$ mice die, while the other half survive through adulthood.

We hypothesize that Tie1 plays a role in cell-cell communication between the endothelium and underlying mesenchyme, and are the first to suggest that such a communication exists and is essential for appropriate valve remodeling. Native Tie1 in the adult is expressed on the arterial side of the valve leaflet in areas of turbulent flow. We also suggest that a loss of Tie1 on the arterial side of the valve in P2Cre Tie1$^{flox/lz}$ mice can lead to further miscommunication as Tie1 may be a directional signal that guides the ECM into proper position during remodeling.

Tie2/Ang1 has been implicated in endothelial-mesenchymal communications in the developing vasculature (Chitra Suri 1996). Endothelial cell migrations and vessel formation require precise cell-cell and cell-matrix interactions. Decreased association of endothelial/smooth muscle cells and the underlying matrix has been reported in the vasculature of Ang1 null animals. A few studies have made direct links between angiopoietins and factors that control endothelial cell survival, migration, morphogenesis and communicative signals (Bazzoni, 1999). We speculate that Tie1 may signal through Tie2/Ang1 in the developing valve to communicate with the underlying matrix.

Future Directions

Since no definitive ligand has been identified for Tie1, studies of downstream signaling targets have been lacking in the literature. Furthermore, the only studies that provide insight into Tie1 signaling targets have arisen from *in vitro* models. Using tissue from P2Cre Tie1$^{flox/lz}$ mice, we hope to first determine the *in vivo* relationship between Tie1 and Tie2 in the valve leaflet. A recent paper sought to examine the interactions between Tie1 and Tie2 *in vitro* (Kim KL SI, 2006). This group looked at expression levels of both Tie1 and Tie2 in endothelial progenitor cells (EPCs) and in HUVECs and found similar levels of expression in each cell type. Tie1 was found to associate with Tie2 in the mature cell type (HUVECs) but not in EPCs, suggesting that Tie1's role in development is not only spatially but temporally context-dependent. In addition, Tie1-Tie2 heterocomplexes inhibit Ang2 from binding and activating Tie2 signaling in HUVEC's.

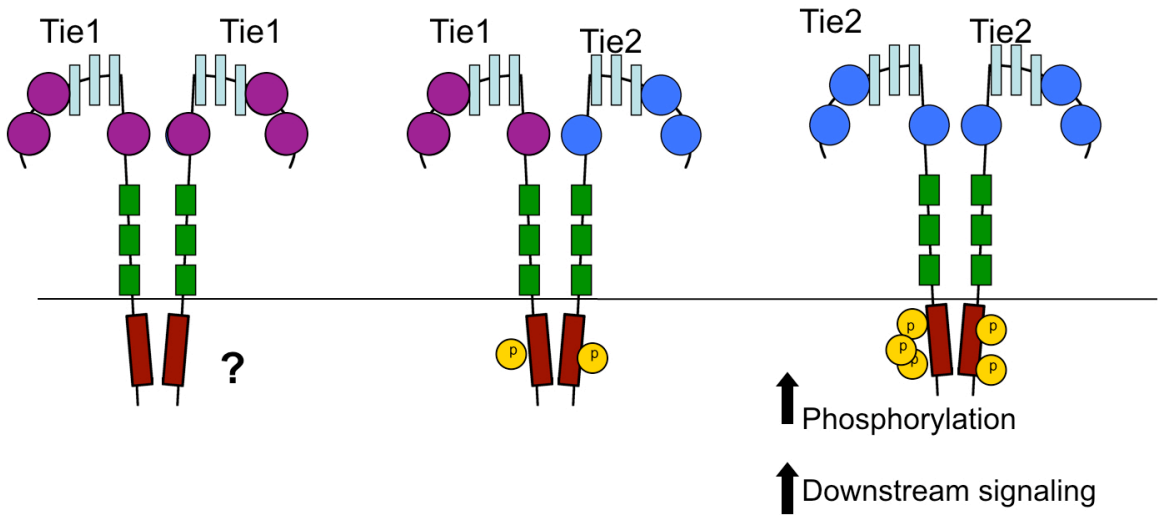

Figure 4.1. ***Potential interaction between Tie1 and Tie2 RTKs***. Tie1 and Tie2 may interact to activate downstream signaling during valve remodeling. It is unknown if Tie1 can homodimerize and signal in the absence of Tie2.

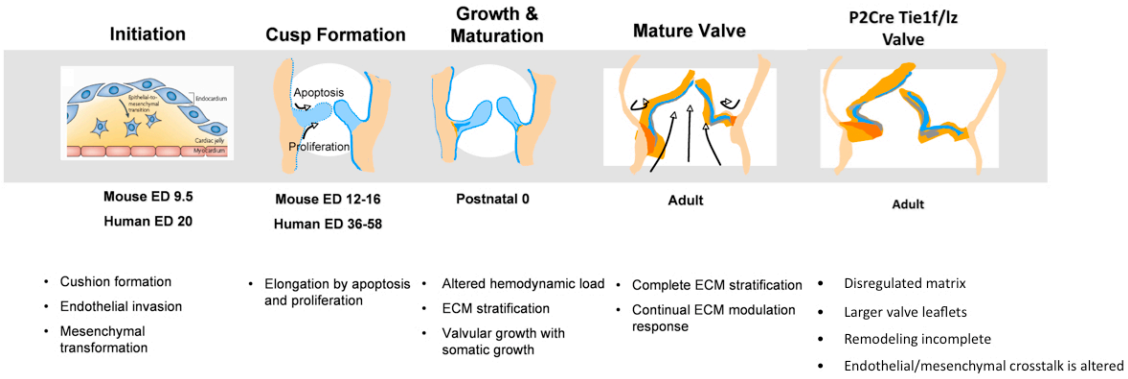

Figure 4.2. ***Abnormal remodeling of P2Cre Tie1$^{flox/lz}$ valves***. Depiction of normal valve development and remodeling, and a representation of normal mature valve leaflets as compared to P2Cre Tie1$^{flox/lz}$ valves. Note that ECM organization is disregulated.

More work needs to be done in this area to determine if similar events take place *in vivo*. We will examine levels of Tie2 phosphorylation in P0 $Tie1^{flox/flox}$ and P2Cre $Tie1^{flox/lz}$ valves to determine if an interaction between these receptors exist in this context. If Tie1 signals through Tie2, then we might expect that there will be changes in Tie2 phosphorylation due to a loss of Tie1. Conversely, if Tie1 signaling is independent of Tie2 in the remodeling heart valve then we would anticipate no alterations in Tie2 phosphorylation when Tie1 is deleted in the valves (Fig. 4.1). Preliminary results suggest that Tie1 is signaling through Tie2 in the developing valve, as Western blot analysis suggests that a loss of Tie1 in the valve endocardium leads to an increase in Tie2 phosphorylation. These data suggest that Tie1 may be responsible for repressing Tie2 activation in this context.

We are currently generating $Tie2^{flox/flox}$ mice in our laboratory using BAC recombineering. The $Tie2^{flox}$ construct contains loxP sites flanking the minimal promoter region, exon1 and a region of intron1. The construct is completed and is currently being injected into blastocysts to generate stable lines. We can use these animals to generate mice that lack both Tie1 and Tie2 (P2Cre $Tie1^{flox/lz}$ $Tie2^{flox/-}$) in the valve endocardium and determine if a genetic interaction exists between these two receptor tyrosine kinases in this context. We anticipate that an interaction exists between Tie1 and Tie2 in the context of the developing valve, therefore double knockouts of the Tie receptors in the valve will likely exacerbate the valve phenotype that we observe in the P2Cre $Tie1^{flox/lz}$ animals.

Furthermore, downstream targets of Tie1 signaling must be identified. $Tie1^{flox/flox}$ and P2Cre $Tie1^{flox/lz}$ valve tissue has been sent to collaborators for RNA

sequencing analysis. We hope that comparisons between the two tissues will provide insight into previously unknown regulatory networks not only for Tie1 but also for normal valve remodeling. We will also be able to make comparisons between wild type pulmonary and aortic valves to determine fundamental differences between these two seeming similar tissues.

We briefly discussed abnormal calcium deposits in 2-3 month old P2Cre $Tie1^{flox/lz}$ valves in Chapter III. We have examined deposition of osteopontin and osteoprotegrin in these valves and have found no differences between $Tie1^{flox/flox}$ and P2Cre $Tie1^{flox/lz}$ valves at any point in development. It is not certain if Tie1 directly regulates pathways involving calcium deposition, or if this is a secondary effect as a result of already weakened valve tissue. The increase in calcium deposition, however, likely contributes to the valvular insufficiency seen in the echocardiograms.

In summary, we have provided evidence to show that Tie1 is highly expressed in the developing heart valve. Aortic valve leaflets lacking Tie1 are larger than $Tie1^{flox/flox}$ leaflets, and more than half of P2Cre $Tie1^{flox/lz}$ animals die, likely due to valvular insufficiencies as a result of abnormal ECM deposition and deficient valve remodeling. We hypothesize that Tie1 is essential for proper cell-cell communication and that this communication may involve Tie2 signaling. The localization of Tie1 to the arterial side of the valve in the adult suggests that Tie1 may act to relay spatial-temporal information to the underlying mesenchyme. Alterations in this communication mechanism may be what results in insufficient ECM stratification. The identification of gene regulatory networks associated with Tie1 signaling and valve remodeling will aid in future efforts to

generate a bio-engineered heart valve that will have the potential to grow and remodel with patients who are not candidates for typical artificial valve replacements.

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