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Role of Fibroblast Growth Factor Signaling in Epicardial Development and Function

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By

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ABSTRACT OF THE DISSERTATION

The Role of FGF Signaling in Epicardial-Derived Cardiac Fibroblast Development and Migration

by

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In this thesis I examined the function of Fibroblast Growth Factor (FGF) signaling in epicardial cells. Epicardial cells serve as the outer layer of the heart and as a signaling center for the growing myocardium. In addition, during development, epicardial cells differentiate into vascular smooth muscle cells (vsmc) and interstitial fibroblasts. Epicardial cells undergo an epicardial to mesenchymal transition (EMT) to give rise to these various cell types, which are termed epicardial derived cells (EPDCs). Epicardial-derived vsmc are an essential component of the arterial network in the myocardium, and the interstitial fibroblasts become part of the fibrous skeleton of the myocardium. To populate the myocardium, EPDCs must migrate through the subepicardial space and into the compact myocardium. Very little is known about how this migration is initiated, maintained and guided.

Although, FGF7 and FGF10 are expressed in the myocardium their function was not known. Biochemically, these FGFs activate the b splice variants of FGFR1 and FGFR2. Here, I show that FGF10 siganls to the epicardium in vivo to induce migration of EPDCs. Furthermore, I found that FGF10 promotes migration of EPDCs that are fated to become interstitial fibroblasts. Embryonic cardiac fibroblasts are important during late heart gestation because they induce proliferation of cardiac myocytes. In hearts in which the FGF10/FGFR2b signaling pathway is disrupted, cardiac fibroblasts fail to migrate into the myocardium. I posit that fewer interstitial cardiac fibroblasts results in decreased cardiac myocyte proliferation and a smaller heart. Other growth factors like PDGFβ had been identified to activate migration of epicardial-derived vsmc but not cardiac fibroblast. Thus it appears that specific extracellular signaling pathways are required to control the migration of EPDC-lineages into the myocardium. These findings are an important contribution to the understanding of epicardial development. Epicardial and EPDC are not only important for heart development, but are thought to be essential for heart repair and regeneration due to the potential of these cells to differentiate in various cell types

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Chapter 1

Introduction

Summary

Epicardial Derived Cells (EPDCs) can differentiate into various cell types and migrate to be recruited in the periphery of endothelial vessels and as part of the fibrous skeleton of the heart. The ability of EPDCs to differentiate into multiple cell types suggests the potential implication for these cells in heart repair. Many scientists are attempting to learn about the developmental pathways required to activate them in the adult heart. The mechanisms that regulate the differentiation and migration of EPDCs are poorly understood. The work in this thesis led to the discovery of a new developmental pathway that regulates EPDC migration and indirectly myocardial proliferation. The observation that embryos lacking a splice variant of FGF receptor 2 that is expressed in the epicardium have small hearts suggested that a signal to the epicardium is required to control heart size. This phenotype prompted us to look closely at the development of the epicardium. To investigate the underlying mechanisms we studied heart development in embryos lacking epicardial FGF receptors and FGF ligands that could signal to these receptors. Our findings suggests that that growth of the myocardium is directly linked to decreased numbers of EPDCs. In addition these studies identify a signaling pathway that specifically regulates migration of epicardial-derived cardiac fibroblasts and supports a hypothesis that EPDC lineages within the myocardium are independently regulated by distinct growth factor signaling pathways. These findings are an important contribution to the understanding of heart development because it provides a basis to support the hypothesis that smooth muscle cell and cardiac fibroblast require the induction of independent signals to migrate into the myocardium.

Elucidating which other signals promote the migration of smooth muscle cells versus cardiac fibroblast could lead to a comprehensive understanding of epicardial cell activation.

This understanding is necessary to further design functional experiments to manipulate epicardial cells for heart repair. Below I summarize the literature on epicardial origins, development, differentiation, migration and function within the heart. In addition, I examine mechanisms of FGF signaling, heart development, the known functions of FGF in heart development and finally focus specifically on discussing the developmental process regulating the formation of the epicardium and epicardial derived cells.

Overview of the Fibroblast Growth Factor Family

The mouse family of Fibroblast Growth Factors (FGFs) is large and diverse. It is comprised of the intracellular FGFs (iFGFs), the canonical FGFs and the hormone-like FGFs (hFGF) (Itoh and Ornitz, 2008). The members of the iFGF family are: *Fgf11*, *Fgf12*, *Fgf13* and *Fgf14* (Smallwood et al., 1996). Intracellular FGFs are similar in sequence and structure to canonical and hormone-like FGFs but differ in function (Olsen et al., 2003). iFGFs exert their function inside the cell where they are known to bind to sodium channels and modulate neuron excitability (Laezza et al., 2007; Laezza et al., 2009; Lou et al., 2005; Xiao et al., 2007). It has been proposed that an iFGF-like protein was the common ancestor to all known FGFs. Unlike the intracellular FGFs, which do not bind to FGF receptors, the canonical and hormone-like FGFs are secreted outside the cells (Hanneken et al., 1994). These are able to signal by the binding of receptor tyrosine kinases from the FGF family of receptors (Rudland et al., 1974). The subfamily of hFGF is comprised of *Fgf15* (being *Fgf19* its ortholog in humans), *Fgf21* and *Fgf23* (Kharitonenkov et al., 2005).

Hormone-like FGFs are thought to arise in a recent event of vertebrate evolution; as a result they lost their high affinity for binding heparin and acquired their endocrine characteristics. A unique feature of hFGFs is the necessity of the cofactors αKlotho and βKloto to enhance signaling through FGF receptors (FGFR) (Kurosu and Kuro, 2009). In contrast, canonical FGFs signal in an autocrine/paracrine fashion due to their high affinity for heparan sulfate (Ornitz and Leder, 1992).

Canonical FGFs can be divided in the following subgroups based on sequence similarity: FGF1 subfamily (comprised of: *Fgf1*, *Fgf2*), FGF4 subfamily (comprised of: *Fgf4, Fgf6 and Fgf5*), FGF7 subfamily (comprised of: *Fgf3, Fgf7, Fgf10 and Fgf22*) and

FGF8 subfamily (comprised of: *Fgf8, Fgf17)* (Ornitz, 2003). Canonical FGFs signal through FGF receptor tyrosine kinases (which are: *Fgfr1*, *Fgfr2*, *Fgfr3* and *Fgfr4*) (Coutts and Gallagher, 1995). FGF receptors have a unique structure composed of three main parts: the extracellular region with three characteristic immunoglobulin-like domains, a single transmembrane domain, and a cytoplasmic domain containing the tyrosine kinase activity (Baird et al., 1988). The mRNA of the receptors bears an alternative splicing site that produces the "c-splice variants" and the "b-splice variant" of receptors *Fgfr1*, *Fgfr2* and *Fgfr3* but not of *Fgfr4* (Fujita et al., 1991).

It has been consistently observed that the c-splice variant is preferentially expressed in mesenchymal-like tissues and the b-splice variant is preferentially expressed in epithelial-like tissues. The ability of the receptors to undergo alternative splicing confers tissue and ligand binding specificity to the signal, making it ideal to control interactions between epithelia and mesenchyme during development (Yeh et al., 2003). To complement the specific pattern of expression of the receptor splice variants, the FGF ligands found within subgroups have higher affinity for specific receptors splice variants (Ornitz et al., 1996). For example, the members of the *Fgf7* family bind FGFR2b with high affinity but do not bind FGFR2c. Ligands within a subfamily and corresponding receptors of the same splice variantare typically found to be expressed simultaneously in adjacent tissues. Ontogeny requires a precise and fine-tuned network of signals to orchestrate the formation of a default outcome; therefore, continued monitoring of signal transduction is necessary to achieve the default state. The complementary expression of FGF ligands and receptors in epithelial and mesenchymal tissues allow the formation of signaling feedback loops that provide a way to monitor the timing, frequency and strength of FGF developmental interaction, ensuring

proper regulation of tissue development and morphogenesis (Niswander et al., 1994; Zhang et al., 2006).

The ligand binding domain is located at the second and third immunoglobulin domain of the FGF receptors (Mohammadi et al., 2005). Although the mechanism is still under investigation, one accepted mechanism of ligand binding is referred to as the "Ligand Dimer". In this mechanism, activation of the receptor occurs when one FGF ligand binds to the cell surface heparin sulphate proteoglycan (HSPG) chain and simultaneously induces receptor dimerization (Ornitz et al., 1995). Receptor dimerization causes a conformational change at the intracellular domain that activates the phosphotyrosine kinase and leads to *trans*-autophosphorilation of the cytoplasmic tails as they come closer to each other (Bae et al., 2010). Activation of the phosphotyrosine binding domain (PTB) in FGFR phosphorylates the scaffolding protein FRS2; this leads to the recruitment of other proteins that will activate downstream effector pathways (Zhang et al., 2008). Recruitment of SOS and GRB2 leads to activation of the downstream pathway RAS/MAPK (Kouhara et al., 1997). On the other hand, recruitment of GAB1 leads to the activation of downstream pathway PI3K/AKT (Ong et al., 2001). A different activation mechanism distinct from FRS2 is the recruitment of PLCγ to a different phosphotyrosine residue within the FGFR cytoplasmic tail which leads to activation of PKC and strengthens the MAPK pathway transduction by phosphorylating RAS (Mohammadi et al., 1991).

Regulation and modification of the FGF signal can occur at various levels of the signaling pathway to render context-dependent signaling. First, the restricted pattern of expression of the ligands, as well as the receptors, limits possible promiscuous interactions. Second, the alternative splicing of the receptors results in selective affinity of ligand-

receptor binding. Third, the interaction of the heparan sulfate proteoglycan with the receptor and ligand can modulate strength and specificity of signal as well as cell type-specific interaction due to the diverse spatial and temporal expression of the many types of HSPG. Finally, the Sprouty proteins can modulate FGF signaling by suppressing the MAPK transduction pathways in a feedback loop dependent manner (Lo et al., 2006). The FGF signal is interpreted based on the spatial-temporal and cellular context of the cell activated (Sivak et al., 2005). The activation of FGF signaling could cause changes in proliferation, differentiation, migration and survival. In this thesis, I investigate functions of FGF10, FGFR2b and FGFR1b in epicardial cells, the outermost layer of the heart.

Overview of Heart Development

The heart is the first organ to develop in the mammal and is required to provide oxygen and nutrient exchange to the growing embryo (Rudolph, 2010). Much research has focused on studying the origins of the cells that make up the heart. Un-committed precursor cells of the heart have been mapped to the primitive streak (Garcia-Martinez and Schoenwolf, 1993). Gastrulation at the primitive streak leads to the migration of the pre-cardiac mesoderm to the anterior side of the embryo (Christiaen et al., 2010). The cardiac mesoderm organizes into two groups of cells in each side of the midline (Nakajima et al., 2009). These cells form the primary heart field (PHF) that later comes together at the midline to form the primary tubular heart (Ramsdell and Yost, 1999).

A secondary heart field originally located below the PHF contributes more cells to the arterial and venous poles of the tubular heart (Moorman et al., 2007; Vincent and Buckingham, 2010). The tubular heart loops to the right, leading its posterior region to the anterior side of the embryo. Looping combined with myocardial expansion leads to the shaping of the cardiac chambers (Taber et al., 2010). Soon after heart looping, another cell population originates at the sinuous venosus — the proepicardium — and travels to the heart to form the epicardial layer (Dettman et al., 1998). Another sprouting of cells from the sinous venosus but distinct from the proepicardium differentiates to the endothelial cells of the coronary vessels (Red-Horse et al., 2010). As development continues, endocardial cells lining the heart lumen undergo epithelial to mesenchymal transition (EMT) to give rise to the cardiac jelly and cardiac cushions (Person et al., 2005). Rapid growth of the myocardium aids with the formation of the chamber septa, these are the interventricular and atrial septae. Another population of cells from the neural crest travels though the pharyngeal

arches into the outflow tract (OFT). These cells contribute to form the septation in the pulmonary trunk and aorta. These cells also contribute to form the electrical network of the heart (Brown and Baldwin, 2006).

FGF Function in Heart Development

FGF signaling is important during different events in heart development. Initially, expression of FGF ligands and FGF receptors was observed within the developing heart, suggesting a putative role for these molecules (Zhu and Lough, 1996). Early on it was shown that FGF2 was expressed in stage six of the chicken embryo and that *Fgf2* antisense oligonucleotides could inhibit proliferation of cultured pre-cardiac anterior avian mesoderm (Sugi et al., 1993). Proliferation of pre-cardiac mesoderm is known to be important in the generation of the tubular heart. Likewise expression of FGF1 and FGF4 were reported in the chicken myocardium from stages eleven to twenty four (Zhu et al., 1996). Expression of *Fgf7* was also reported in cardiomyocytes as early as embryonic day 10.5 (E10.5) but to date no functional *in vivo* evidence has been examined to attribute a role of FGF7 in heart development (Mason et al., 1994).

The first *in vivo* evidence of FGF signaling controlling myocyte proliferation came from a study in chicken where an *Fgfr1* dominant negative retroviral vector introduced during the first week of chicken development was able to decrease myocardial proliferation (Mima et al., 1995). FGF signaling was further implicated in heart development when a null mutant embryo of *Heartless,* a Drosophila FGF receptor homolog was found unable to induce the pre-cardiac mesoderm and yielded a heartless fly (Beiman et al., 1996). Similarly, in vertebrates, *Fgf8* was found to be expressed in the cardiac endoderm that lays contiguous with the cardiac-mesoderm. Physical removal of this endoderm caused downregulation of cardiac markers; complementary to this observation, external addition of FGF8 could restore the expression of these markers (Alsan BH, 2002). After the discovery of the secondary heart field in 2001 it was shown that expression of an *Fgf10* enhancer trap

bearing β-galactosidase mapped myocytes derived from the secondary heart field in the OFT and right ventricle (Waldo et al., 2001). This observation suggested that FGF10 expressing cells were exclusively part of the secondary heart field lineage proposed to be of independent origin from the PHF (Kelly et al., 2001). Although these studies implicated a role for FGF8 and FGF10 in development and expansion of the cardiac fields, *Fgf8* null mutants only displayed disruption in OFT and right ventricle formation but no major defect in heart development and *Fgf10*$^{-/-}$ only showed a defect in abnormal positioning of the ventricular apex. Recent studies conditionally knocking both *Fgf8* and *Fgf10* from the mesoderm confirmed that these ligands have overlapping functions at the secondary heart field and gene dosage is important for the penetrance of OFT defect and pharyngeal arch artery formation (Watanabe et al., 2010). Specific SHF deletion of *Fgf8* and FGF receptors have also been generated to conclude that FGF signaling in the SHF acts in an autocrine manner.

Similarly to its functional effects in early heart development, FGF signaling functions in other stages of heart development. For example, it has been found that *Fgf4* expressed in cardiac cushion mesenchyme can cause proliferation of these cells. Microinjection of *Fgf4* protein *in vivo* to chicken embryos resulted in increased proliferation of cushion mesenchyme, providing evidence of functional requirement of *Fgf4* during this process (Sugi et al., 2003). Heart growth can happen by proliferation of cardiac myocytes and other cell types within the heart or by cellular hypertrophy. Proliferation of cardiomyocytes is the preferred mechanism of heart growth during heart development. It has been postulated that two significant myocardial expansions happen after heart looping. One happens immediately after looping and another one during late gestation (Lavine and Ornitz,

2008). The midgestational bout of myocardial proliferation happens simultaneously with the formation of the epicardial layer (Lavine and Ornitz, 2008). This coincident development has been proposed to be part of the mechanism of myocardial proliferation during midgestation. The epicardium is considered a center of proliferative signals for the myocardium (Sucov et al., 2009).

The first observation pointing to such a mechanism came from studies of the retinoic acid receptor alpha (RXRα). Deletion of this receptor resulted in hypoplastic ventricles. RXRα was shown to function in the epicardium and proposed to regulate secretion of growth factors from the epicardium that in turn could stimulate myocardial proliferation (Chen et al., 2002). These growth factors were later determined to be FGF9 and FGF16. Studies characterizing the *Fgf9*$^{-/-}$ embryos revealed these embryos had a small heart due to a decrease in myocardial proliferation. Complementary deletion of both *Fgfr1* and *Fgfr2* specifically in the myocardium recapitulated the phenotype observed in *Fgf9*$^{-/-}$hearts. Moreover, FGF9 and FGF16 were found to be expressed in the epicardium and endocardium, leading to the hypothesis that FGF signaling contributions from epicardium and endocardium regulate cardiac growth during midgestation (Lavine et al., 2005). Consistent with these findings *Fgf16*$^{-/-}$ hearts also display decreased embryonic proliferation consistent with a synergistic role with FGF9 during heart development (Hotta et al., 2008). Another important role of FGF signaling during midgestation is that it regulates the timing of formation of the primitive vascular plexus by indirectly activating SHH signaling. It is still unknown how FGF regulates SHH in the epicardium (Lavine et al., 2006).

FGF signaling is also important during homeostasis and maintenance of the adult heart. The earliest observation of FGF function in the adult heart came from studies of gene

expression. For example, expression of different FGF ligands in cardiomyocytes was observed after induced cardiac stenosis (Bernotat-Danielowski et al., 1993). These data suggested that FGF signaling was required during heart homeostasis. More recently, FGF2 has become the center of much research since it has been found to have cardioprotective capabilities (Kardami et al., 2007). The cardioprotectiveness by FGF2 was first observed in isolated models of ischemia-reperfusion. In this study, administration of FGF2 after ischemia lead to improvements in mechanical function (Padua et al., 1995). Consistent with the observed protective role of FGF2 during heart ischemia, transgenic hearts overexpressing *Fgf2* displayed higher myocyte viability (Sheikh et al., 2001).

Overexpression of FGF2 was also protective in cardiac infarct models (House et al., 2003; House et al., 2005; House et al., 2007). Administration of FGF2 to myocardial infarcted hearts resulted in a decrease of ischemia activated cell dead and arrhythmias. FGF2 can be translated in two different isoforms; one is low molecular weight FGF2 (lo-FGF2), and the other is high molecular weight FGF2 (hi-FGF2) (Liao et al., 2009). The specific role of each isoform in cardioprotection is unclear (Liao et al., 2007). Currently, knockouts for both isoforms have been generated for cardioprotection. Both isoforms have similar effects immediately after acute ischemia. The main difference found was that hi-FGF2 had a stronger induction of PKCζ and p70 S6 kinases (Jiang et al., 2009). Another possible FGF involved in adult heart homeostasis is FGF16. Interest in FGF16 has increased due to its expression peak in the perinatal heart. Particularly intriguing is the existence of an NF-κB element in FGF16's promoter sequence. The NF-κB site has been found to be responsive to NF-κB induction by isoproterenol, suggesting that it could be activated upon NF-κB binding (Sofronescu et al., 2010).

Overview of Epicardial Development

Epicardial development initiates with the formation of the proepicardium (Figure 1). Development of the proepicardium has been studied in: *Xenopus laevis*, zebrafish, *Acipenser naccari* and mammals, suggesting that it is an evolutionarily conserved biological process. (Icardo et al., 2009; Jahr et al., 2008; Serluca, 2008). The proepicardium is a cauliflower-like bundle of cells located anterior to the inflow of the heart (Nesbitt et al., 2006). Proepicardial cells travel to the atrioventricular grove of the heart. Further migration over the myocardial surface results in the formation of the epicardial mantle (Mikawa and Gourdie, 1996). These now-termed epicardial cells undergo an epithelial to mesenchymal transition to invade the myocardium where they differentiate into cardiac fibroblast and vascular smooth muscle cells.

Proepicardial and epicardial cells have the potential to differentiate into various cell types, making them plausible candidates to elicit repair in the adult heart (Winter and Gittenberger-de Groot, 2007). Studies have tried to trace the lineage of the proepicardial cells. Using Cre-loxP lineage tracing it was observed that proepicardial cells are derived from *Nkx2-5* and *Isl1* progenitors (Zhou et al., 2008b). Consistent with this observation, *Nkx2-5* null mutants fail to form a proepicardial structure, in contrast, the proepicardium forms in the *Isl1* knockout mice. Similar to *Nkx2-5*$^{-/-}$ the *Gata4*$^{-/-}$ do not form a proepicardial organ, as a result the heart does not develop an epicardial layer (Watt et al., 2004). These studies give insight into the lineage origin of proepicadial cells, but do not elucidate how proepicardial cells are specified to the proepicardial fate from myocardial precursors (van Wijk and van den Hoff, 2010). Recent studies in chicken indicated that a balance between

BMP2 and FGF2 signaling is responsible for the early separation between myocardial progenitors and proepicardial progenitors (van Wijk et al., 2009). BMP is responsible for driving myocardial cell fate and FGF is responsible for driving proepicardial cell fate.

Once proepicardial cells are specified, they express markers for Wt1, TBX18, TCF21 and capsulin, amongst others (Hatcher et al., 2000; Lu et al., 1998; Moore et al., 1999; Robb et al., 1998). Expression of these markers was used as a symbol of lineage commitment in zebrafish studies. These studies suggested that TBX5a and BMP4 are important for proepicardial commitment (Hatcher et al., 2004). Mutant fish of either *tbx5*a or *acvr1l* (the receptor for BMP4) repressed expression of proepicardial markers TCF21 and TBX18. Normal expression of BMP4 in *tbx5a* mutants lead to the hypothesis that TBX5a promotes competency of the lateral plate mesoderm to commit to the proepicardial cell fate. Complementary BMP4 signaling was responsible for guaranteeing their commitment later on (Liu and Stainier, 2010).

Proepicardial development has been studied in chickens and in mice, therefore, it is important to mention key differences between proepicardial and epicardial development between these species. In the chicken, the proepicardium develops from bilaterally symmetrical buds of proepicardial cells that form a final asymmetrical structure on the right side of the embryo (Nahirney et al., 2003). In the mouse, both proepicardial buds develop uniformly and symmetrically (Schulte et al., 2007). FGF8 and Snail1 were found to control the left-right (L-R) asymmetry of the chicken proepicardium. Inhibition of *snail* in the right side prevented proepicardial formation. Overexposure of FGFR1 and FGF8 on the left side of the proepicardial field was sufficient to drive ectopic proepicardial formation (Schlueter and Brand, 2009). Proepicardial asymmetry is lost in the mouse, but it would be interesting

to investigate if manipulation of these signals could generate an asymmetric proepicardium in the mouse.

Once the proepicardium has been specified and formed, proepicardial cells have been reported to express markers of different cell fates. Although it is not proven yet, this observation has promoted the idea that cells within the proepicardium are already specified to become cardiac fibroblast or smooth muscle cells prior to their migration to the epicardium. Another possibility is that EPDCs differentiate as they migrate into the myocardium. More research in this area of epicardial development is needed to discern which of these two possibilities is correct.

Migration of proepicardial cells to the atrioventricular grove has been studied in chickens and mice using SEM and histological analysis. In chickens, proepicardial cells start migrating at stage HH14 and form an extracellular matrix bridge made of proteoglycans, heparin sulfate and fibronectin that guides the proepicardial cells into the heart (Nahirney et al., 2003). Migration of proepicardial cells in mouse starts at E9. In areas of the proepicardium that are closest to the bare myocardium, cells start to swell and form multicellular villous clusters that extend towards the heart. Once these clusters have achieved sufficient length to contact the heart, the tip of the cluster touches the nude myocardium. Beating pulls the tip off the cluster leaving the epicardial cells attached to the myocardium. Villous tips detach and float to reach the myocardium at areas where its unable to touch and attach to the nude myocardium (Rodgers et al., 2007).

Very little is known about the molecules directing the migration of proepicardial cells towards the myocardium. Evidence that adhesion is important for proepicardial

migration can be found in the phenotype of the integrin *α4β1* knockout mouse. α4β1 is a cell adhesion molecule. *α4β1* null mice display defects in villous budding (Sengbusch et al., 2002). VCAM1, the ligand for α4β1, can recapitulate the defects seen in the receptor null embryos (Pae et al., 2008). Another molecule that presents a similar phenotype when inactivated is *RXRα. This* nuclear receptor has been found to have a role during proepicardial migration. Proepicardial cells of $Rxr\alpha^{-/-}$ hearts have a higher rate of apoptosis compared to controls and display a decreased number of villous clusters contacting the myocardium. Furthermore, formation of the epicardial layer is disrupted due to detachments of epicardial cells and incomplete coverage of the myocardium. These defects could be attributed to a decreased number of epicardial cells migrating to envelop the heart (Jenkins et al., 2005). Deletion of the glycoprotein Podoplanin results in a smaller proepicardial organ, which impairs migration of proepicardial cells, upregulates ecadherin at the epicardium and results in decreased amounts of Epicardial Derived Cells within the myocardium (Mahtab et al., 2009). Also, both overexpression and repression of TBX5 in chicken leads to inhibition of proepicardial migration to cover the myocardium (Hatcher et al., 2004).

After proepicardial cells have migrated, they need to cover the heart and attach their basement membrane to the myocardium. It is hypothesized that adhesion molecules are important to mediate attachment of the epicardial layer to the myocardium. Interestingly, in addition to problems of proepicardial migration disruption of *α4β1, VCAM1, rxrα*, *podoplanin* and t*bx5* function also results in problems adhering to the nude myocardium. Typically, a characteristic phenotype of epicardial detachment is the formation of epicardial

sacs that lack direct contact between the epicardium and the myocardium (Jenkins et al., 2005; Pae et al., 2008; Sengbusch et al., 2002).

After the epicardium has covered the heart and properly adhered to the myocardium, a subset of epicardial cells undergo epicardial to mesenchymal transitions. Epicardial cells become mesenchymal and delaminate into the subepicardial mesenchyme (Gittenberger-de Groot et al., 2010; Perez-Pomares et al., 1998). Once in the subepicardial mesenchyme, epicardial derived cells migrate further into the myocardium. These cells are named EPDCs.

The observation that epicardial mesenchyme gives rise to a large part of the adult heart was made during the 1990s by several different laboratories using clonal retrovirals to trace the lineage of these cells (Dettman et al., 1998; Mikawa and Gourdie, 1996; Perez-Pomares et al., 1997). EPDCs differentiate into vascular smooth muscles cells and pericytes which outline the endothelial vascular plexus. They also give rise to interstitial fibroblasts, a cell type that has been shown to control myocyte proliferation (Weeke-Klimp et al., 2010). Therefore, failure to properly adhere to the myocardium, undergo EMT, migrate into the myocardium and differentiate could result in cardiac heart defects. Researchers in the field are trying to elucidate how epicardial cells become activated and competent to undergo EMT. Canonical EMT occurs by the dissolution of cell-cell junctions, loss of apical-basal polarity, and finally the modification of cytoskeletal proteins to a mesenchymal phenotype that permits — in the case of epicardial cells — to coalesce within the subepicardial space (Thiery et al., 2009).

It is not known yet if epicardial EMT happens throughout canonical mechanisms of EMT. The epicardium as discussed earlier is not a typical epithelial tissue because its

lineage is traced to a mesothelial origin. Support for this view comes from data identifying expression of mesenchymal markers like vimentin in the epicardial layer (Wu et al., 2010). In an effort to understand epicardial EMT various laboratories have looked at prototypical proteins of EMT in the epicardium. Downregulation of e-cadherin is considered a hallmark event to initiate EMT. In the epicardium it has been found that upregulation of e-cadherin leads to disruption of epicardial EMT. Two molecules in the epicardium seem to regulate e-cadherin expression, VCAM1 and Wt1. VCAM1 was observed to promote the epithelial-like state of epicardial cells by decreasing the generation of stress fiber and maintaining expression of e-cadherin. The effects of VCAM1 in epicardial cells were found to counteract the EMT promoting effects of TGFβ3 (Dokic and Dettman, 2006). The other molecule found to regulate epicardial EMT though modulation of e-cadherin expression is Wt1. Mouse embryos lacking Wt1 specifically in the epicardium by deletion with Gata5Cre were found to have defects in EMT yielding a smaller heart and malformation of the coronary vessels. Hearts lacking Wt1 were shown to have an increase in amounts of e-cadherin expression within the epicardial layer, suggesting that downregulation of e-cadherin is important for Wt1-induced EMT. Wt1 binding sequences were found within the e-cadherin promoter and ChIP analysis confirmed the existence of the interaction *in vivo*. Additionally, Wt1 directly binds and regulates expression of Snail1, another gene that is typically involved in canonical EMT (Martinez-Estrada et al., 2010). Other known regulators of EMT are found to be expressed in the epicardial layer. For example, Slug is expressed in all cells of the epicardium and it has been proposed to promote competency of epicardial cells to undergo EMT, but no functional experiments have been done to prove this hypothesis (Carmona et al., 2000). Another regulator or epicardial EMT in chicken is

ETS1/2. Antisense oligonucleotides for ETS1/2 halted EMT of epicardial cells and lead to multiple cardiac defects including a thinner myocardium. Growth factors have also been shown to control epicardial EMT (Lie-Venema et al., 2003). TGFβ signaling has been implicated in modulating EMT of epicardial cells, but it is not yet clear whether it stimulates or inhibits EMT since studies from different researchers are contradictory. Studies in the chicken embryo showed that addition of TGFβ2 and TGFβ3 inhibit EMT of epicardial monolayers and EMT of explanted hearts. This contrasts with other results that showed that treatment with TGFβ3 to chicken explanted hearts activated EMT. It is important to mention that most studies suggested that TGFβ signaling promotes EMT and inhibits the epicardial phenotype (Compton et al., 2006; Dokic and Dettman, 2006; Morabito et al., 2001). These findings support the idea that epicardial EMT occurs via a canonical EMT mechanism. Recent reports on the effect of β-catenin in epicardial EMT argue that asymmetrical cell division is part of the mechanism. β-catenin epicardial knockouts were found to have decreased amounts of EMT causing defects in cardiac morphology. This decrease in EMT was due to the structural effect of β-catenin in regulating the spindle orientation of epicardial asymmetrical divisions (Wu et al., 2010). This data taken together raises new questions. How are the mechanisms of asymmetrical cell division coupled with the prototypical mechanisms of EMT? Traditionally defects in epicardial EMT have been interpreted in the field as a failure of the epicardial-derived cell to coalesce into the subepicardium and/or invade the myocardium. This interpretation makes it very difficult to distinguish between varied phenotypes that could account for this result. For example, EMT defects, migration defects or both. Another interesting question is what could be the role, if any, of the subepicardial mesenchyme during EMT?

Subsequent to EMT, the epicardial derived cell migrates further into the myocardium to reach their final destination within the heart architecture. Smooth muscle cells and pericytes migrate to the periphery of the coronary vessels and the interstitial fibroblasts migrate within the cardiac myocytes. Mechanisms regulating this migration are poorly understood. One could imagine that these cells require a very complicated array of signals to move toward their final destination. These signals should control polarity, differentiation and chemotacticity of the EPDCs. Not to mention that the length of the migration could, in certain cases, be very long; therefore, extended mechanisms of induction are required to reach the final developmental goal. Very few researchers have examined the mechanisms of epicardial-derived cells migration. One researcher showed that absence of connexin 43 caused decrease migration of EPDCs. This was due to the disruption of cell polarity (Rhee et al., 2009). The obstruction of migration in these cells leads to secondary heart malformations. The final fate of cells lacking connexin43 was not determined. PDGFRβ was also found to regulate epicardial-derived cell migration (Mellgren et al., 2008). Defects in formation of the coronary vessels and absence of regional vascular smooth muscle in hearts with epicardial deletion of PDGFRβ lead to the hypothesis that PDGFRβ is important for migration of specifically vsmcs. Expression of EphrinB1 and EphrinB3 localized to the epicardium of the chick embryo. Explant cultures of epicardial monolayers treated with EprhinB1 was able to induce migration of the monolayer (Wengerhoff et al., 2010).

Many questions arise from these experiments. How is migration of smooth muscle cells versus interstitial fibroblast directed towards different compartments of the heart? When does terminal differentiation of EPDCs occur? Are the epicardial derived cells predetermined to a cell fate before undergoing EMT? What are the signals that direct

differential migration and differentiation of migratory EPDCs? How do epicardial-derived smooth muscle cells couple their insertion into the coronary vessels' architecture and vice versa? What is the function of the epicardial-derived interstitial fibroblast during development and after? Importantly, many of the molecules found to play a role in epicardial mesenchymal transformation or migration have also been implicated in regulating differentiation of these cells into smooth muscle cells, but none have been correlated with the generation of cardiac fibroblast (Mahtab et al., 2009; Mellgren et al., 2008; Wengerhoff et al., 2010). It is important to mention that epicardial-derived cells also have been found to give rise to cardiac muscle in mice. Recently, two labs reported that epicardial-derived cells in the mouse could give rise to myocardial cells. They used inducible Cre-Loxp lineage tracing to follow the fate of the epicardial cells in mouse embryos. Although their conclusions are very interesting, the results are controversial because the Cre markers used are not exclusive to the epicardium and continue to be expressed in the mouse heart at later stages (Cai et al., 2008; Zhou et al., 2008a).

Previously, I mentioned that the developing heart has different mechanisms to induce myocardial proliferation throughout development. During midgestation, for example, FGFs emanating from the epicardium activate FGF receptors directly in the cardiac myocytes to induce proliferation (Lavine et al., 2005). Little was known about the mechanism of cardiac expansion during the late embryonic stages. However, it was recently shown that cardiac fibroblasts are required for the rapid proliferation of cardiomyocytes during late gestation. A surge in the development of embryonic cardiac fibroblast correlates with the late gestation cardiac myocyte proliferation. Srivastava et al showed that embryonic cardiac myocytes, but not adult cardiac myocytes, could induce myocardial proliferation. It was elucidated that

fibronectin and collagen produced by the cardiac fibroblast signals to the cardiac myocyte through binding the β1 integrin receptor. This interaction is promoted by HBEGF produced by cardiac fibroblast. This data point to HBEGF as the growth factor needed during late gestation to induce myocardial proliferation. Conditional deletion of β1 integrin in the myocardium lead to the formation of a smaller heart suggesting that the interaction between ECM and β1 integrin is seminal to cardiomyocyte proliferation during late embryonic development (Ieda et al., 2009). Interestingly, the major source of embryonic cardiac fibroblasts is provided by the epicardial-derived cells. Furthermore, epicardial EMT and migration occurs actively from E13.5 to E17.5 in the embryo which correlates with the time of ventricular compaction. As with the myocardial knockout of β1 integrin, many mouse models of proteins defective in epicardial EMT and migration mentioned previously develop a smaller heart. This observation suggests that the amount of embryonic cardiac fibroblasts migrating into the heart might be critical to reach the proper size of the heart. The role of cardiac fibroblasts during heart development is not well studied. In the adult, cardiac fibroblasts comprises the largest non-myocyte population of cells in the heart and is known to be necessary for extracellular matrix synthesis, a very important component of the cardiac skeleton (Snider et al., 2009).

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Figure 1.

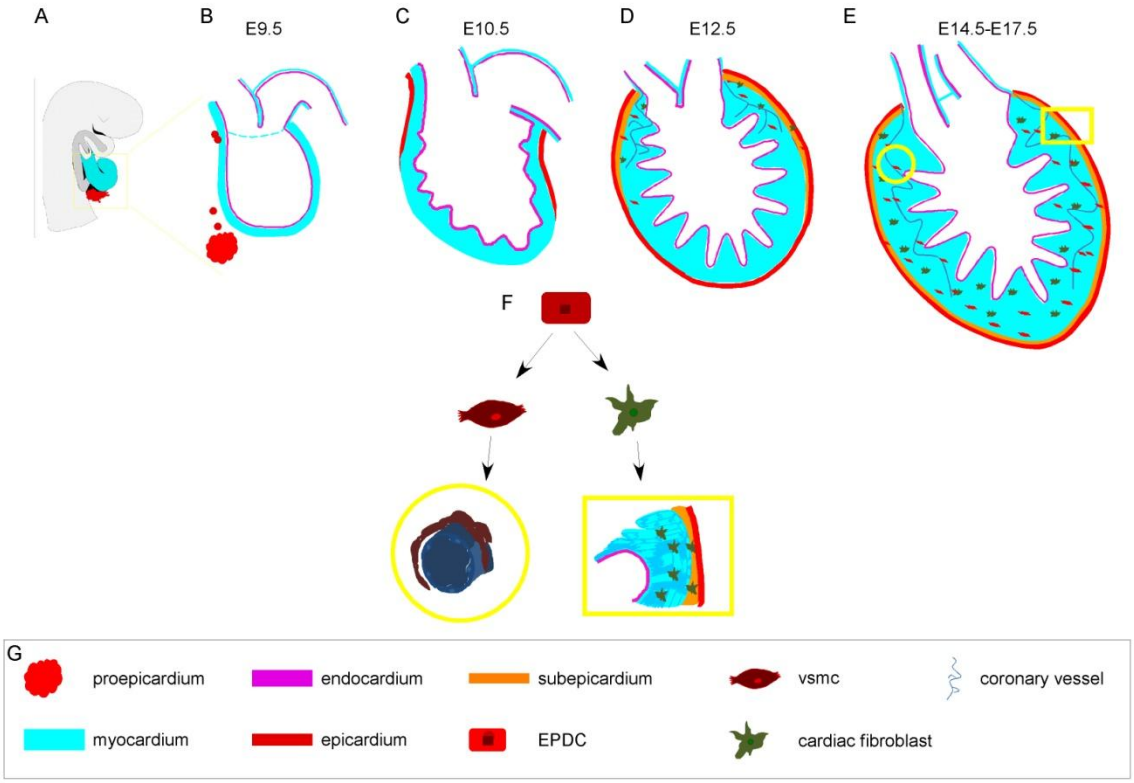
A
B
E9.5
C
E10.5
D
E12.5
E
E14.5-E17.5
F
G
proepicardium
endocardium
subepicardium
vsmc
coronary vessel
myocardium
epicardium
EPDC
cardiac fibroblast

Figure 1.

Overview of epicardial development. (A) At E9.5 the heart is looped and the proepicardium is a bundle of cells attached to the ventral body wall. The yellow square marks the area zoomed in (B). (B) The heart is only composed of two layers: the endocardium (pink) and the myocardium (cyan). Proepicardial villous cysts (red) grow allowing proepicardial cells to contact the heart or travel through the pericardial fluid to contact the heart. (C) Proepicardial cells migrate in a gradient from base to apex over the surface of the heart covering it. (D) As the epicardium covers the heart, the subepicardial mesenchyme (orange) forms to provide a space for a subset of epicardial cells to undergo an epithelial to mesenchymal transition. The cells that undergo EMT and migrate are termed EPDCs. Epicardial cells differentiate into either vascular smooth muscle cells (red) or cardiac fibroblasts (green). Simultaneously, the primitive vascular tree formed by sprouting of the sinous venosus also grows in a gradient from base to apex (blue). (E) The heart continues growing and the vascular plexus remodels by incorporating the vascular smooth muscle cells. The yellow circle and square marks a zoomed area shown in (F). (F) Shows the lineage fate of the epicardial cells and their final location in the heart. Vascular smooth muscle cells are recruited to the endothelial tubes and cardiac fibroblast become part of the fibrous skeleton of the heart. (G) Legend.

Chapter 2

FGF10/FGFR2b signaling is essential for cardiac fibroblast development and growth of the myocardium

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Abstract

The epicardium serves as a source of growth factors that regulate myocardial proliferation and as a source of epicardial-derived cells, interstitial cardiac fibroblasts and perivascular cells, which populate the compact myocardium. In addition to epicardial-derived growth factors, such as fibroblast growth factor 9 (FGF9), cardiac fibroblasts are also necessary for myocardial growth. The mechanisms that regulate epicardial function during development and the mechanisms that regulate the formation of epicardial-derived cells are poorly understood. Here, we identify a myocardial to epicardial fibroblast growth factor (FGF) signal, mediated by FGF10 and FGFR2b that is essential for movement of cardiac fibroblasts into the compact myocardium. Inactivation of this signaling pathway results in fewer epicardial derived cells within the compact myocardium, decreased myocardial proliferation and a resulting smaller, thin-walled heart.

Introduction

The epicardium comprises the outer layer of the heart and provides a source of cardiac fibroblasts, vascular smooth muscle cells and pericytes during heart development (Cai et al., 2008; Dettman et al., 1998; Marguerie et al., 2006; Merki et al., 2005; Mikawa and Fischman, 1992 ; Mikawa and Gourdie, 1996; Snider et al., 2009). The formation of the epicardial layer begins after heart looping at E9.5 in the mouse (Kalman et al., 1995). Epicardial cells arise from the proepicardial organ, which is a transient structure located close to the sinus venosus on the ventral body wall (Hiruma and Hirakow, 1989; Vincent and Buckingham, 2010). Cells from the proepicardium migrate to the atrioventricular groove and then from the base of the heart to the apex, covering the heart as a single cell layer. Concurrently, a capillary plexus grows from the dorsal atrioventricular groove and expands towards the apex and ventrally to envelope the entire heart (Kattan et al., 2004; Lavine et al., 2006; Red-Horse et al., 2010). As epicardial cells migrate to cover the heart, a subset of epicardial cells undergo epithelial to mesenchymal transitions (EMT) and delaminate from the epicardium. These cells are termed epicardial derived cells (EPDCs). Once EPDCs acquire mesenchymal phenotype they migrate further into the compact myocardium where they differentiate into smooth muscle cells and cardiac fibroblasts. PDFGRβ, Alk5 are factors that regulate epicardial-derived vascular smooth muscle cells (Mellgren et al., 2008; Sridurongrit et al., 2008). In contrast, factors that regulate migration of cardiac fibroblast have not been identified. These differentiated smooth muscle cells and cardiac fibroblasts become part of the mature coronary vasculature and interstitial mesenchyme of the heart.

The FGF family is comprised of 18 signaling ligands and four receptors (FGFRs) (Itoh and Ornitz, 2008; Ornitz and Itoh, 2001; Turner and Grose, 2010). *Fgfr1*, *Fgfr2* and *Fgfr3* undergo alternative splicing that results in b and c splice variants (Dell and Williams, 1992; Werner et al., 1992). The b splice variants are preferentially expressed in epithelial and epithelial-like tissues, such as the epicardium (Marguerie et al., 2006). In contrast, c splice variants are preferentially expressed in mesenchymal tissues. FGF ligands are classified in subfamilies based on phylogenetic similarities. Members of each subfamily of FGFs share similar biochemical properties, such as affinity for specific FGFRs and FGFR splice variants (Itoh and Ornitz, 2004). FGFR2c is efficiently activated by members of the FGF9 subfamily (FGF9, FGF16 and FGF20) (Ornitz et al., 1996; Zhang et al., 2006). In contrast, FGF3, FGF7, FGF10 and FGF22 are ligands that activate FGFR2b. Downstream FGF signal transduction can proceed via three main pathways: Ras/MAPK pathway, phospholipase Cγ (PLCγ)/Ca^{2+} pathway, and the PI3 kinase/Akt pathway (Eswarakumar et al., 2005; Lemmon and Schlessinger, 2010).

The expression of several FGFs and FGFRs in cardiac and vascular mesoderm, mesothelium and endoderm suggests an important role for these molecules in development of the heart. In zebrafish and avian models, FGF signaling has been implicated as important for epicardial cells to undergo EMT, to enter the myocardium, and potentially to differentiate into coronary smooth muscle cells, interstitial cardiac fibroblasts, coronary endothelial cells and cardiomyocytes (Lepilina et al., 2006; Mikawa and Gourdie, 1996; Morabito et al., 2001; Perez-Pomares et al., 2002). The FGF ligands, FGF1, FGF2 and FGF7 were shown to stimulate EMT in cultured epicardial cells (Morabito et al., 2001) and pharmacological inhibition of FGF signaling impaired

epicardial EMT (Pennisi and Mikawa, 2009); however, retroviral expression of a dominant negative FGFR1 in epicardial and endothelial precursors in the proepicardial organ did not affect epicardial EMT but did impair the progeny of proepicardial-derived cells from invading the myocardium (Pennisi and Mikawa, 2009). These studies suggest that FGF signaling is necessary for epicardial and endothelial development but do not define the precise FGF signaling pathways that regulate each of these lineages or determine whether signaling is direct or indirect.

In previous studies, we identified an epicardial to myocardial FGF signaling pathway, in which FGF9, expressed in the epicardium, signals to FGFR1c and FGFR2c in the myocardium to control myocardial proliferation and indirectly, vascular formation (Lavine et al., 2005). Several studies have identified expression of *Fgf7* and *Fgf10* in the developing myocardium and one study showed that mice lacking the b splice variant of *Fgfr2 (Fgfr2b*$^{-/-}$*)* developed a thin-walled heart (Marguerie et al., 2006; Morabito et al., 2001). These observations suggest that FGF signals emanating from the myocardium might directly regulate epicardial development or function. In this study, we show that FGF10 signals to the epicardium through FGFR1 and FGFR2b. In turn, these receptors control movement of EPDCs into the compact myocardium. Inactivation of this pathway results in fewer EPDCs within the compact myocardium and results in reduced cardiomyocyte proliferation and a smaller heart.

Results

FGF10 signaling to the epicardium regulates heart size.

The phenotype of *Fgfr2b*$^{-/-}$ mice and the presence of appropriate ligand expression in the heart suggested that FGF signaling might regulate epicardial function and indirectly myocardial development. Such a signal, from cardiomyocytes, fibroblast or vascular cells in the compact myocardium may constitute a feedback loop to the epicardium to control heart size during development. To test the hypothesis that a myocardial to epicardial signal could regulate development of the heart, we measured the cross-sectional area of the whole heart and the thickness of the compact myocardium in *Fgfr2b*$^{-/-}$, *Fgf7*$^{-/-}$ and *Fgf10*$^{-/-}$ embryos at several developmental time points. At earlier stages (E13.5 to E15.5), *Fgfr2b*$^{-/-}$ hearts appeared normal in external morphology (data not shown), but at embryonic day 17.5 (E17.5), *Fgfr2b*$^{-/-}$ embryos and *Fgf10*$^{-/-}$ embryos both appeared smaller (Figure 1A-D). The width of the compact myocardium of *Fgfr2b*$^{-/-}$ embryos as shown by (Marguerie et al., 2006) and *Fgf10*$^{-/-}$ embryos were significantly ($p<0.02$, $p< 0.003$, respectively) thinner than age-matched control embryos (Figure 1A'-D'). *Fgf7*$^{-/-}$ embryos did not show a significant difference in thickness of the compact myocardium. We were able to generate two *Fgf7*$^{-/-}$*;Fgf10*$^{-/-}$ embryos at E17.5, and the hearts of both appeared smaller in size compared to *Fgf10*$^{-/-}$ hearts, suggesting possible redundancy with FGF7. *In utero* echocardiography also showed a decrease in diastolic wall thickness in E17.5 *Fgfr2b*$^{-/-}$ hearts when compared to control littermates (Figure 1G-J and supplemental Figure 1). Consistently, the interventricular septum of *Fgfr2b*$^{-/-}$ hearts was also thinner (Figure 1K). We also examined the formation of coronary vessels in *Fgfr2b*$^{-/-}$ and *Fgf10*$^{-/-}$ hearts. Endothelial vessels formed normally compared to controls

(Supplemental Figure 2). Taken together, these data suggest that FGF10 signals to FGFR2b during late gestation to control heart size.

Based on these phenotypes, we hypothesized that FGFR2b should be expressed in epicardial cells and FGF10 should be expressed in cardiac myocytes or other cell-types within the compact myocardium. *In situ* hybridization localized *Fgf10* mRNA expression within the myocardium of wild type hearts at E17.5 (Figure 2A-B). No expression was observed in $Fgf10^{-/-}$ hearts or with a sense probe. FGFR2 protein expression was examined using an antibody that detects both the b and c splice forms (Figure 2C-D). In wild type hearts, FGFR2 expression was observed in both the epicardial layer and the myocardial layer. In contrast, in $Fgfr2b^{-/-}$ hearts, the expression of FGFR2 was absent in the epicardial layer but present throughout the myocardium. This expression pattern supports a model in which myocardial derived FGF10 signals to FGFR2b in the epicardium to control heart size. Additionally, FGF10 could signal to other unidentified FGFR2b-expressing cells within the myocardium.

$Fgfr2b^{-/-}$ is a germline knockout with multiple developmental defects. Therefore, to determine whether FGFR2 signaling in epicardial cells and EPDCs could be responsible for the observed cardiac phenotypes in $Fgfr2b^{-/-}$ embryos, we used *Wt1-Cre* to inactivate a floxed allele of *Fgfr2* in the epicardium and in EPDCs (Figure 3). Because FGF10 can also signal to FGFR1b and FGFR1 and FGFR2 often show functional redundancy, we simultaneously inactivated conditional alleles of both *Fgfr1* and *Fgfr2*. Mice with the genotype, *Wt1-Cre*, $Fgfr1^{f/f}$, $Fgfr2^{f/f}$ (referred to as $Fgfr1/2^{Wt1\text{-}Cre}$) showed a thin-walled compact myocardium similar to that seen in $Fgfr2b^{-/-}$ and $Fgf10^{-/-}$ embryos (Figure 3E). However, mice conditionally lacking only *Fgfr1* or *Fgfr2* did not show a

significant decreased wall thickness, demonstrating functional redundancy of these receptors. In addition, total heart size in *Fgfr1/2*$^{Wt1\text{-}Cre}$ embryos was decreased compared to heterozygous control embryos when normalized to body weight (Figure 3F). The more severe phenotype of *Fgfr2b*$^{-/-}$ hearts, compared to *Fgfr2*$^{Wt1\text{-}Cre}$ hearts, could be a consequence of developmental defects intrinsic or extrinsic to the heart.

To account for the observed small size of the heart in *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ embryos, we examined myocardial proliferation and cell death. Examination of BrdU incorporation showed a significant decrease in proliferation when compared to controls at E15.5 and E17.5 (Figure 4) in *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. These results suggest that FGF10 controls epicardial development or function that in turn indirectly regulates myocardial growth. Immunostaining for active Caspase 3 expression did not show any differences between controls and *Fgfr1/2*$^{Wt1\text{-}Cre}$ or *Fgf10*$^{-/-}$ hearts (Supplemental Figure 3).

Regulation of epicardial development by FGF signaling.

To determine whether loss of epicardial FGFR1 and FGFR2 in *Fgfr1/2*$^{Wt1\text{-}Cre}$ mice affects epicardial development we examined the rate of proliferation of epicardial cells and the number of epicardial-derived cells localized within the compact myocardium in *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ were counted. At E17.5, there was no change in proliferation of epicardial cells between *Fgfr1/2*$^{Wt1\text{-}Cre}$ mice and control littermates (Figure 4D). To determine if epicardial EMT, delamination from the epicardium, or EPDC migration was defective in *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts, we examined the expression of Wt1, a protein expressed in epicardial cells and EPDCs (Figure 5A-D). In control hearts at E17.5, 15% of cells within the compact myocardium expressed Wt1. In contrast, in *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts there were significantly ($p< 0.005$ and $p< 0.03$,

respectively) fewer (9%) cells in the compact myocardium that expressed Wt1. In addition, Wt1 expression at E13.5 was also significantly reduced in the myocardial area of *FGFR2b*$^{-/-}$ and *Fgf10*$^{-/-}$ hearts (Supplemental Figure 4). However, Wt1 expression in the epicardium appeared normal. To further determine whether epicardial EMT could be impaired, expression of E-cadherin, a factor involved in epicardial EMT, was examined. Like Wt1, E-cadherin expression appeared normal in the epicardium of *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts (Supplemental Figure 5).

A prediction of these observations is that activation of the epicardial FGF signaling pathways would increase migration of EPDCs within the compact myocardium. We therefore examined the expression of Wt1 in embryos induced to overexpress FGF10 (Figure 5E-G). Embryos containing the *Rosa26-rtTA; TetO-Fgf10* alleles were induced with doxycycline from E15.5 to E17.5 to upregulate expression of *Fgf10* throughout most embryonic tissues. Hearts from these embryos showed a 10% ($p < 0.05$) increase in the number of Wt1 positive cells within the myocardium compared to wild type or heterozygous littermate controls.

To further characterize the EPDCs within the myocardium, hearts were stained with an antibody to vimentin, a marker of cardiac fibroblasts (Figure 5H-K). Consistent with the decreased number of Wt1-positive cells within the myocardium of *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts, the number of vimentin-positive cells was also decreased in *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts compared to controls. These loss-of-function and gain-of-function studies support a model in which FGF signaling regulates migration of a subset of EPDCs (that will become cardiac fibroblasts) into the compact myocardium.

FGF signaling regulates EPDC migration.

To determine whether FGF10 signaling regulates migration of EPDCs into the myocardium, hearts were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) to label epicardial cells (Morabito et al., 2001), allowing their location to be imaged following explant culture. CFSE is permeable to cells, but once inside a cell, esterases cleave the molecule trapping it in the cytosol. To determine whether epicardial cells could be specifically labeled, dissected E17.5 wild type hearts were treated with CFSE for 1 hr and then fixed, sectioned and immunostained for Wt1. CFSE and Wt1 were co-localized in the epicardial cell layer, and Wt1 was also present in EPDCs that had already migrated into the myocardium prior to labeling with CFSE (Figure 6A-C, arrows highlight $Wt1^+$ cells that have already migrated into the myocardium). To determine whether FGF10 activated FGFR signaling in CFSE-labeled cells, CFSE-labeled and FGF10-treated explants were stained for p-Erk, a downstream target of activated FGFRs. Exposure to FGF10 for 48 hrs resulted in an increase in p-Erk labeling of $CSFE^+$ cells in the epicardium and myocardium, but not of $CFSE^-$ cells within the compact myocardium (Figure 6D-F). This increase in p-Erk labeling in response to FGF10 was blocked by treatment with the FGFR inhibitor PD173074.

To determine if EPDC migration into the myocardium responded to FGF10, E17.5 heart explants were treated with CFSE for 1 hr, washed, and then cultured for 48 hr with or without FGF10 and PD173074. In addition, to determine the specificity of FGF signaling, explants were also treated with FGF9, a ligand that is expressed in the epicardium that signals to cardiomyocytes (Figure 6G-K). In response to treatment with FGF10, explants showed a significant ($p< 0.002$) increase in CFSE-labeled cells within the sub-epicardial space and compact myocardium. Addition of the FGFR inhibitor,

PD173074, along with FGF10 resulted in a significant ($p < 0.005$) decrease in CFSE-labeled cells within the sub-epicardial space and compact myocardium, while treatment of explants with FGF9 had no effect on migration of CFSE-labeled epicardial cells (Figure 6K, O). Taken together, these data demonstrate that FGF10 is sufficient to increase CFSE-labeled cell movement into the myocardium.

To determine whether FGF10 had an effect on EPDCs that had already migrated into the myocardium prior to CFSE labeling, the number of $Wt1^{+}$, $CFSE^{-}$ cells in FGF10-treated explanted hearts were counted. Consistent with a model in which FGF10 signals only to FGFR1b/FGFR2b in epicardial cells, there was no change in the number of Wt^{+}, $CFSE^{-}$, EPDCs following FGF10 treatment (Figure 6L-O).

FGF10 regulates formation of cardiac fibroblasts

During heart development, epicardial cells give rise to cardiac fibroblasts and smooth muscle cells that populate the compact myocardium. Under specialized conditions, such as following injury, epicardial cells may also give rise to cardiomyocytes and endothelial cells. To determine the fate of epicardial cells that respond to FGF10, explants labeled with CFSE and treated with FGF10 for 48 hr were sectioned and immunostained for markers of specific cardiac lineages including myocytes, endothelial cells, smooth muscle cells and fibroblasts (Figure 7). CFSE-labeled cells did not co-immunostain with antibodies to myocytes, endothelial cells, or smooth muscle cells, but did co-label with an antibody to vimentin, a marker expressed on fibroblasts (Figure 7D-F). These data suggest that FGF10 promotes formation and movement of EPDCs that preferentially differentiate into cardiac fibroblasts.

To determine whether FGF signaling could affect the motility of epicardial cells, live-imaging was used to monitor movement of isolated epicardial cells from *Fgfr2b*$^{-/-}$ and wild type hearts and hearts treated with vehicle or PD173074 (Figure 8). Epicardial cells from *Fgfr2b*$^{-/-}$ hearts showed a significantly ($p< 0.02$) shorter displacement, but similar distance traveled when compared to wild type epicardial cells. Consistent with this result, epicardial cells treated with PD173074 also showed a significantly ($p< 0.001$) shorter displacement and no change in the distance traveled. Addition of FGF10 to epicardial cell cultures did not increase epicardial cell motility (data not shown), indicating that FGF signaling was likely saturated in these cultures.

Discussion

Epicardial derived cells give rise to several cell types that populate the compact myocardium. These include interstitial fibroblasts, perivascular cells and smooth muscle cells. EPDCs also regulate growth of the myocardium but the factors that regulate their differentiation and their migration into the myocardium are poorly understood. We show that during late embryonic development, FGF10 signals to epicardial and epicardial derived cells through FGFR2b to induce their migration into the myocardium.

In mice conditionally lacking *Fgfr1* and *Fgfr2* in epicardial cells, or lacking *Fgf10*, significantly fewer EPDCs were observed within the compact myocardium. Several mechanisms could result in this phenotype including: defects in epicardial EMT; failure of EPDCs to migrate into the compact myocardium; or increased death of EPDCs. EMT is a complex process that requires the dissolution of cell-cell junctions, loss of apical-basal polarity, and finally the modification of cytoskeletal proteins to a mesenchymal phenotype that permits, in the case of epicardial cells, movement into the

subepicardial space (reviewed in Thiery et al., 2009). Although the precise signals and mechanisms governing epicardial EMT are not known, epicardial EMT requires β-catenin-dependent asymmetrical cell division (Wu et al., 2010) and Wt1 mediated repression of E-cadherin and upregulation of snail (Martinez-Estrada et al., 2010). In mice lacking FGF10 or epicardial FGFR1/2, epicardial EMT appears to occur normally, as proliferation and Wt1, snail and β-catenin expression were not changed in epicardial cells. Furthermore, increased apoptosis of EPDCs, which could also explain fewer of these cells within the myocardium, is also unlikely, since staining for activated caspase 3 revealed no increase in cell death in *Fgfr1r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts.

Following epicardial EMT, EPDCs migrate further into the myocardium and differentiate into either smooth muscle cells or cardiac fibroblasts. The mechanisms that direct EPDCs into the compact myocardium are not known, however, our data suggests that FGF10/FGFR2b signaling may regulate epicardial movement into the myocardium. In primary epicardial cell cultures lacking *Fgfr2b* or treated with FGFR kinase inhibitors, we observed a reduction in cell displacement. These results could be explained by FGF10 functioning as a chemotactic factor or regulating cell motility (displacement in epicardial cultures). *In vivo*, FGF10 is unlikely to regulate directional migration of EPDCs in the heart, because of its diffuse expression throughout the compact myocardium. In contrast, in the lung, *Fgf10* is expressed focally in mesenchyme where it functions to induce epithelial branching and migration towards the source of FGF10 (Weaver et al., 2000). In addition, in heart explants, addition of FGF10 protein to the media induced EPDC migration into the compact myocardium, suggesting that focal expression of FGF10 is not required. Recently, it was demonstrated that FGF-regulated increases in cell motility

could have net positive effects on directional cell movements required for embryonic axis elongation (Benazeraf et al., 2010). It is thus possible that FGF10 regulated cell motility could account for the specific influx of cardiac fibroblasts into the compact myocardium. Other factors like PDGFRβ and Alk5, that regulate either epicardial migration or EMT, have been found to specifically affect vascular smooth muscle cell recruitment or differentiation, but do not have reported effects on cardiac fibroblasts (Mellgren et al., 2008; Sridurongrit et al., 2008). We posit that FGF preferentially regulates migration of cardiac fibroblasts and PDGFβ preferentially regulates migration of vascular smooth muscle cells.

FGFs often signal bidirectionally during organogenesis, for example in limb bud and lung development (Morrisey and Hogan, 2010; Yang, 2009; Zeller et al., 2009). During midgestation heart development, communication between the epicardium and myocardium appears necessary to regulate the ultimate size of the heart. Although reciprocal FGF signaling between mesenchymal and epicardial tissues is important for heart development, other signaling molecules, direct cell-cell contact, and physiological factors are likely to interact with FGF signaling to coordinate heart size with growth of the embryo and its physiological requirements.

Multiple signals regulate growth of the myocardium (Sucov et al., 2009). Of these, epicardial derived FGF9 and FGF16 are factors that directly signal to FGFRs expressed in cardiomyocytes. Although myocardial proliferation is reduced in mice lacking FGF9 (Lavine et al., 2005), FGF16 (Hotta et al., 2008) or lacking both FGF9 and FGF16 (unpublished), proliferation is clearly not arrested. This indicates that other factors must act in parallel to FGF9/16 to regulate myocardial proliferation. Other factors

could include other growth factors such as WNT9b (Merki et al., 2005), or direct interactions between cells. Recently, cardiac fibroblasts were shown to directly induce myocardial proliferation through a mechanism involving HBEGF and integrin signaling (Ieda et al., 2009). The major source of embryonic cardiac fibroblasts during development is EPDCs. In our model, inactivation of FGF signaling in EPDCs leads to a decrease in EPDCs that specifically give rise to cardiac fibroblasts within the compact myocardium. Interestingly, we also observed a coincident decrease in myocardial proliferation and a reduction in heart size. We posit that decreased myocardial proliferation in hearts lacking epicardial FGF signaling could result from indirect consequences of decreased numbers of interstitial cardiac fibroblasts. This is consistent with small heart size phenotypes observed in other mutations that disrupt proepicardial migration, defects in epicardial EMT and EPDC migration into the myocardium (Martinez-Estrada et al., 2010; Rhee et al., 2009; Wu et al., 2010).

In the studies presented here, the *Fgfr2b*$^{-/-}$ hearts appear to have a more severe (smaller heart) phenotype than *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. *Fgfr2b*$^{-/-}$ is a germline knockout; therefore deletion of *Fgfr2b* is complete and can act over a longer period of time compared to a conditional knockout. In addition, other developmental defects could indirectly contribute to the cardiac phenotype in *Fgfr2b*$^{-/-}$ hearts.

Understanding mechanisms that regulate myocardial growth have historically been the focus of much research because of the importance of the cardiomyocyte to heart homeostasis and response to injury. One of the challenges that have slowed advances in the treatment of the injured heat is the limited ability of adult cardiomyocytes to proliferate. Recent studies on epicardial cells and their ability to differentiate into various

cell types and communicate with cardiac myocytes have suggested new therapeutic targets to treat heart disease. Future studies are needed to determine whether FGF10/FGFR2b signaling occurs in the adult heart under homeostatic or pathological conditions and whether this signaling pathway could be therapeutically manipulated to promote cardiac protection or regeneration.

Materials and Methods

Mice

Mouse lines used: *Fgfr2b*$^{-/-}$ (Revest et al., 2001), *Fgf7*$^{-/-}$ (Guo et al., 1996), *Fgf10*$^{-/-}$ (Min et al., 1998), *Wt1-Cre* (Min et al., 1998), *Fgfr1*$^{f/f}$ (Trokovic et al., 2003), *Fgfr2*$^{f/f}$ (Yu et al., 2003), *Rosa26-rtTA* (Belteki et al., 2005), *TetO-Fgf10* (Clark et al., 2001).

Histology

Paraffin sections (5μm) were stained with hematoxilin and eosin (H&E) for general visualization. Myocardial area was calculated with the contouring tool using Canvas X software. Cross-sectional area of the heart was defined as the measure of total muscle including both chambers in one mid-frontal section. Atrial area was not included. In *Fgfr1*$^{Wt1\text{-}Cre}$, *Fgfr2*$^{Wt1\text{-}Cre}$ and *Fgfr1/2*$^{Wt1\text{-}Cre}$ values were normalized to body weight by dividing area by total body weight. Heart wall thickness was calculated with the linear dimensioning tool in Canvas X software. Compact myocardial thickness was determined by averaging three measurements of the length from the subepicardial layer to the edge of the compact myocardium in histological sections from each embryo examined. Statistical significance was determined using the student's t-test, with n representing number of embryonic hearts examined.

Immunohistochemistry and Immunofluorescence

For immunohistochemistry, paraffin sections (5μm) were dewaxed, rehydrated, incubated in methanol/hydrogen peroxide, antigen unmasked, and blocked in 10% goat serum. Antigen unmasking was performed by incubating sections in 1% trypsin for 5 min at room temperature or by pressure cooking in citrate buffer for 15 min. Primary antibodies used were FGFR2 (rabbit IgG, Santa Cruz, sc-122), BrdU (mouse IgG, Becton and Dickinson, 1:100), activated caspase 3 (BD Pharmigen cat# 557035) and Snail1 (a gift from A. García de Herreros Madueno). Expression was visualized using the Histostatin SP broad spectrum (DAB) kit from Invitrogen (95-9643). Immunofluorescence was performed the same way excluding blocking endogenous peroxidase. Primary antibodies used were Wt1 (mouse $IgG_{1\kappa}$, Dakocytomation, M3561), vimentin (mouse IgM, Abcam, ab20346), pERK (mouse IgG_{2a}, Santa Cruz, sc-7383), desmin (mouse $IgG_{1,}$ Research Diagnostics Inc, RDI-PRO10519), pecam (rabbit IgG, Abcam, ab28364), smooth muscle cell actin (mouse IgG_1-Cy3, Sigma, c-6198), E-cadherin (mouse IgG_{2a}, BDTransduction, #610181), b-catenin (mouse IgG_1, BDTransduction, #610153) and troponin (mouse $IgG_{2a,}$ Developmental Studies Hybridoma Bank, CT3-s). Secondary antibodies were incubated for 1hr and visualized with a Zeiss confocal microscope or Zeiss apotome microscope.

Staining for β-galactosidase was performed as described (Soriano, 1999).

In Situ Hybridization

Tissues were fixed in 4% formaldehyde, embedded in paraffin and sectioned (5 μm). In situ hybridization was performed as previously described (Wilkinson, 1992). The Fgf10 in situ probe was provided by B. Hogan (Bellusci et al., 1997).

Proliferation Analysis

For embryos, pregnant females at E15.5 and E17.5 were injected IP with BrdU (50 µg/g body weight), 30 minutes prior to sacrifice. BrdU immunohistochemistry was performed as described above. Sections were counterstained with hematoxylin. For statistical analysis, two areas from three different specimens were analyzed per stage. The number of BrdU-positive nuclei relative to the total number of nuclei was counted from two 63x fields per section. Data is shown as mean ± SD.

For organ cultures, 6.4 ng/ml BrdU was added to the culture media 30 min prior to fixation. BrdU incorporation was detected by immunohistochemistry. After fixation, tissues were embeded in paraffin and sectioned. Immunohistochemistry was performed as described above.

Heart explant culture

Hearts were dissected under aseptic conditions at E17.5. Hearts were then labeled with 50mM CFSE (5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate succinimidyl ester, Invitrogen, cat# C1165) for one hour and placed in glass scintillation vials containing 1 ml of media (DMEM, 2 µg/ml Heparin, antibiotic and antimycotic). Vehicle (0.25 µl/ml DMSO), FGF10 (10 nM, Peprotech Inc.), FGF9 (10 nM, Peprotech Inc.) or PD173074 (25 nM, Pfizer Inc.) was added to the vials. Vials were incubated for 48 h on a rocker at 37°C/5% CO_2 with loose caps. Hearts were harvested, fixed in 10% formalin or 4% formaldehide and embeded in paraffin prior to sectioning.

Epicardial live imaging

Hearts were dissected under aseptic conditions at E15.5 and set in 1% collagen coated delta T dishes (Fisher) over night in 350 µl of media (DMEM, 5% horse serum, 2 µg/ml Heparin and antibiotic and antimycotic). Hearts were then removed from the dish

leaving foci of epicardial cells attached to the dish. Adherent cells were washed and 2 ml of media was added to the cultures. FGF inhibitor (PD173074, 22 nM) was added as indicated. Cultures were placed in a live imaging chamber on Leica DMI 6000B microscope and maintained at 37°C in 5% CO_2. 20x images were taken every 10 min for a period of 24 h. Image series were taken with a camera Retiga Exi. Images were prepared and exported using the CIMAT software (C. Little, UMKC, Kansas City) .Images were analyzed using the Manual Tracking plugin for Image J software. X and Y coordinates and scaling were used to calculate the distance, displacement, speed and velocity of cells in culture.

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Figures and Figure Legends

Figure 1.

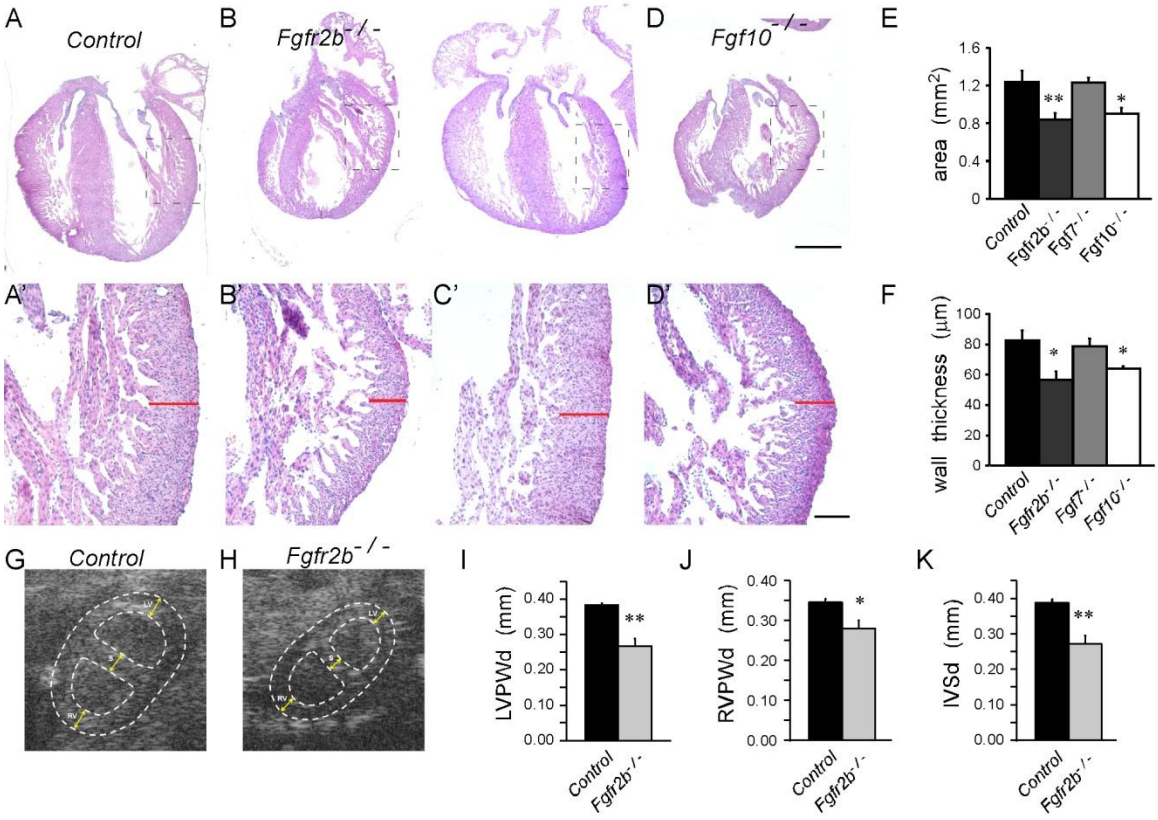


Figure 1.

Fgfr2b and *Fgf10* regulate heart size. (A-D, A'-D') H&E staining of hearts at E17.5. Control (A,A'), *Fgfr2b*$^{-/-}$ (B,B'), *Fgf7*$^{-/-}$ (C,C') and *Fgf10*$^{-/-}$ (D,D'). Dashed rectangle in (A-D) denotes magnified area in (A'-D'). (E) Quantification of the relative area of the heart. Control, n=8; *Fgfr2b*$^{-/-}$, n=8, ** p<0.001; *Fgf10*$^{-/-}$, n=8, * p<0.02; *Fgf7*$^{-/-}$, n=5. (F) Quantification of left ventricular wall thickness (red line in A'-D'). *Fgfr2b*$^{-/-}$, n=6, *p<0.02; *Fgf10*$^{-/-}$, n=9, * p<0.003; *Fgf7*$^{-/-}$, n=5. (G,H) Short axis *in utero* echocardiogram at E17.5 of control (G) and *Fgfr2b*$^{-/-}$ heart (H). Area measured (dashed white lines) and wall thickness measured (yellow lines) are placed at end diastole according to the movie shown in supplemental Figure 1. LV (left ventricle); S (septum); RV (right ventricle). (I-K) Quantification of left ventricular posterior wall diameter at end diastole (LVPWd), n=7, ** p<0.0003; right ventricular posterior wall diameter at end diastole (RVPWd), n=7, *p<0.02; interventricular septum diameter at end diastole, n=7, ** p<0.001. Scale bar: (A-D) 500μm, (A'-D') 100μm. Control hearts are a mix of wild type, *Fgfr2b*$^{+/-}$ and *Fgf7*$^{+/-}$, *Fgf10*$^{+/-}$. Asterisk indicates statistically significant difference compared with controls.

Figure 2.

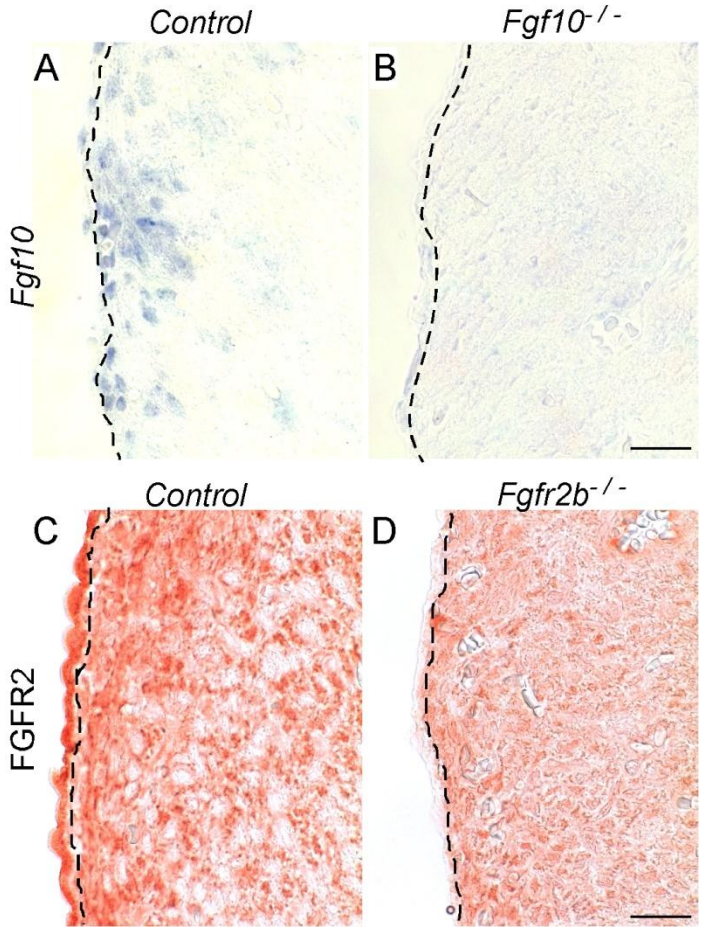


Figure 2.

Expression of *Fgfr2b* and *Fgf10* in the left ventricle of the heart at E17.5. (A-B) *Fgf10 in situ* hybridization showing *Fgf10* mRNA in cells within the myocardial area in controls, no expression of FGF10 was observed in *Fgf10*$^{-/-}$ hearts. (C-D) Immunohistochemistry showing expression of FGFR2 throughout the heart in controls (C). In *Fgfr2b*$^{-/-}$ heart (D), FGFR2b is absent from the epicardial layer. Dashed lines denotes border between myocardial and epicardial layer. Scale bar: 20 μm.

Figure 3.

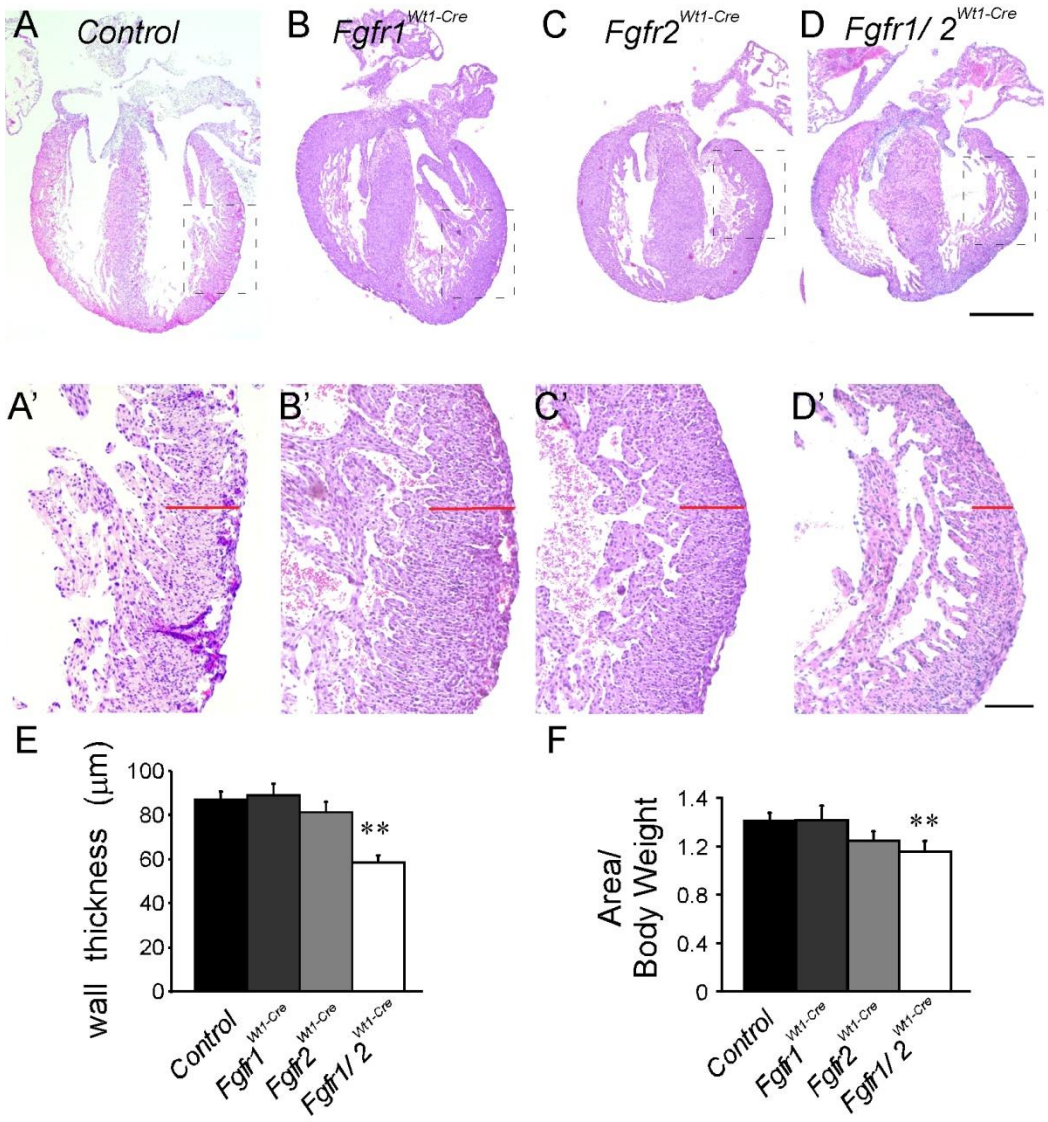

A Control
B Fgfr1 Wt1-Cre
C Fgfr2 Wt1-Cre
D Fgfr1/ 2 Wt1-Cre
A'
B'
C'
D'
E
wall thickness (μm)
100
80
60
40
20
0
**
Control
Fgfr1 Wt1-Cre
Fgfr2 Wt1-Cre
Fgfr1/ 2 Wt1-Cre
F
Area/ Body Weight
1.4
1.2
0.8
0.4
0
**
Control
Fgfr1 Wt1-Cre
Fgfr2 Wt1-Cre
Fgfr1/ 2 Wt1-Cre

Figure 3.

Decreased heart size after epicardial conditional inactivation of *Fgfr1* and *Fgfr2* with *Wt1-Cre*. (A-D, A'-D') H&E staining of E17.5 control (A,A'), *Fgfr1*$^{Wt1\text{-}Cre}$ (B,B'), *Fgfr2*$^{Wt1\text{-}Cre}$ (C,C'), and *Fgfr1/2*$^{Wt1\text{-}Cre}$ (D,D') hearts. *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts are smaller compared to controls and display a thinner compact myocardium. Dashed rectangle in (A-D) denotes magnified area in (A'-D'). (E) Quantification of the left ventricle wall thickness (red line in A'-D'). *Control*, n=14; *Fgfr1*$^{Wt1\text{-}Cre}$, n=5; *Fgfr2* $^{Wt1\text{-}Cre}$, n=10; *Fgfr1/2*$^{Wt1\text{-}Cre}$, n=9, ** $p<0.001$. Scale bar: (A-D) 500µm, (A'-D') 100µm. (F) Quantification of heart cross-sectional area normalized to body weight. *Fgfr1/2*$^{Wt1\text{-}Cre}$, n=10, $p<0.003$; *Fgfr1*$^{Wt1\text{-}Cre}$, n=5; *Fgfr2*$^{Wt1\text{-}Cre}$, n=9. Control hearts contain *Wt1-Cre* and are a combination of wild type and floxed alleles of *Fgfr1 and Fgfr2*. Asterisk indicates statistically significant difference compared with controls.

Figure 4.

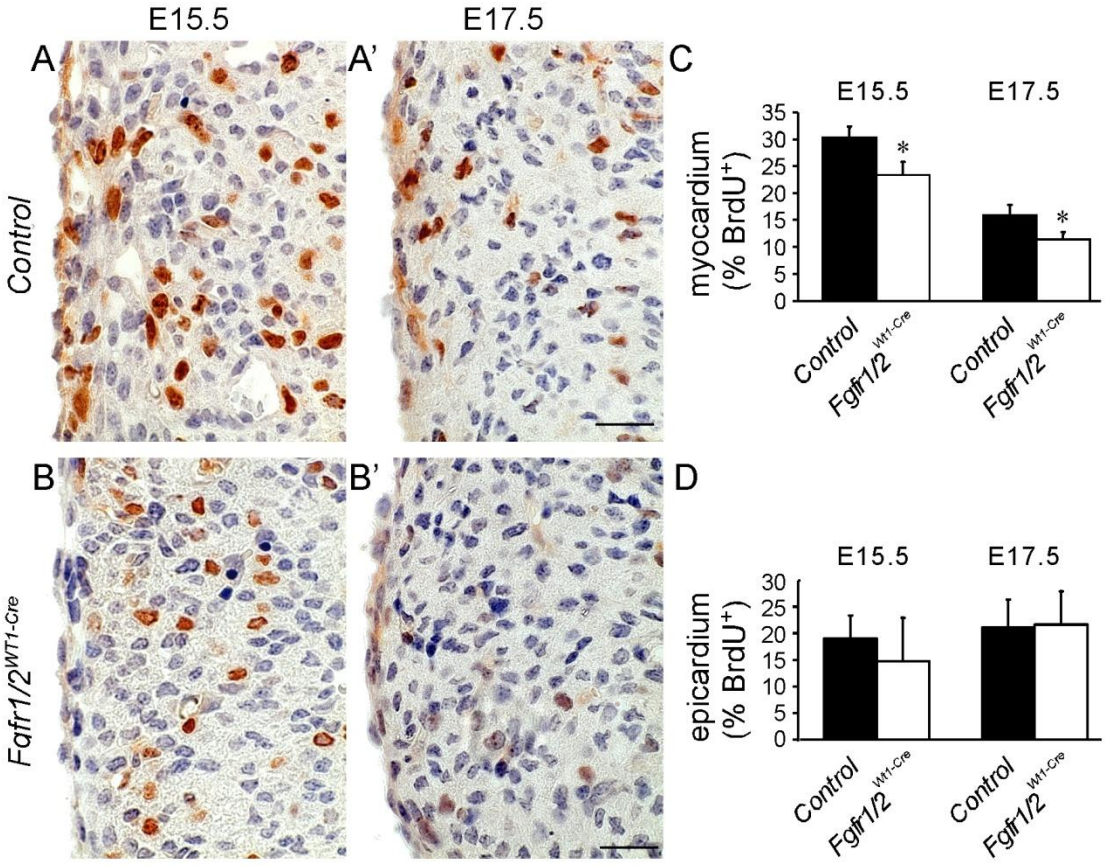

E15.5
E17.5
A
A'
C
Control
B
B'
D
Fafr1/2WT1-Cre
myocardium (% BrdU+)
epicardium (% BrdU+)
E15.5
E17.5
Control
Fgfr1/2 Wt1-Cre
Control
Fgfr1/2 Wt1-Cre
*
*

Figure 4.

Reduced myocardial, but not epicardial, proliferation in *Fgfr1/2*$^{Wt1\text{-}Cre}$ heart. (A-B) BrdU incorporation at E15.5. (A'-B') BrdU incorporation at E17.5. (C) Quantification of the percent of BrdU positive cells within the myocardium, showing decreased proliferation with age and decreased proliferation in *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts compared to control hearts. E15.5, n=3, * $p<0.04$; E17.5, n=5, * $p<0.03$. (D) Quantification of the percent of BrdU positive cells within the epicardium showing no difference between controls and *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. Scale bar: 20μm.

Figure 5.

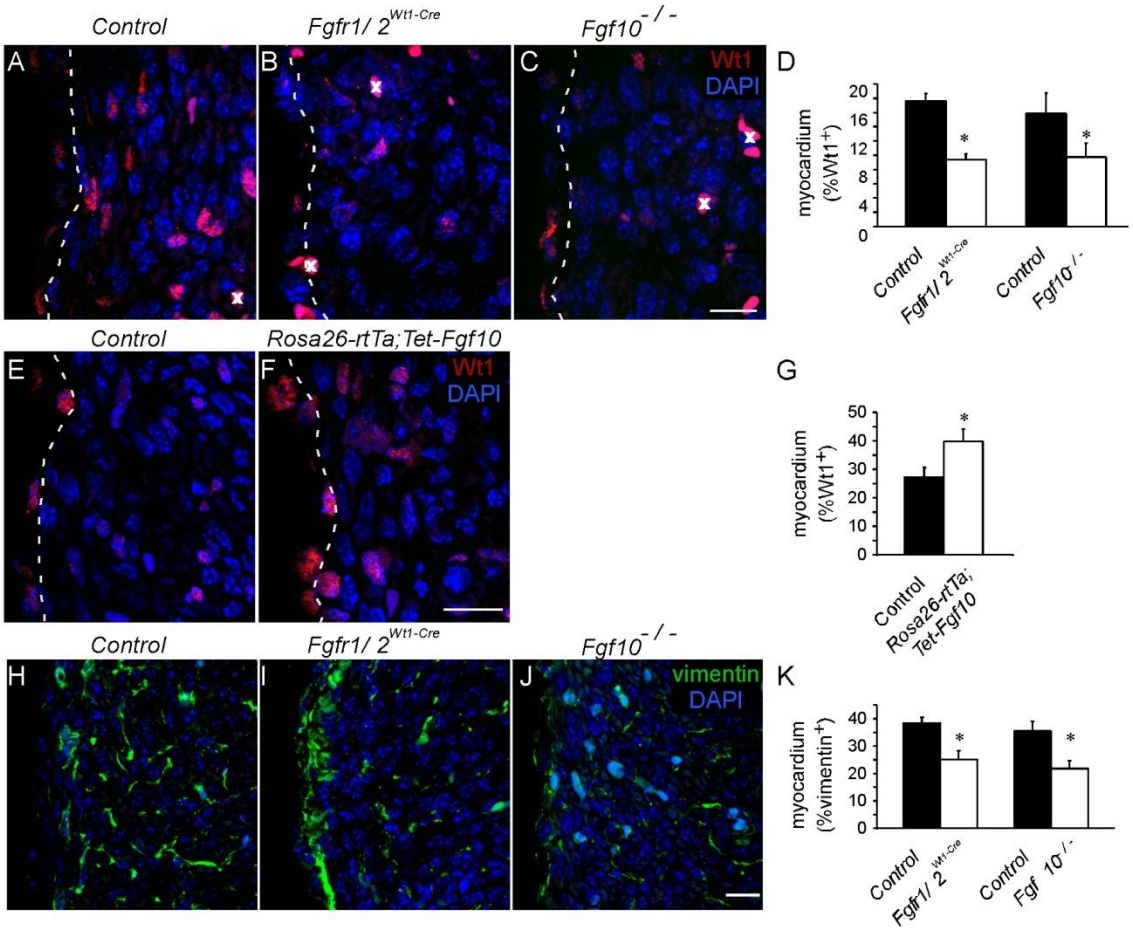
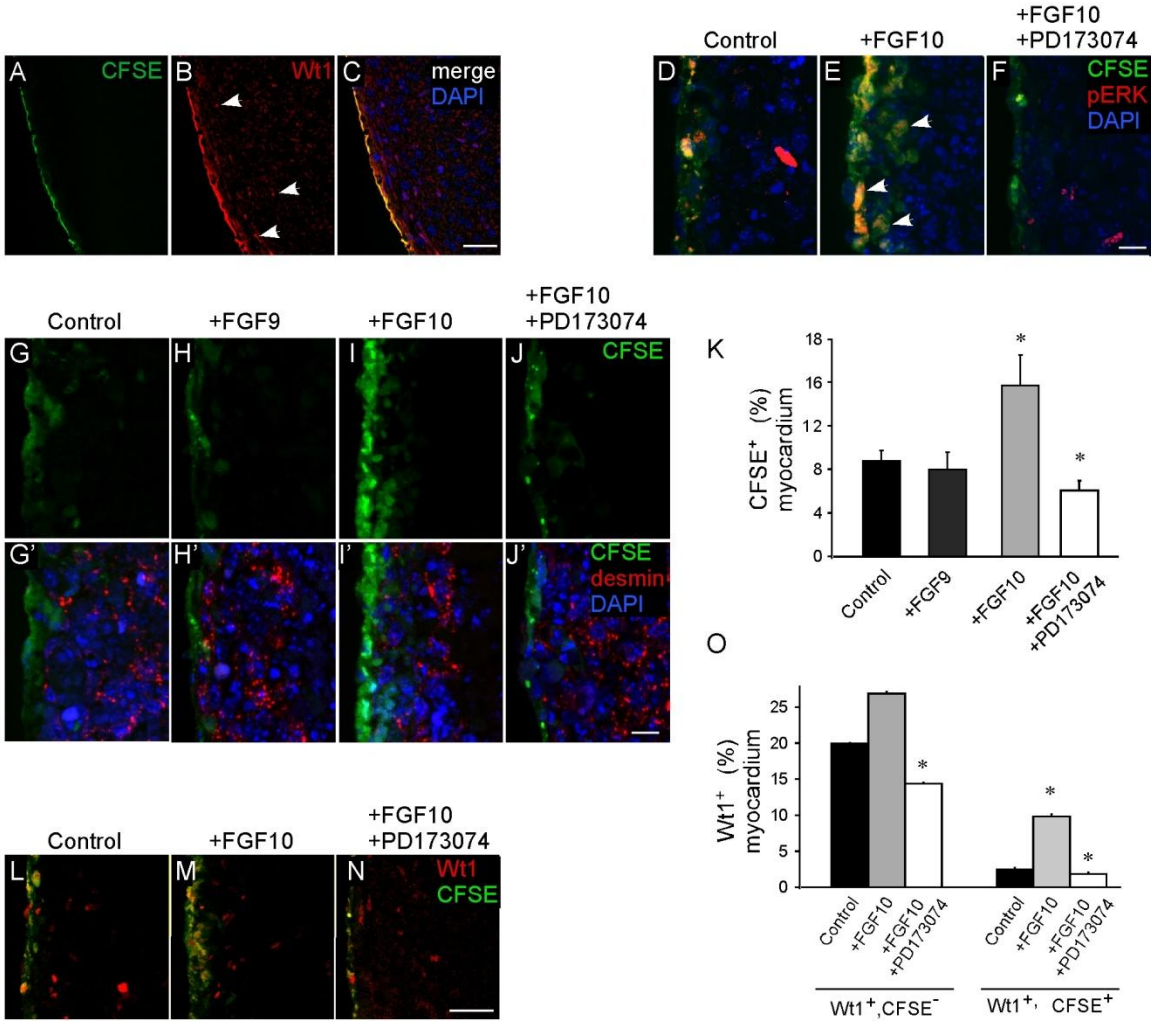

Control
Fgfr1/ 2Wt1-Cre
Fgf10-/-
A
B
C
Wt1
DAPI
D
myocardium (%Wt1+)
20
16
12
8
4
0
*
*
Control
Fgfr1/ 2Wt1-Cre
Control
Fgf10-/-
Control
Rosa26-rtTa;Tet-Fgf10
E
F
Wt1
DAPI
G
myocardium (%Wt1+)
50
40
30
20
10
0
*
Control
Rosa26-rtTa; Tet-Fgf10
Control
Fgfr1/ 2Wt1-Cre
Fgf10-/-
H
I
J
vimentin
DAPI
K
myocardium (%vimentin+)
40
30
20
10
0
*
*
Control
Fgfr1/ 2Wt1-Cre
Control
Fgf 10-/-

Figure 5.

FGF signaling to epicardial cells regulates migration of EPDCs into the myocardium. (A-C) Wt1 immunofluorescence at E17.5 showing fewer $Wt1^{+}$ cells within the myocardium of *Fgfr1/2*$^{Wt1\text{-}Cre}$ (B) and *Fgf10*$^{-/-}$ (C) hearts. Wt1, red; DAPI, blue. (D) Quantification of the percent of $Wt1^{+}$ cells in the myocardium. *Fgfr1/2*$^{Wt1\text{-}Cre}$, n=4, * $p<0.005$, *Fgf10*$^{-/-}$, n=7, * $p< 0.03$. (E-F) Wt1 immunofluorescence at E17.5 of control (E) and *Rosa26-rtTA;TetO-Fgf10* (F) induced with doxycycline from E15.5 to E17.5. (G) Quantification of the percentage of $Wt1^{+}$ cells in the myocardium. n=5, * $p<0.05$. (H-K) Vimentin immunofluorescence at E17.5 showing fewer $vimentin^{+}$ cells within the myocardium of *Fgfr1/2*$^{Wt1\text{-}Cre}$ (I) and *Fgf10*$^{-/-}$ (J) hearts. Vimentin, green; DAPI, blue. (K) Quantification of the percent of $Vimentin^{+}$ cells in the myocardium. *Fgfr1/2*$^{Wt1\text{-}Cre}$, n=8, * $p<0.005$; *Fgf10*$^{-/-}$, n=10, * $p< 0.02$. Scale bar in F, I, M, 20μm. White (x) denotes red blood cells and dashed white line denotes the epicardial boundary.

Figure 6.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CFSE
B
Wt1
C
merge
DAPI
Control
+FGF10
+FGF10
+PD173074
D
E
F
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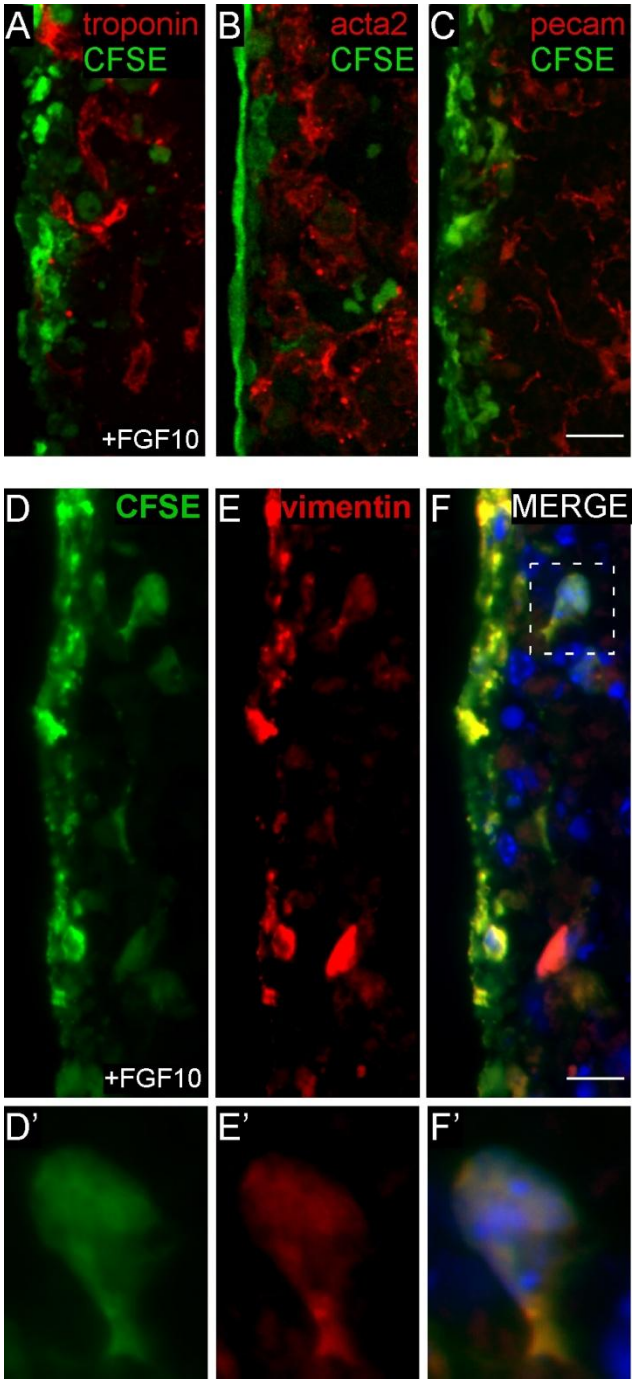
pERK
DAPI
Control
+FGF9
+FGF10
+FGF10
+PD173074
G
H
I
J
CFSE
G'
H'
I'
J'
CFSE
desmin
DAPI
K
CFSE+ (%) myocardium
18
16
12
8
4
0
*
*
Control
+FGF9
+FGF10
+FGF10
+PD173074
Control
+FGF10
+FGF10
+PD173074
L
M
N
Wt1
CFSE
O
Wt1+ (%) myocardium
25
20
15
10
5
0
*
*
*
Control
+FGF10
+FGF10
+PD173074
Control
+FGF10
+FGF10
+PD173074
Wt1+,CFSE-
Wt1+, CFSE+

Figure 6.

Fgf10 induces migration of EPDCs in explant culture. (A-C) Hearts explanted at E17.5 and treated with CFSE for 1 h labels only epicardial cells (A) and not EPDCs that have already migrated into the myocardium (B,C). (D-F) pErk immunohistochemistry of CFSE-labeled explanted hearts treated with FGF10 (E) and FGF10 plus PD173074 (F) for 48 h. Arrows indicates cells positive for pErk and CFSE. (G-J, G'-J') Migration of CFSE-labeled epicardial cells into the myocardium following treatment with FGF9, (H,H'); FGF10, (I,I'); and FGF10 plus PD173074, (J,J'). CFSE, green; desmin, red (to identify the boundary between myocardium and epicardium), DAPI, blue. (K) Quantification of number of $CFSE^{+}$ within the myocardium after 48 h in culture. +FGF9, n=8; +FGF10, n=19, * $p<0.003$, significant increase compared to control; +FGF10, +PD173074, n=10, * $p<0.006$, significant decrease compared to control.

(L-N) FGF10-induced CFSE-labeled EPDCs in the myocardium are positive for Wt1. Explanted hearts labeled with CFSE were treated with FGF10 (M) or FGF10 plus PD173074 (N) for 48 h and then sectioned and immunostained for Wt1. CFSE, green; Wt1, red. (O) Quantification of the number of $Wt1^{+}$ cells within the myocardium. FGF10 treatment significantly increased the number of $CFSE^{+}$,$Wt1^{+}$ double positive cells within the myocardium, n=10, * $p<0.002$; but did not affect the number of pre-existing ($CSFE^{-}$) $Wt1^{+}$ within the myocardium. Treatment with FGF10 and PD173074 significantly decreased the number of $CFSE^{-}$,$Wt1^{+}$ cells within the myocardium, n=10, * $p<0.004$ and the number of $CFSE^{+}$,$Wt1^{+}$ within the myocardium, n=10, * $p<0.002$. Scale bar: (A-B), 100µm (D-F, G-J, L-N) 20µm

Figure 7.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troponin
CFSE
+FGF10
B
acta2
CFSE
C
pecam
CFSE
D
CFSE
+FGF10
E
vimentin
F
MERGE
D'
E'
F'

Figure 7.

Migratory EPDCs become cardiac fibroblasts. Explanted hearts were labeled with CFSE and treated with FGF10. After 24 h, hearts were sectioned and stained with markers for (A) cardiac muscle, troponin (red); (B) smooth muscle actin, acta2 (red); (C) endothelial cells, pecam (red); and (D-F) fibroblasts, vimentin (red). (D'-F') CFSE is only co-expressed with vimentin. Scale bar: 20μm. Dashed squared denotes the magnified insert (D'-F')

Figure 8.

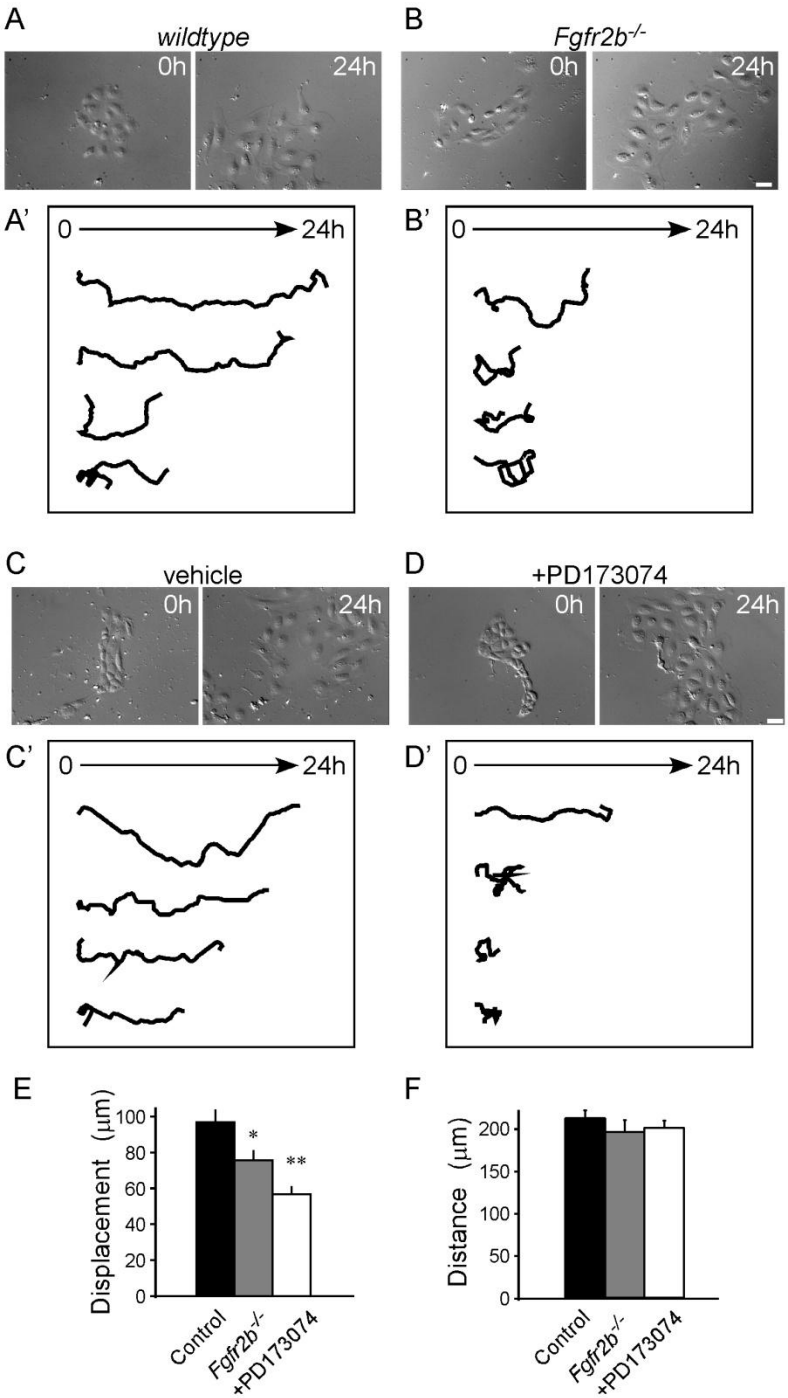
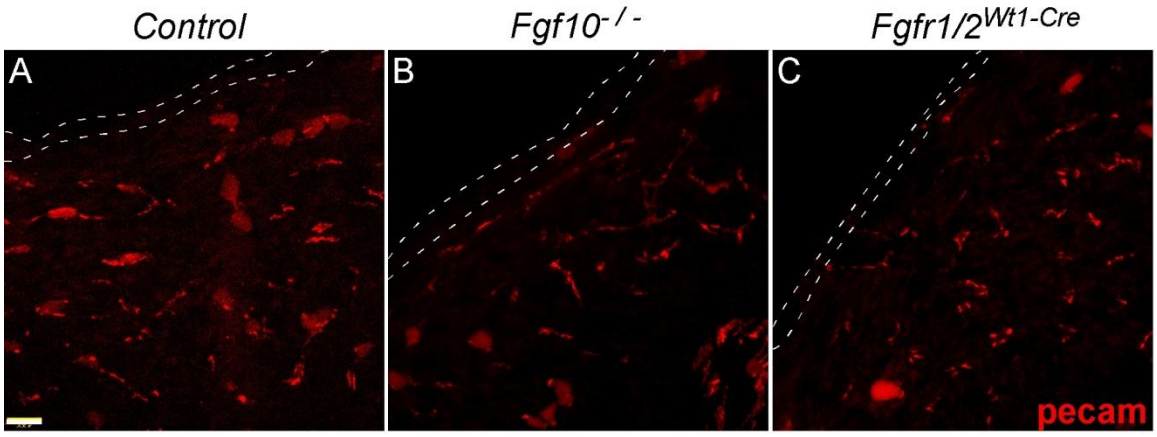

A
wildtype
0h
24h
B
Fgfr2b-/-
0h
24h
A'
0
24h
B'
0
24h
C
vehicle
0h
24h
D
+PD173074
0h
24h
C'
0
24h
D'
0
24h
E
Displacement (μm)
100
80
60
40
20
0
*
**
Control
Fgfr2b-/-
+PD173074
F
Distance (μm)
200
150
100
50
0
Control
Fgfr2b-/-
+PD173074

Figure 8.

FGF signaling regulates displacement of epicardial cells in culture. (A-D) Representative cells and cell paths during the 24 h culture period. (E) Quantification of cell displacement over 24 h. (F) Quantification of distance of distance traveled over 24 h. Net movement (displacement) of (A') wildtype and (C') vehicle treated cells is greater than that of *Fgfr2b*$^{-/-}$ cells, n=79, * $p<0.02$; and cells treated with PD173074, n=77, ** $p<0.001$. Scale bar: 20μm.

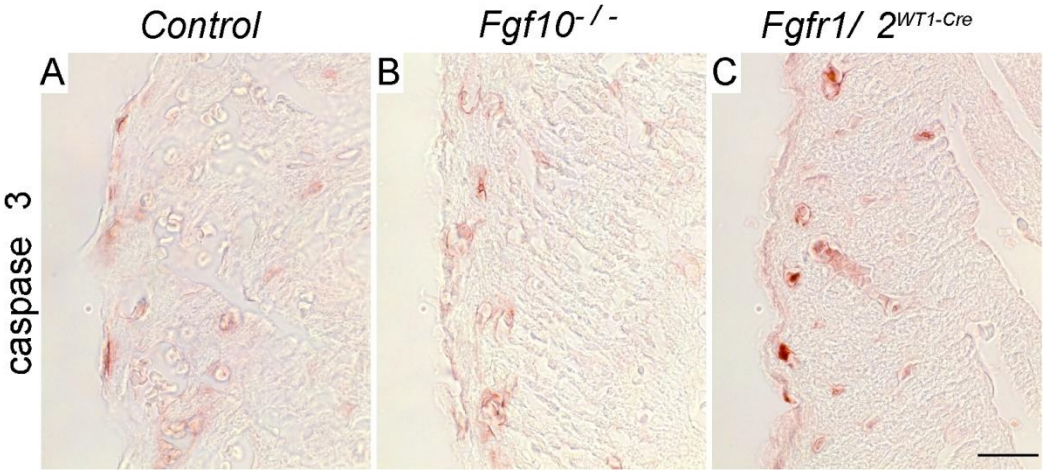
Supplemental figure 1.



Supplemental figure 1.

Coronary vasculature in E17.5 hearts. Immunostaining for PECAM/CD31 (red) in control, *Fgf10*$^{-/-}$ and *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. (A) Control , (B) *Fgf10*$^{-/-}$, and (C) *Fgfr1/2*$^{Wt1\text{-}Cre}$. Compared to controls, the vascular plexus in *Fgfr1/2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts appears normal. Scale bar: 10µm. Dashed lines contours the epicardial layer.

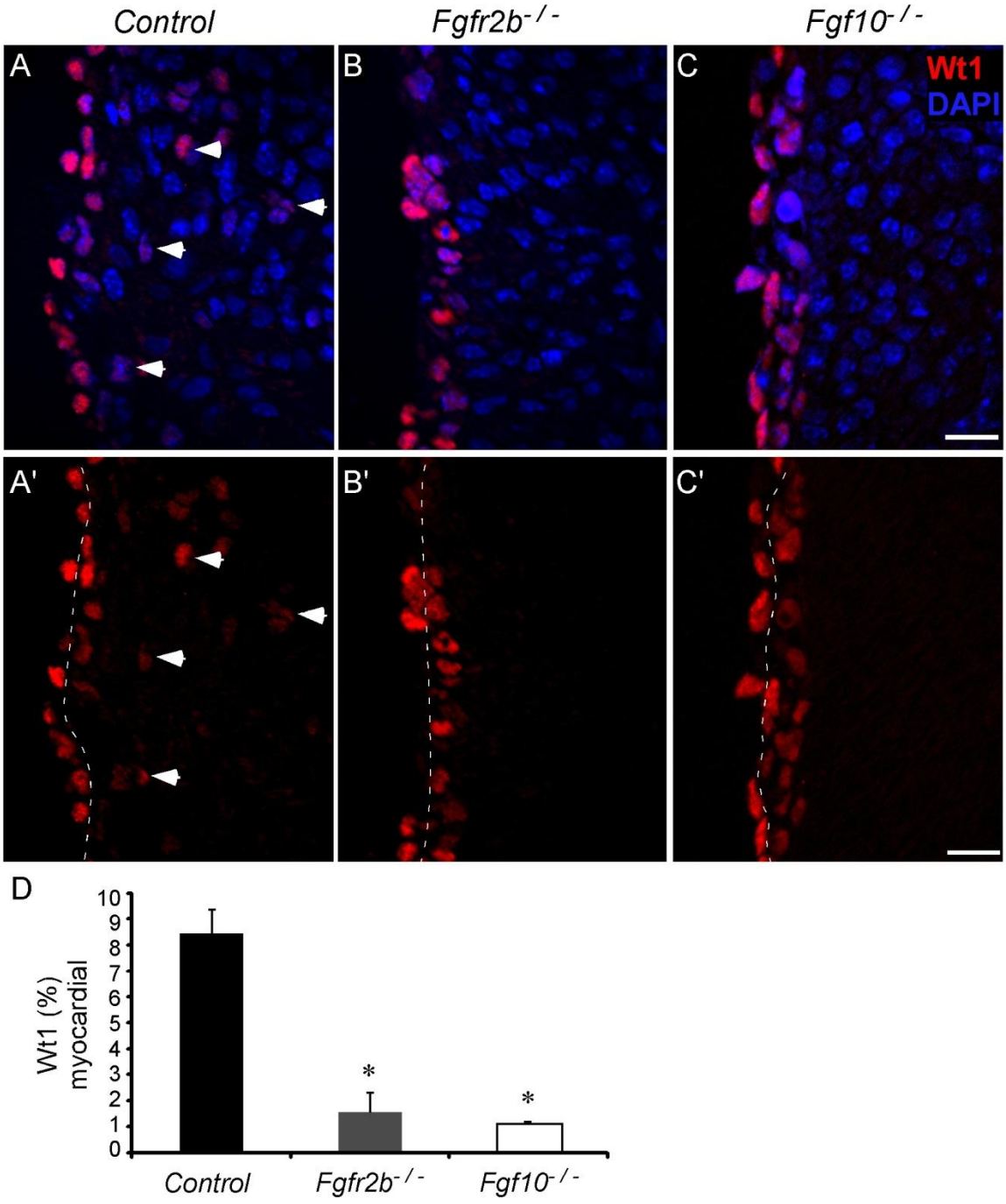
Supplemental figure 2.



Supplemental figure 2.

Assay for cell death in E17.5 hearts. Immunostaining for activated caspase 3 in control, *Fgf10*$^{-/-}$ and *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. (A) Control, (B) *Fgf10*$^{-/-}$, and (C) *Fgfr1/2*$^{Wt1\text{-}Cre}$. No change in capase 3 staining was observed. Scale bar: 20μm.

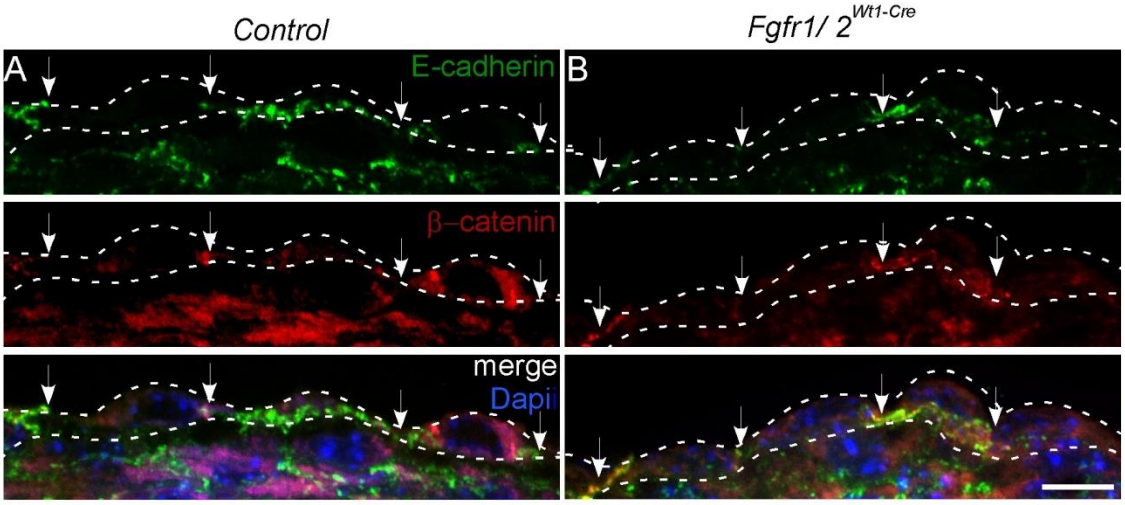
Supplemental figure 3.

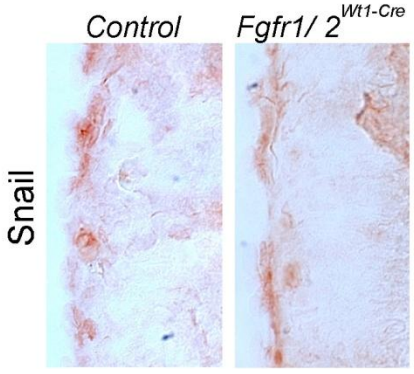


Supplemental figure 3.

Identification of EPDCs within the myocardium at E13.5. (A-C) Immunostaining for Wt1 (red) showing fewer $Wt1^{+}$ cells within the myocardium in *Fgfr2b*$^{-/-}$ (B) and *Fgf10*$^{-/-}$ (C) hearts, compared to control (A) hearts. (D) Quantification of Wt1+ cells located within the myocardium. *Control*, n=5*; Fgfr2b*$^{-/-}$, n=4, * p< 0.001; *Fgf10*$^{-/-}$, n=3 ** p<0.01. DAPI, blue. White line denotes the boundary between epicardium and myocardium and white arrows indicate $Wt1^{+}$ cells within the myocardium. Scale bar: 20μm.

Supplemental figure 4.





Supplemental figure 4.

EMT is not impaired in *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. Immunostaining for E-cadherin (green) and β-catenin (red) at E17.5 in control (A) and *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts (B). Arrows indicate points of adhesion of epicardial cells were both E-cadherin and β-catenin are normally co-expressed. Immunostaining for Snail1 at E15.5 (bottom) shows normal expression in the epicardial cell layer of *control* and *Fgfr1/2*$^{Wt1\text{-}Cre}$ hearts. Dashed line contours the epicardial layer. Scale bar: 10μm.

Chapter 3

Future Directions

Summary

In this thesis I explored the role of FGF signaling during development of epicardial-derived cells (EPDCs). I found that FGF10, secreted from the myocardium, signals to FGFR2b in the epicardium to activate migration of epicardial cells into the myocardium. Epicardial derived cells populate the heart with vascular smooth muscle cells (vsmc) and cardiac fibroblasts (Mikawa and Gourdie, 1996; Vrancken Peeters et al., 1999). These cell types are needed in the heart to complete the formation of the vascular tree and the fibrous skeleton. I observed that FGF10 specifically signaled to cardiac fibroblasts but not to vsmc to induce their migration into the myocardium. In addition, inactivation of FGF10/FGFR2b/ signaling in mouse embryos resulted in a smaller heart visible by embryonic day 17.5 (E17.5). It has been shown that cardiac fibroblasts are important for cardiac myocyte proliferation during late gestation (Ieda et al., 2009). The largest contributors of cardiac fibroblasts during development are epicardial-derived cells (Krenning et al., 2010). Therefore, defective migration of cardiac fibroblasts could result in a decreased number of cardiac fibroblast within the myocardium, impeding their interaction with myocytes. In turn, decreased interaction affects proliferation, leading to the formation of a smaller heart. Taking this into account, we hypothesize that when the FGF10/FGFR2b/ signaling is disrupted we observe a decrease in heart size due to the decrease in cardiac fibroblast migration and subsequent signaling to the cardiomyocyte.

These observations contribute to the knowledge we have about epicardial cells. Epicardial cells are important for development and because of their potential to

serve as a progenitor cell during cardiac injury. Because developmental pathways normally required for the formation of epicardial and epicardial-derived cells may be necessary for homeostasis and reactivation of the epicardium in the adult, it remains important to learn how to manipulate this cell type in the adult context (Gittenberger-de Groot et al., 2010; Limana et al., 2010; Shrivastava et al., 2010; Wessels and Perez-Pomares, 2004). Therefore, FGF10 could potentially be an important factor during heart repair, and future studies are necessary to determine if this is so. Next I am going to present various questions that stem from the research done for this thesis and discuss potential directions we can take to investigate them.

Involvement of FGF7 in epicardial development.

Fgfr2b$^{-/-}$ hearts have a thinner compact myocardium. FGFR2b is expressed in the epicardium, therefore we hypothesized that members of the FGF7 subfamily could potentially signal to FGFR2b. To examine this possibility, we performed rt-PCR of whole wildtype hearts at E17.5. From the members of the FGF7 subfamily of ligands, we detected *Fgf10* and *Fgf7* mRNA expression but not of *Fgf3* or *Fgf22* (data not shown). We next examined the overall phenotype of both *Fgf10*$^{-/-}$ and *FgF7*$^{-/-}$hearts. We found that *Fgf7*$^{-/-}$ by itself does not develop a smaller heart, but *Fgf10*$^{-/-}$ does, suggesting that FGF10 is the main ligand for FGFR2b in the epicardium. We also were able to generate two *Fgf7*$^{-/-}$ and *Fgf10*$^{-/-}$ double germline knockouts which yielded an even smaller heart compared to the *Fgf10*$^{-/-}$ (Figure 1). A simple way to explain the differences in the phenotypes of *Fgf7*$^{-/-}$, *Fgf10*$^{-/-}$ and the *Fgf7*$^{-/-}$, *Fgf10*$^{-/-}$, double knockout could be that FGF7 and FGF10 pathways are redundant, which leads to a more severe phenotype when both are absent in the double knockout. The differences in the severity of the phenotype of the individual knockouts could be explained by the different binding specificities of FGF7 and FGF10 to FGFR2b in the heart. Binding of FGF ligands to their receptors are regulated by heparan sulfate proteoglycans (HSP). HSP epitopes are found to be expressed in a tissue-specific and developmentally regulated fashion (Mohammadi et al., 2005). It is possible that FGF10, but not FGF7, is favored to bind FGFR2b due to the specific HSP present. In this scenario, FGF7 could still bind FGFR2b but not predominantly, yielding a very week phenotype when deleted on its own. When deleted along with FGF10, the phenotype becomes more severe.

In the future, we need to generate more *Fgf7* and *Fgf10* double knockouts to characterize their phenotype and investigate the extent to which *Fgf7* is necessary in the epicardium for migration of the epicardial cells. It is also possible that deletion of *Fgf7* along with *Fgf10* could uncover other functions of FGF in the development of epicardial cells, such as differentiation and EMT that might require a combination of both ligands.

Initiation and promotion of epicardial cell migration by FGF10

In studies performed for this thesis, we found that fewer epicardial-derived cells, specifically EPDCs that differentiated into cardiac fibroblasts, had migrated into the myocardium of *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts by late gestational stages. In addition, we found that addition of FGF10 to explanted hearts could induce migration of labeled epicardial cells into the myocardium. We interpreted that these results could reflect either abnormal EMT or migration. We were unable to find evidence that EMT was defective in our embryonic mouse models. Instead, we observed that wild type isolated epicardial cells move longer net distances than *Fgfr2b*$^{-/-}$ epicardial cells and epicardial cells treated with an FGFR inhibitor. This observation suggests that FGF10 can signal to epicardial cells within the epicardial layer. It is still unclear if FGF10 acts as a competence factor to potentiate epicardial cells to begin migration or if it acts as a signal that is required to direct or promote motility during the course of migration. It has been established that FGF10 signals to FGFR2b and that when EMT occurs there is a shift in expression from FGFR2b to FGFR2c (Savagner et al., 1994). If epicardial cells shifted expression of FGFR2b to FGFR2c during EMT, FGF10 could only continue signaling to the EPDCs for as long as they retained FGFR2b on their cell surface. In other words, can FGF10 signal to epicardial cells and/or the delaminated EPDCs? The simplest way to examine this possibility is to determine the dynamic domains of expression of *Fgfr2b* and *Fgfr2c* in the heart. Unfortunately, no antibody has been developed to specifically detect each isoform. One way is to use a general FGFR2 antibody on tissue from individual isoform knockouts. Using this method, one can delineate the expression of FGFR2

by absence of staining in each specific isoform knockout. Another way to investigate if FGF10 could functionally signal to EPDCs is to isolate epicardial-derived cells. We know that epicardial-derived cells continue expressing the marker Wt1 as they migrate into the myocardium; therefore, cells that are express Wt1 could either be epicardial cells or EPDCs (Perez-Pomares et al., 2002; Rhee et al., 2009). We also know that an adhesion molecule important for long-term attachment of epicardial cells is α4β1 integrin; this means that epicardial cells, but not EPDCs, will be positive for both α4β1 and Wt1 (Sengbusch et al., 2002). We could use cell sorting to isolate epicardial-derived cells based on their being immunopositive for Wt1 and immunonegative for α4β1 integrin ($Wt1^{+}$; $\alpha4\beta1^{-}$). Isolated EPDCs from *Fgf10*$^{-/-}$ and wild type hearts can be cultured and visualized using live imaging to calculate the net displacement and distance of their movement in the presence of FGF10 protein. also, look at ability of different FGF ligands to bind to the cell surfeace (iodinated or fluorescent tagged)

Whether FGF10 is able to signal to EPDCs as they migrate is important in investigating how it elicits that migration. FGF10 could promote migration in various ways. FGF10 could act as a chemotactic molecule. It has been shown that FGF10 can act as a chemotactic factor for distal lung epithelia; therefore, it is possible that it could act in the same way in the heart (Bellusci et al., 1997; Park et al., 1998). Also FGF10 could create a gradient of random cell motility without directionality that could result in global directed movement (Benazeraf et al., 2010). In addition FGF10 could signal to the EPDC to maintain establishment of the polarized lamellipodia.

In our experiments FGF10 does not appear to be acting as a chemotactic factor. In the lung, were FGF signaling is chemotactic, the expression of FGF10 is focally located in the mesenchyme. This ensures proper directionality for the migration of lung distal epithelium. In contrast, in the heart the expression of FGF10 appears to be spread throughout the compact myocardium but not focally located. In addition, induction of heart explants with FGF10 added to the media; resulted in an increased migration of cells into the myocardium when compared with controls. These data do not support a chemotactic role for FGF10 in the heart. Nonetheless, we have designed experiments to examine directionality of EPDCs. We can isolate epicardial cells in colonies, embed them in soft agar, and place a FGF10-coated bead in the vicinity of the colony of cells. In the future, we will investigate whether cells within these colonies move towards the bead coated with FGF10 or not. We will also try isolating colonies of cells from *Fgfr2b*$^{-/-}$ hearts and treat wild type colonies with FGFR inhibitor.

Our live imaging experiments showed that cells from wildtype, *Fgfr2b*$^{-/-}$ and control cells treated with FGFR inhibitor could all move similar distances, suggesting that motility was not impaired by inactivation of FGF signaling in epicardial cells. In contrast, only wild type cells treated with vehicle were able to show the longest net displacement. This observation implies that *Fgfr2b*$^{-/-}$ cells and wildtype cells treated with inhibitor are able to move as much as controls but that their movement is not organized to reach a migratory endpoint. In this experiment wildtype epicardial cells did not move in the same direction but were able to move successfully from one point to another. In contrast, *Fgfr2b*$^{-/-}$ cells and wildtype cells

treated with inhibitor were not able to move from one point to another, instead they remained in the same area. This observation suggests that FGF10 may function to create a gradient of random cell motility without directionality that can result in global directed movement.

Interestingly, in the live imaging movies, we observed that wildtype cell were able to maintained a polarized lamellipodia, but, *Fgfr2b*$^{-/-}$ cells and wildtype cells treated with inhibitor did not. This observation suggests that FGF10 could control migration of EPDCs by the maintenance of a polarized lamellipodia independent from directionality. In the future we could investigate this possibility by looking at the actin filament organization of *Fgfr2b*$^{-/-}$ and wildtype cells treated with inhibitor during live imaging. In addition we could treat cells with an inhibitor of actin filament motor function like blebbistatin (Benazeraf et al., 2010) to observe if it has a similar effect to inactivation of FGF signaling.

FGF signaling restriction to epicardial-derived cardiac myocytes.

In our study, we found that the FGF10/FGR2b signaling activates only EPDCs that will become cardiac fibroblast, not vsmc, cardiomyocytes or endothelial cells. Consistent with this observation, we found a decrease in cardiac fibroblasts within the myocardium of *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts. This is the first time a signal has been identified that specifically targets cardiac fibroblasts versus other epicardial-derived cells. In the past, other researchers have reported that PDGFRβ, β-catenin, Alk5 and Wt1, amongst other genes, are involved in either migration or EMT of epicardial-derived cells that specifically develop into vascular smooth muscle cells (Martinez-Estrada et al., 2010; Mellgren et al., 2008; Sridurongrit et al., 2008; Zamora et al., 2007). Mouse models from these genes present two very distinct phenotypes of defective EPDC development: poor formation of the coronary vasculature due to decreased recruitment or differentiation of vsmcs and thinned compact myocardium. In contrast, *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts do not display problems in the recruitment of vsmc in view of the properly formed vascular plexus. Future experiments will concentrate on studying how the FGF signal is restricted to only cardiac fibroblasts. Possible mechanisms of specificity could entail a balance between PDGFβ signaling and FGF signaling. For example, epicardial cells could receive both signals but only able to interpret and respond to one. In this case, many other genes could be involved in making the epicardial cell competent to receive or suppress one or the other signal. Other genes like Sprouty or Cbl which modulate downstream pathways or recycling of receptor tyrosine kinases (RTKs) could also play a role (Aranda et al., 2008; Miyake et al., 1999). Another plausible mechanism

is timing, for example PDGFβ signaling could occur prior or after FGF signals to epicardial cells. Alternatively, all epicardial cells may be fated to become vsmc, but activation of FGF10/FGFR2b signaling results in differentiation into cardiac fibroblasts, or vise versa.

Indirect induction of cardiomyocyte proliferation through the cardiac fibroblast

Our study provides evidence that migration of cardiac fibroblast into the myocardium plays a role in cardiomyocyte proliferation. We observed a decrease in proliferation of cells within the myocardium in *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts that result in a smaller heart, visible at late gestation. There are a couple of ways that FGF10/FGFR2b signaling could regulate proliferation. One is by signaling to the FGF9/FGFR1c/FGFR2c pathway during midgestation or by inducing other factors made in the epicardium. Another possible way is by inducing migration of cardiac fibroblast during ventricular compaction (Ieda et al., 2009; Lavine et al., 2005). The FGF9/FGFR1c/FGFR2c pathway has been established as the main mechanism for cardiac myocyte expansion after heart looping and throughout midgestation. In this pathway, retinoic acid receptor promotes expression of FGF9 and FGF16 in the epicardium and endocardium. FGF9 and FGF16 signal to FGFR1c and FGFR2c in the myocardium to directly activate proliferation and inhibit differentiation. This pathway indirectly induces SHH expression in the epicardium which results in the activation of VEGF and ANG2. VEGF and ANG2 promote formation of the vascular plexus. Originally we thought that FGF10 signaling to FGFR2b might function in a reciprocal feedback loop with FGF9/FGFR1c/FGFR2c to ensure sustained signaling to cardiomyocytes and induction of SHH during midgestation. When we examined development of the primitive vascular plexus, we found no delay in its formation. We hypothesized that if the reciprocal signal was abrogated in *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts we would see changes in the expression of FGF9. In the future, we need to examine the expression of FGF9 in these mouse models to find out if FGF9

is regulated. Correspondingly, we did not observe any changes in heart size in *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ embryos at midgestation when the FGF9/FGFR1c/FGFR2c signaling is active. Instead, we observed changes in heart size at late gestation starting at E17.5 coincident with the time of ventricular compaction. Our proliferation analysis revealed that proliferation was decreased at E15.5 and E17.5 in *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts. Taken together, these data suggest that FGF10/FGFR2b signaling pathways do not have a function along with the mechanism of cardiomyocyte proliferation during midgestation. The data points to a function of FGF10/FGFR2b in the later stages of cardiomyocyte expansion.

Recently it has been acknowledged that embryonic cardiac fibroblasts are important to promote β1-integrin activated-proliferation of cardiac myocytes by secretion of HBEGF. β1-integrin can induce activation of PI3k/Akt and MEK/ERK1/2 in cardiomyocytes and promote cell division. This has been regarded as the mechanism of heart growth during endpoint heart development. The main source of embryonic cardiac fibroblasts is thought to arise from the epicardial-derived cell. Other studies also have implicated not only differentiated cardiac myocytes but also EPDCs as a cell type that can physically interact with cardiac myocytes to induce cell proliferation, cellular alignment and contraction (Eid et al., 1992; Weeke-Klimp et al., 2010) .

We hypothesize that in the absence of proper EPDC migration, fewer epicardial-derived fibroblasts are going to invade the myocardium. This will result in a decrease of β1-integrin- activated cardiomyocyte proliferation and lead to the formation of a smaller heart. The phenotype of the *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ is

consistent with this hypothesis. In *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts, we observed a decrease in the migration of cardiac fibroblasts. Furthermore, we observed decreased proliferation of cardiac myocytes. We hypothesize that as a result of decreased epicardial-derived fibroblasts, migration β1-integrin signaling in the cardiac myocytes of *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts is impaired. In the future, we need to explore this hypothesis. The downstream pathways controlling β1-integrin cell division are PI3k/Akt and MEK/Erk1/2. A simple way to evaluate the activation of these downstream pathways is to compare the expression of Akt and/or Erk1/2 in *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ hearts with controls. A more functional approach would be to co-culture isolated cardiac fibroblasts from *Fgfr1/r2*$^{Wt1\text{-}Cre}$, *Fgf10*$^{-/-}$ and controls with isolated cardiac myocytes and ask whether proliferation of cardiomyocytes is decreased in the mouse models. It is already known that increased titration of isolated embryonic cardiac fibroblasts in co-cultures with myocytes increases proliferation of cardiac myocytes; therefore, we will only see a difference using this approach if we originally isolate fewer cardiac fibroblasts from *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ compared to control hearts. On the other hand, if we isolate similar amounts of cardiac fibroblast from *Fgfr1/r2*$^{Wt1\text{-}Cre}$, *Fgf10*$^{-/-}$ and controls and see no difference in proliferation, it could mean that cardiac fibroblasts from *Fgfr1/r2*$^{Wt1\text{-}Cre}$ and *Fgf10*$^{-/-}$ are unable to signal. In this case, we could look at the ability of these cells to induce β1-integrin-activated proliferation through the growth factor HBEGF. We also could explore the ability of the cardiac myocytes to produce Fibronectin1 and Collagen 3, two ECM proteins known to bind β1-integrin to induce cardiomyocyte proliferation.

FGF10/FGFR2b in adult heart repair

The study of heart repair is important for the development of therapies that could help treat heart disease, one of the most prevalent causes of mortality worldwide. One important area of investigation is the repair of damaged tissue due to myocardial infarction. Competent cardiac healing requires the proliferation of myocytes, formation of new vessels and regulation of vascular remodeling. One of the approaches taken in the past is reperfusion with known cardioprotective and angiogenic factors like VEGF and FGF2 (Molin and Post, 2007). The expectation is that these cardioprotective molecules will cause proliferation of cardiomyocytes, vascularization and reduction of the fibrotic response. Although these approaches have been successful in isolated ischemic heart models and transgenic mouse models, they have been disappointing in clinical trials (Ludman et al., 2010). Failure of these approaches seems to stem from the quantity and sustainability of the growth factor and the targeting and delivery of the cells.

Another approach undertaken in the field is the use of cardiac progenitor cells. These cells are typically grafted into the injured heart with the expectation that it will divide and generate progeny to supply the healing myocardium (Di Nardo et al., 2010). If developed properly, this method could overcome the targeting and dosage problems found with angiogenic therapies, but it could be challenging to bypass the surveillance of the immune system for rejection. In the past, several stem cell types have been studied, such as the cardiac progenitor cell and the mesenchymal stem cells, with some success of protection to the injured mouse and rat heart. (Christoforou et al., 2010; Jin et al., 2009; Yi et al., 2009).

Recently this approach has been used to study the effects of epicardial cells in the injured heart. Epicardial cells are considered to be undifferentiated cardiac progenitor cells (Wessels and Perez-Pomares, 2004). These cells have been found to differentiate into vascular smooth muscle cells, interstitial fibroblasts and cardiomyocytes, although the latter is still controversial. Recently a study isolated epicardial cells from adult human hearts, labeled them and injected into a mouse heart (myocardial infarct) MI model. This injection resulted in increased vascularization, cardiac function and decreased remodeling, suggesting that adult epicardial cells are able to promote cardiac protection (Winter et al., 2007). In this study, epicardial cells did not differentiate into cardiomyocytes, but engrafted rapidly in the heart suggesting that factors secreted by the epicardial cells could be important for cardioprotection. Additionally, Thymosin β4 has been shown to promote epicardial cell migration that results in differentiation to vsmc and endothelial cells in the adult mouse MI models (Smart et al., 2010).

In our studies, we observed that FGF10/FGFR2b signaling can induce migration of epicardial cells *in vivo* and *in vitro*. In the future, it would be important to investigate whether administration of FGF10 in the adult injured mouse heart can elicit similar cardioprotective effects to Thymosin β4. Many experiments could be designed to examine the role of FGF10 in repair. First, it would be important to see if FGF10 can elicit migration of adult epicardial cells in pathological and pathophysiological conditions. We could generate adult mice bearing GFP-labeled epicardial cells by crossing Gata5Cre with a R26R-EGFP reporter mouse. FGF10 could be administered either locally or systemically, and epicardial cells of Gata5-

EGFP hearts could be monitored for migration and differentiation. In parallel, we could use FGF10-inducible mice (*Rosa26-rtTA, TetO-Fgf10*) to promote FGF10 expression in the adult. To follow epicardial cells, we could inject very low doses of CFSE into the pericardium of the heart. If migration of adult epicardial cells was successfully activated using any of these two approaches, we would expect to see an increase in cardiac myocyte proliferation. We also expect FGF10 to promote epicardial cell migration; specifically of cardiac fibroblasts, but not vsmc or endothelial cells. The effects of treatment with FGF10 could differ from Thymosin β4. Thymosin β4 is known to induce vascularization but not proliferation of cardiomyocytes. Next we could repeat the same experiments using the adult MI model heart to uncover the response of epicardial cells to FGF10 during repair.

If adult epicardial cells are unable to respond to FGF10 we could examine the role of embryonic epicardial cell grafts pre-treated with FGF10 in the adult. For these experiments, we could isolate embryonic epicardial cells, label them with CFSE and treat them with FGF10. We can inject labeled-induced cells into adult infarcted mouse hearts and monitor the role of these cells in myocardial repair.

In summary, FGF10 signaling to FGFR2b in epicardial cells is one of the first signaling pathways found to be required for epicardial cell migration. It is very important to examine and study the potential of this pathway to indirectly affect myocyte proliferation. Understanding of this pathway and the pathways it interacts with will aid in formulating a more comprehensive view of heart development.

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Figures and Figure Legends

Figure 1.

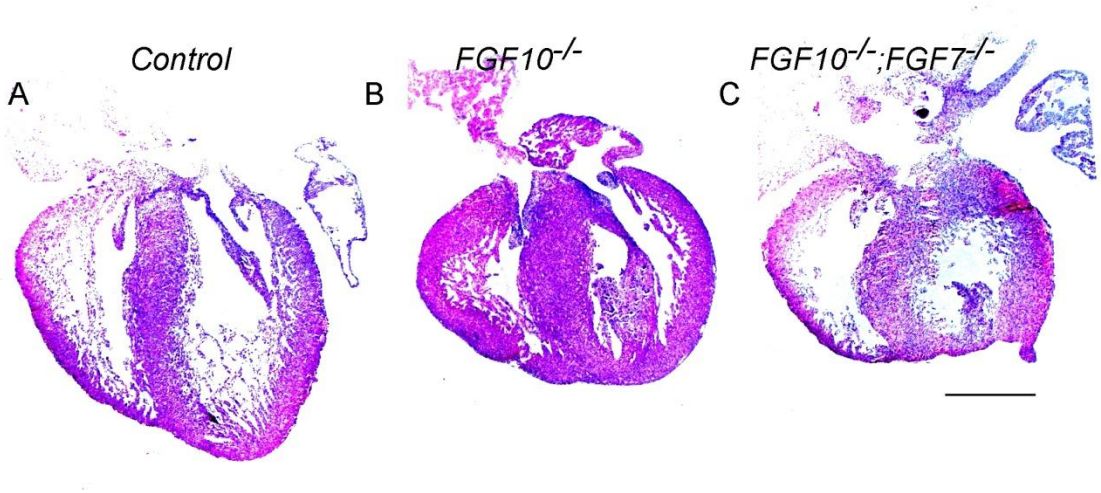

Figure 1.

H&E staining of double knockout of *FGF7*$^{-/-}$ *; FGF10*$^{-/-}$. (A) H&E of *control*. (B) H&E of *Fgf10*$^{-/-}$. (C) H&E of *FGF7*$^{-/-}$ *; FGF10*$^{-/-}$.

Appendix A

Role of TGFβ in coronary vascular formation

Mónica Vega Hernández, David M. Ornitz

Abstract

We have shown that initiation of vessel tube formation during coronary vascular development requires the expression of VEGF ligands and receptors. We know that induction of VEGF expression is regulated by a FGF9/FGFR1c/FGFR2c pathway that leads to the indirect activation of SHH. In turn, SHH induces expression of VEGF through Patched 1 activation in the myocardium. Here we explore alternative mechanisms of VEGF activation. In *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ embryos, coronary artery formation is only delayed, therefore, we hypothesized that other RTKs could be part of the normal VEGF activation and that these elicit a compensatory mechanism observed in the *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ mouse model. Alternatively we hypothesized that other transcription factors could directly regulate expression of VEGF independent from SHH signaling. In this study we look at TGFβ signaling as a candidate pathway to induce VEGF expression during coronary vascular formation. TGFβ signaling could act in a compensatory mechanism by inducing SHH in a synergistic manner with FGF. Alternatively TGFβ could directly regulate expression of VEGF. We failed to find evidence that would implicate TGFβ signaling as important to regulate VEGF expression and in turn vascular formation.

Introduction

One of four Americans suffers from some sort of heart disease. Elucidating the different molecular cascades which interplay to form the cardiovascular system can lead to a better understanding of adult heart injury and healing. FGF's, SHH, VEGF's and TGFβ's are molecules that have been shown or proposed to be important in myocardial proliferation and vasculogenesis. In this investigation we evaluate the possibility that TGFβ could act downstream or synergistically during FGF induced coronary vascular formation. We also look at the role of TGFβ in coronary vascular development by deleting *Tgfβr2* in the myocardium.

The Transforming Growth Factor Beta Super Family can be divided in two sub groups, the Activin receptor response members and the Bone Morphogenetic Protein (BMP) receptor response members (Moustakas and Heldin, 2009). Secreted ligands from both groups signal through distinctive type I (signal transducing) and type II (ligand binding) serine/threonine receptor complexes (Mokrosinski and Krajewska, 2008). This signal is transduced intracellularly by the Smad family of transcription factors. Smads are classified as, receptor regulated Smads (R-Smads), common mediator Smad (Co-Smad) and Inhibitor Smad (I-Smads). The R-Smads are activated through the receptor, consequently heterodimerizes with Co-Smad and enter the nucleus where they can form transcriptional complexes (Miyazawa et al., 2002).

TGFβ super family has been found to have an important function during heart development. The role of TGFβ isoforms during heart development is not clearly understood. Not much is known about the TGFβ3 although it is present in the epicardium along with TGFβ1 and TGFβ2. TGFβ2 deficient mice have defects in valve development and septation. In addition data suggest that ALK5 play a role in heart looping (Sanford et al., 1997). TGFβ1, 2 and 3 are reported to be immunolocalized in all tissues of the heart. TGFβ2 being the most prominent in cardiomyocytes is also localized at the media and adventitia layer of the blood vessels and in the outflow track (OFT) of the heart. TGFβ1 is expressed in the endocardial layer and TGFβ3 is expressed in the cardiac cushions and cardiac fibroblasts (Molin et al., 2003). TGFβ signaling has been implicated in epithelial to mesenchymal transition not only of the cardiac cushion but also of epicardial derived cells but it is not yet clear whether it stimulates or inhibits EMT of epicardial derived cells . Studies from different laboratories are contradictory. In the chicken embryo addition of TGFβ2 and TGFβ3 inhibit EMT in epicardial monolayers. This contrasts with previous reports that showed treatment with TGFβ3 to chicken explanted hearts induced EMT. Although debatable, most reports suggest TGFβ signaling can induce EMT (Compton et al., 2006; Dokic and Dettman, 2006; Morabito et al., 2001).

VEGF ligands and receptors are important during coronary vasculogenesis. Induction of VEGF expression is controled by a FGF9/FGFR1c/FGFR2c pathway

that leads to the indirect activation of SHH. SHH expressed in the epicardium induces expression of VEGF in the myocardium signaling to its receptor Patched1. Induction of VEGF signaling by FGF is not completely abrogated, therefore, we hypothesized that other RTKs could be part of the normal VEGF activation and that these could elicit a compensatory mechanism observed in the *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ mouse model. Another possibility is that RTK's could directly regulate expression of VEGF, independent of SHH signaling. In this study we explored TGFβ signaling as a candidate signaling pathway to induce VEGF expression during coronary vascular formation. TGFβ signaling could be one compensatory pathway by which VEGF activation is induced along with FGF. Alternatively, TGFβ could directly regulate expression of VEGF through it downstream effectors Smad2/3.

Results

In *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ embryonic hearts, the vascular plexus basal to apical migration is delayed. This delay is attributed to a decrease in VEGF expression in knockout hearts. We hypothesize that TGFβ signaling could be increased in *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ embryonic hearts as a compensatory mechanism to induced VEGF expression and in turn vascular tube formation. In order to examine this hypothesis we performed western blots of control and *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ hearts to look at the expression of activated Smad 2/3 the transcription factors downstream of TGFβ signaling normalized to endogenous Smad2/3 at E12.5 and E13.5 (Figure 1A-1B). We quantified the amount of protein but we were unable to detect any differences between controls and *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ hearts suggesting that TGFβ does not act as a compensatory mechanism to assure the proper formation of the primitive vascular plexus (Figure 1C.)

Next we hypothesize that TGFβ signaling could directly regulate the expression of VEGF by the binding of Smad2/3 to VEGF regulatory sequences. We observed that various VEGF ligands are expressed in a similar spatio temporal manner during coronary heart development. *vegf-b* and *vegf-c* are expressed in the same gradient fashion as previously describe for *vegf-a*. This concerted spatio-temporal expression suggests that all three ligands could be co-regulated during vasculogenesis. To look at known regulatory motifs that could explain these gene expression patterns, we used the program Promoter Analysis Pipeline. With this program you can examine the coincidence of binding sites of known transcription factors between possible co-regulated genes. We analyzed the promoter regions of

vegf-a, *vegf-b* and *vegf-c* and observed that they shared conserved and non-conserved Smad2 binding motifs (Figure2A-2C). This finding suggested that TGFβ signaling is capable of regulating VEGF ligand expression in a coordinated manner.

To test if Smads could control VEGF expression directly by binding to VEGF ligand regulatory sequences we created a myocardial deletion of *Tgfβr2*. TGFβR2 binds all three TGFβ ligands and is necessary to phosphorylate the receptor type I complex. We hypothesize that TGFβ ligands expressed in endocardium, epicardium and/or myocardium could bind TGFβR2 in cardiac myocytes and elicit the activation of Smad2/3 in myocardial cells. Smad2/3 in turn could directly bind to VEGF sequences in cardiac myocytes. Binding of Smad2/3 could induce VEGF expression in cardiac myocytes and VEGF ligands emanating from cardiac myocytes could activate VEGF receptors localized at endothelial cells to induce endothelial tube formation. To test this hypothesis, we stained controls and $Tgf\beta r2^{Mlc2v\text{-}Cre}$ from E12.5 and E13.5 with Pecam, a marker for endothelial cells that marks the primitive vascular plexus. We observed no change in the density or rate of vascular formation in $Tgf\beta r2^{Mlc2v\text{-}Cre}$ compared to control hearts (Figure 3).

Discussion

Our laboratory has demonstrated that FGF signaling promotes proliferation of the cardiomyoblast cell population and coronary vascular development (Lavine et al., 2005). FGF9 and FGF16 signal to FGFR1 and FGFR2 in the myocardium to induce proliferation and simultaneously promoting SHH activity. SHH signaling directly regulates VEGF and in turn coronary vascular formation (Lavine and Ornitz, 2008). In the *Fgfr1/r2*$^{Mlc2v\text{-}Cre}$ mouse model, the delay in vascular development is moderate suggesting that other growth factors might play a role in vasculogenesis. TGFβ has been proposed to be an angiogenic factor and a regulator of FGF signal *in vitro*, but there is a lack of data to link TGFβ to coronary vascular formation (Hildner et al., 2010).

Here we tried to find evidence that TGFβ could have a role in coronary vascular formation through interaction with FGF signaling by either a compensatory mechanism or a synergistic mechanism. It has been reported that FGF and TGFβ have both opposing and combinatorial effects in different biological processes (Bosse et al., 2006; Ko et al., 2009; Lee et al., 2010; Ramos et al., 2010). We were unable to observe a differential expression of TGFβ upon deletion of FGF receptors in the myocardium, suggesting that TGFβ is unlikely to compensate for the lack of FGF signaling during midgestation. One possible explanation is that many other receptor tyrosine kinases are recruited to ensure proper growth and formation of the coronary vasculature. This could be a mechanism of heart development to protect

organ survival via a compilation of redundant pathways. Other possible RTK's involved in myocardial growth are IGF2, Erb and PDGF signaling.

The observation that TGFβ is unchanged upon FGF signaling ablation does not eliminate the possibility that TGFβ signaling could control vessel development directly in cardiac fibroblasts. Although all three VEGF ligands have binding sequences for Smad in their promoter region we did not observed any defect in the formation of the vasculature, suggesting that VEGF ligands are able to signal properly in the absence of TGFβ signaling from the cardiomyocytes. This result suggests that TGFβ signaling is dispensable in the cardiac myocyte because no other growth or morphological defects were observed. Consistent with this observation deletion of Alk5 (a TGFβ type I receptor) in the myocardium results in no phenotype (Sridurongrit et al., 2008). Alternatively, TGFβ action could be necessary in other cell type of the heart during coronary vascular formation. TGFβ ligands are expressed in many other cell types such as the epicardium, endocardium, cushion mesenchyme, cardiac fibroblast and smooth muscle cells, and endothelial cells. Elucidating which cell type requires the activation of TGFβ will require conditional inactivation of these genes. Another possibility is that another TGFβ receptor type II could be acting redundantly within the cardiac myocyte.

To conclude, we found that FGF signaling does not interact with TGFβ to contribute to formation of the coronary vasculature and that TGFβ signaling in the

myocardium is dispensable for coronary vascular development. This conclusion is consistent with published results were deletion of *Alk5* (a receptor type 1) in the myocardium does not play a major role in the myocardium during development. Taken together this information supports that TGFβ signaling in the myocardium is not necessary for heart development (Sridurongrit et al., 2008).

Materials and Methods

Western blot

Homogenize heart in RIPA buffer with protease inhibitor cocktail. Measure protein concentration using Bradford test and boil sample for 5min at 95°C. Run gel using precast biorad gels and Biorad running gel buffer. Transfer 1h and 30min at 80 volts at 4°C. Block for 1h with 5% powder milk at 4°C. Primary antibody (Santa Cruz Biotechnologies, sc-11769) 1h. Incubate in secondary antibody for 1h. Develop using luminescent reagent. Strip and reprobe.

Promoter analysis pipeline (PAP)

Is a software developed by the Washington University Biomedical Informatics Core. The software analyzes a set of co-expressed genes (in this case VEGF ligands co-expressed in the heart) to identify possible transcription factor that could be orchestrating their co-expression in vivo. The software identifies shared transcription factor motifs that are found in the promoter region of the genes of interest. To use this software you create an account in the following website

http://bioinformatics.wustl.edu/webTools/PromoterAnalysis.do;jsessionid=2D5029FD0BAF20BC7E82F452B8CD97CD

Once created you are able to input the accession numbers of your genes of interest. The software runs remotely so you do not have to download the program. The results are displayed with an option to export them to your personal files.

Whole mount immunohistochemistry of pecam

Tissue is fixed in 4%PFA overnight at 4°C. Tissue is dehydrated in methanol series and block from endogenous peroxidase using 4 to1 volumes of methanol to 30% hydrogen peroxide for 3h. Tissue is rehydrated in methanol series. Tissue is incubated with ProteinaseK 10µg/ml for 30 min at room temperature. Block with 2% skim milk, 5% serum, 0.1%BSA, 0.1%triton-x for 2h. Primary (Abcam, ab28364) antibody in blocking overnight. Secondary antibody byotinilated. Develop in DAB.

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Figures and Figure Legends

Figure 1.

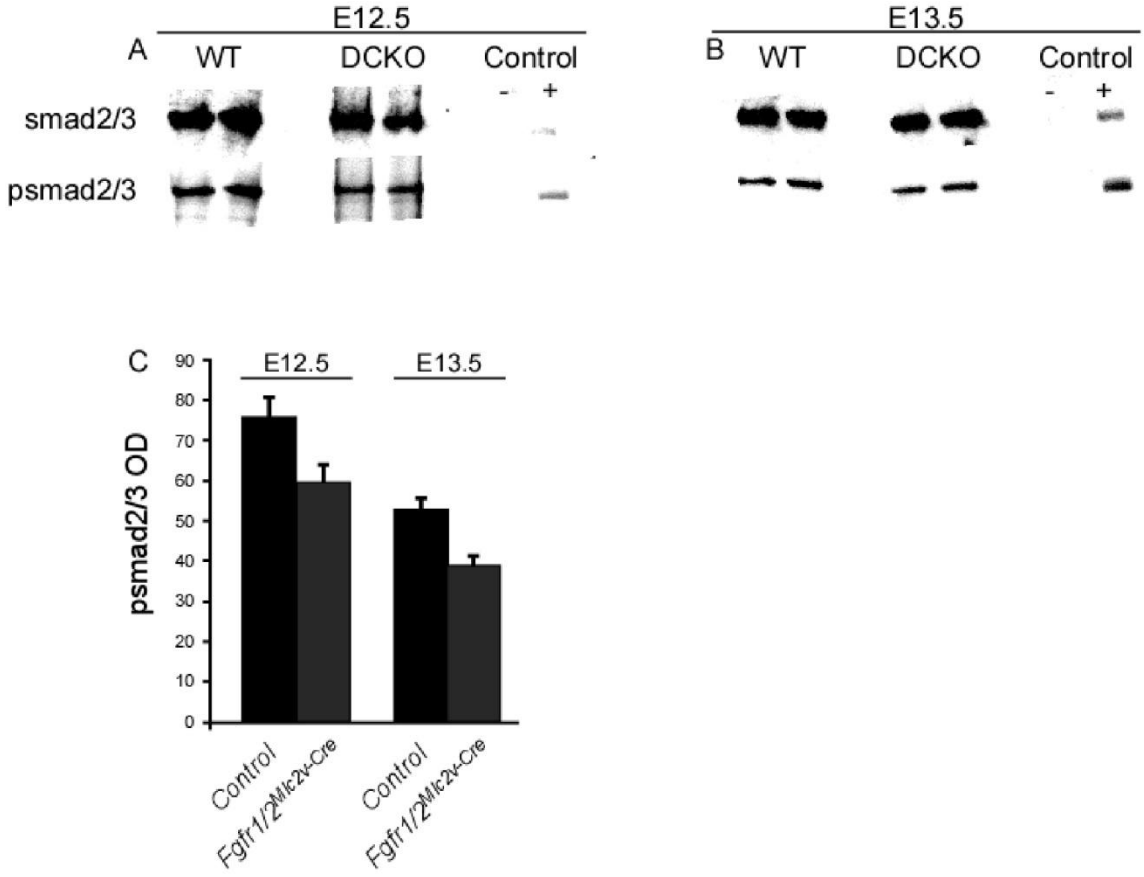
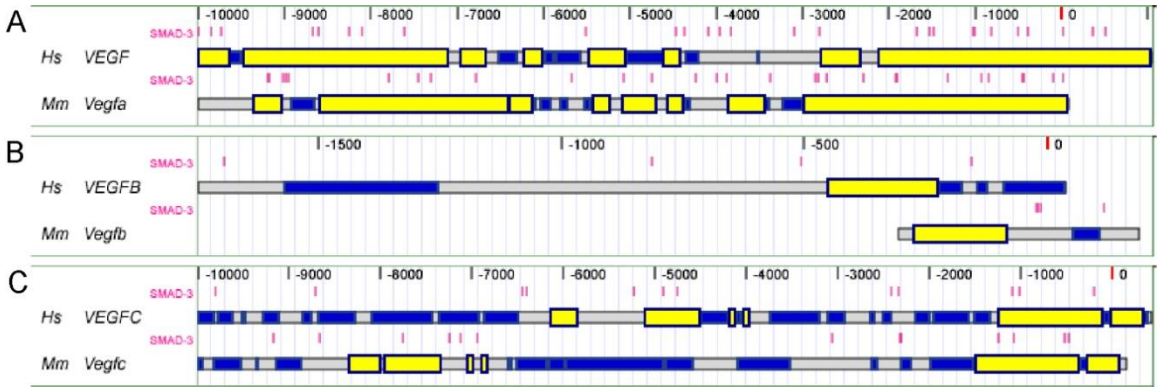


Figure 1.

Western blot for Smad2/3 and pSmad2/3 at embryonic stages E12.5 and E13.5. (A) Shows the expression of Smad2/3 (top row) and pSmad2/3 (bottom row) of control and *Fgfr1/r2*$^{Wt1\text{-}Cre}$ knockout hearts. Expression of *Fgfr1/r2*$^{Wt1\text{-}Cre}$ is similar to controls in both the endogenous Smad2/3 and pSmad2/3 at E12.5. (B) Shows the expression of Smad2/3 (top row) and pSmad2/3 (bottom row) of control and *Fgfr1/r2*$^{Wt1\text{-}Cre}$ knockout hearts. Expression of *Fgfr1/r2*$^{Wt1\text{-}Cre}$ is similar to controls in both the endogenous Smad2/3 and pSmad2/3 at E13.5. HepG cells were used as negative control. Positive control was obtained by inducing HepG cells with TGFβ3 protein to induce Smad phosphorilation. (C) Quantification of optical density for western blots resulted in no change once normalized to endogenous levels of Smad2/3. Controls and *Fgfr1/r2*$^{Wt1\text{-}Cre\ +}$ n=4.

Figure 2.


A
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-9000
-8000
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-6000
-5000
-4000
-3000
-2000
-1000
0
SMAD-3
Hs VEGF
SMAD-3
Mm Vegfa
B
-1500
-1000
-500
0
SMAD-3
Hs VEGFB
SMAD-3
Mm Vegfb
C
-10000
-9000
-8000
-7000
-6000
-5000
-4000
-3000
-2000
-1000
0
SMAD-3
Hs VEGFC
SMAD-3
Mm Vegfc

Figure 2.

Shared promoter sites for Smad3 in *vegf-a*, *vegf-b* and *vegf-c*. (A) Shows conserved and non conserved Smad3 transcription binding site on (hs) human and (Mm) mouse for *vegf-a*. (B) Shows conserved and non conserved Smad3 transcription binding site on (hs) human and (Mm) mouse for *vegf-b*. (C) Shows conserved and non conserved Smad3 transcription binding site on (hs) human and (Mm) mouse for *vegf-c*

Figure 3.

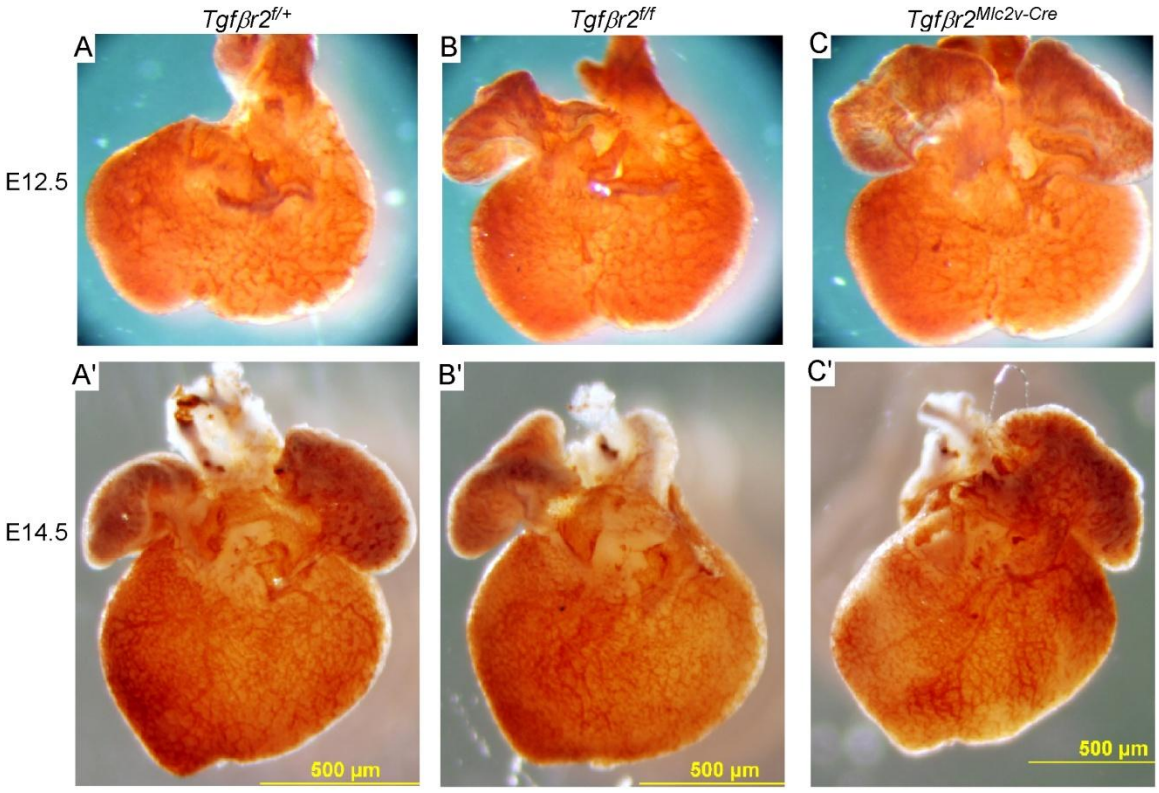
Figure 3.
Tgfβr2f/+
Tgfβr2f/f
Tgfβr2Mlc2v-Cre
A
B
C
E12.5
A'
B'
C'
E14.5
500 µm
500 µm
500 µm

Figure 3.

Whole mount pecam immunohistochemistry. (A-A') *Tgfβ*$^{f/+}$, n=3 , E12.5 and E13.5 respectively . (B-B') *Tgfβ*$^{f/f}$ (C-C') E12.5 and E13.5 respectively. n=3 *Tgfβr2*$^{Mlc2v\text{-}Cre}$ n=3 E12.5 and E13.5 respectively. Scale: 500μm

Appendix B

Inactivation of *Fgfr1* and *Fgfr2* using the epicardial specific Gata5-Cre does not affect, epicardial development or coronary vascular formation during midgestation

Mónica Vega Hernández, David M. Ornitz

Abstract

Previously we found that deletion of *Fgfr2b* in the germline leads to decrease cardiomyocyte proliferation and as a smaller heart. Germline inactivation causes deletion of *Fgfr2b* in all the cells of the mouse embryo, therefore we cannot distinguish in which cell type is the action of *Fgfr2b* required. We hypothesized that *Fgfr2b* and or *Fgfr1b* could be functioning in the epicardial layer of the heart. Here we deleted *Fgfr2b* and *Fgfr1b* specifically from the epicardium using Gata5-Cre. We found that unlike deleting *Fgfr1* and *Fgfr2* with another epicardial Cre (Wt1-Cre) or deleting *Fgfr2b* in the germline deletion of *Fgfr2b* and *Fgfr1b* during the spatio-temporal context of Gata5-Cre activation is dispensable for heart development. Differences in the effect of $Fgfr2b^{-/-}$ and $Fgfr1/r2^{Wt1\text{-}Cre}$ in heart development vs $Fgfr1/r2^{Gata5\text{-}Cre}$ could be due to various reasons: for example, target of cell type within the epicardium, effective cre recombination and temporal expression of the Cre-line.

Introduction

The epicardium is the outermost layer of the heart. Epicardial cells travel from the proepicardium to the posterior base of the heart and migrate and extend over the heart as a single cell layer. Later they undergo epicardial to mesenchymal transitions to give rise to vascular smooth muscle cells (vsmc), cardiac fibroblasts and pericytes (Cai et al., 2008; Dettman et al., 1998; Merki et al., 2005; Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996; Snider et al., 2009). These cells are very important to the formation of the adult heart. Vascular smooth muscle cells become part of the arterial wall and cardiac fibroblasts interact with myocytes to induce proliferation (Kattan et al., 2004; Lavine et al., 2006; Red-Horse et al., 2010). Epicardial differentiation is not well understood, it is hypothesized that these cells express different cell fate markers as early as in the proepicardium. In addition, how these cells migrate into the myocardium is not very well studied either.

FGF signaling is very important during development. The FGF family is composed of fifteen canonical ligands and four receptors (Itoh and Ornitz, 2008; Ornitz and Itoh, 2001; Turner and Grose, 2010). FGFR1, FGFR2 and FGFR3, but not FGFR4, undergo alternative splicing and give rise to alternative splice variants c and b. FGFR splice variant b is consistently expressed in epithelial like tissues and FGFR splice variant c is preferentially expressed in mesenchymal like tissue. The ligands are classified in subgroups due to their sequence similarity and ligands within a group activate the same receptor splice variant with similar affinity (Itoh and Ornitz, 2004). FGF signal transduction can proceed through the activation of three main

pathways: Ras/MAPK pathway, phospholipase Cγ (PLCγ)/Ca^{2+} pathway, and the PI3 kinase/Akt pathway (Eswarakumar et al., 2005; Lemmon and Schlessinger, 2010).

FGF signaling has been shown to be important during heart development. FGF9 emanating from the epicardium signals to receptors FGFR1c and FGFR2c in the myocardium to induce cardiomyocyte proliferation (Lavine et al., 2005). In addition FGFR2b, which is tought to be expressed in the epithelial cell types of the heart, is also important for the control of heart size. The main epithelial cell types and epithelial-like cell types of the heart are endothelial cells, endocardial cells and epicardial cells (Marguerie et al., 2006). We have deleted FGFR1 and FGFR2 using two endothelial specific Cre alleles (Tie2-Cre and Flk1-Cre) and found that both animal models to undergo normal heart development. Hence, we hypothesized that FGFR2b could function in epicardial cells. To test this hypothesis we used Cre-Loxp recombination to inactivate *Fgfr2b* in the epicardium using an epicardial specific Cre (Gata5-Cre) that we refer to here as $Fgfr1/r2^{Gata5\text{-}Cre}$. We found that $Fgfr1/r2^{Gata5\text{-}Cre}$ hearts do not develop a smaller heart, in addition we found that the coronary vasculature in these hearts is normal. This suggests that FGFR1b and FGR2b in the epicardium do not exert a function during the spatial and temporal domain of Gata5 expression. Alternatively, recombination by Gata5-Cre could be innefficient or restricted to a non-fibroblast lineage.

Results

To determine if Gata5-Cre would induce recombination in the epicardial layer, we crossed Gata5-Cre mice with the Rosa26 reporter mice (hereafter refer as *Rosa26;Gata5-Cre*). We then examined if recombination led to expression of β-galactosidase in the epicardium (Figure 1). We observed that expression of β-galactosidase was present in *Rosa26;Gata5-Cre* but not in control littermates lacking either or both: *Gata5-Cre* and or *Rosa26.* The expression of β-galactosidase in the epicardium suggested that *Gata5-Cre* is able to induce recombination in epicardial cells.

We then created the *Fgfr1/r2*$^{Gata5\text{-}Cre}$ mice and looked at the general histology by H&E staining (Figure 2). The H&E showed that at E13.5 *Fgfr1/r2*$^{Gata5\text{-}Cre}$ embryonic hearts seemed to have less subepicardial space but the thickness of the compact myocardium was comparable to control littermates. Decrease subepicardial mesenchyme could result in defects in epicardial EMT, migration and differentiation. Disruption of these processes can lead to defects in coronary vascular formation because epicardial derived cells contribute to the media layer of coronary arteries.

We hypothesize that deletion of *Fgfr1* and *Fgfr2* with Gata5-Cre could lead to improper formation of the coronary vasculature. We examined the endothelial vascular plexus of controls and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ hearts and found that coronary artery formation proceeds normal at E13.5 (Figure 3). We looked at the formation of subepicardial vessels and intramyocardial vessels and found that both are present in controls and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ hearts (Figure 3A'-3B'). We next followed the growth

of these heart at later stages and found that both, their size and coronary vessel development is normal (Figure 3C-3D).

We measured the relative area of controls and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ at E13.5 and E17.5 and found no significant difference between them (Figure 3E). We next wanted to see if the apparent decrease in subepicardial mesenchyme, observed in Fgfr1/r2$^{Gata5\text{-}Cre}$ hearts would lead to defects in EMT. We looked at two main EMT markers, cytokeratin and vimentin (Figure 4). When cells are undergoing EMT they shift their cytoskeleton from epithelial-like (cytokeratin) to mesenchymal –like (vimentin). We observed no difference between controls and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ hearts in their expression of cytokeratin in the epicardial layer or vimentin within the myocardial area. We measured the relative pixel intensity (mgv) of the cytokeratin expression in the epicardium and failed to find any significant change between littermate controls and *Fgfr1/r2*$^{Gata5\text{-}Cre}$hearts. Next, we checked Wt1 expression because this gene has been shown to induce epicardial to mesenchymal transition (Figure 5). We measured the total number of Wt1^{+} cells within the epicardium, sub epicardium and myocardium and found no difference in the total number of Wt1^{+} cells when compared to controls and *Fgfr1/r2*$^{Gata5\text{-}Cre+}$ embryonic hearts.

Discussion

We expected that ablation of *Fgfr1* and *Fgfr2* in the epicardium with *Gata5-Cre* would lead to a smaller heart similar to $Fgfr2b^{-/-}$ phenotype, but failed to detect any differences between controls and $Fgfr1/r2^{Gata5\text{-}Cre}$ hearts. This result is surprising because it differs from the results we observed in chapter two of this thesis using the Wt1-Cre allele. Using Wt-1Cre, we observed a very similar phenotype to the $Fgfr2b^{-/-}$ mouse. We propose that these differences could be due to the following reasons:

1. The time of expression of *Gata5-Cre*. The germline knockout of *Fgfr2b* is deleted throughout development, therefore, the function of FGFR2 is abrogated at all time points in development. On the other hand *Gata5-Cre* starts to be expressed at E9.25 in proepicardial cells and continues to be expressed in the epicardial cell. Alternatively, *Wt1-Cre* expression starts in the proepicardium at E9.5 and continues to be expressed in the epicardium all throughout development, similar to *Gata5-Cre.* These differences in timing of ablation could lead to different phenotypic results.

2. The cell type that expresses *Gata5-Cre.* The germline knockout targets every single cell in the mouse embryo but *Gata5-Cre* only targets the proepicardial and epicardial cells but not the epicardial derived cells. In contrast *Wt1-Cre* continues its expression in epicardial derived cells making it not only an epicardial deletion but also an epicardial derived cell deletion of FGFR1 and FGFR2. These differences could account for the discrepancy in the phenotypes.

3. The type of Cre. *Gata5-Cre* is a transgenic Cre insertion but *Wt1-Cre* is a knock-in of Wt1 sequences driving cre recombinase cDNA inserted at the *Wt1* gene locus. This insertion results in the expression of just on normal allele of Wt1. It is possible that haploinsufficiency of Wt1 could create a more sensitive genetic background and result in the different phenotypes we described here and in chapter two. However, alone, Wt1-Cre does not affect EPDC migration into the myocardium.

4. Background of mouse strains. Although both *Gata5-Cre* and *Wt1-Cre* have been mated into mix backgrounds there still a possibility that the genetic makeup of the lines is different and susceptibility mutations could be generating the contrasting phenotypes.

5. The phenotype in *Gata5-Cre* might not be as severe. The phenotype could still be present in $Fgfr1/r2^{Gata5\text{-}Cre}$ hearts but differences like the ones mentioned: mouse strain, timing and temporal expression could be responsible for a less severe phenotype that we don't have the sensitivity to detect.

6. In addition, the effectiveness of Cre recombination could be compromised in *Gata5-Cre.*

Materials and Methods

LacZ staining

Staining for β-galactosidase was performed as described (Soriano, 1999).

Histology

Paraffin sections (5μm) were stained with hematoxilin and eosin (H&E) for general visualization. Myocardial area was calculated with the contouring tool using Canvas X software. Area of the heart was defined as the measure of total muscle including both chambers in one whole mount picture. Atrial area was not included. In *Fgfr1* $^{Wt1\text{-}Cre}$, *Fgfr2* $^{Wt1\text{-}Cre}$ and *Fgfr1/2* ^{Wt1Cre}. Statistical significance was determined using the student's t-test, with n representing number of embryonic hearts examined.

Whole mount immunohistochemistry of pecam

Tissue is fixed in 4%PFA overnight at 4°C. Tissue is dehydrated in methanol series and block from endogenous peroxidase using 4 to1 volumes of methanol to 30% hydrogen peroxide for 3h. Tissue is rehydrated in methanol series. Tissue is incubated with ProteinaseK 10μg/ml for 30 min at room temperature. Block with 2% skim milk, 5% serum, 0.1%BSA, 0.1%triton-x for 2h. Primary (Abcam, ab28364) antibody in blocking overnight. Secondary antibody byotinilated. Develop in DAB.

Immunofluorescence

For immunofluorescence, paraffin sections (5μm) were dewaxed, rehydrated, incubated in methanol/hydrogen peroxide, antigen unmasked, and blocked in 10% goat serum. Antigen unmasking was performed by incubating sections in 1% trypsin for 5 min at room temperature or by pressure cooking in citrate buffer for 15 min. Primary antibodies used were cytokeratin (Dako, M3515),vimentin (mouse IgM, abcam, ab20346), Wt-1 (mouse $IgG_{1\kappa}$, Dakocytomation, M3561), Secondary antibodies were incubated for 1hr and visualized with a Zeiss confocal microscope or Zeiss apotome microscope.

Acknowledgement

We acknowledge Pilar-Lozano for sending the Gata5-Cre strain.

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Figure and Figure Legends

Figure1.

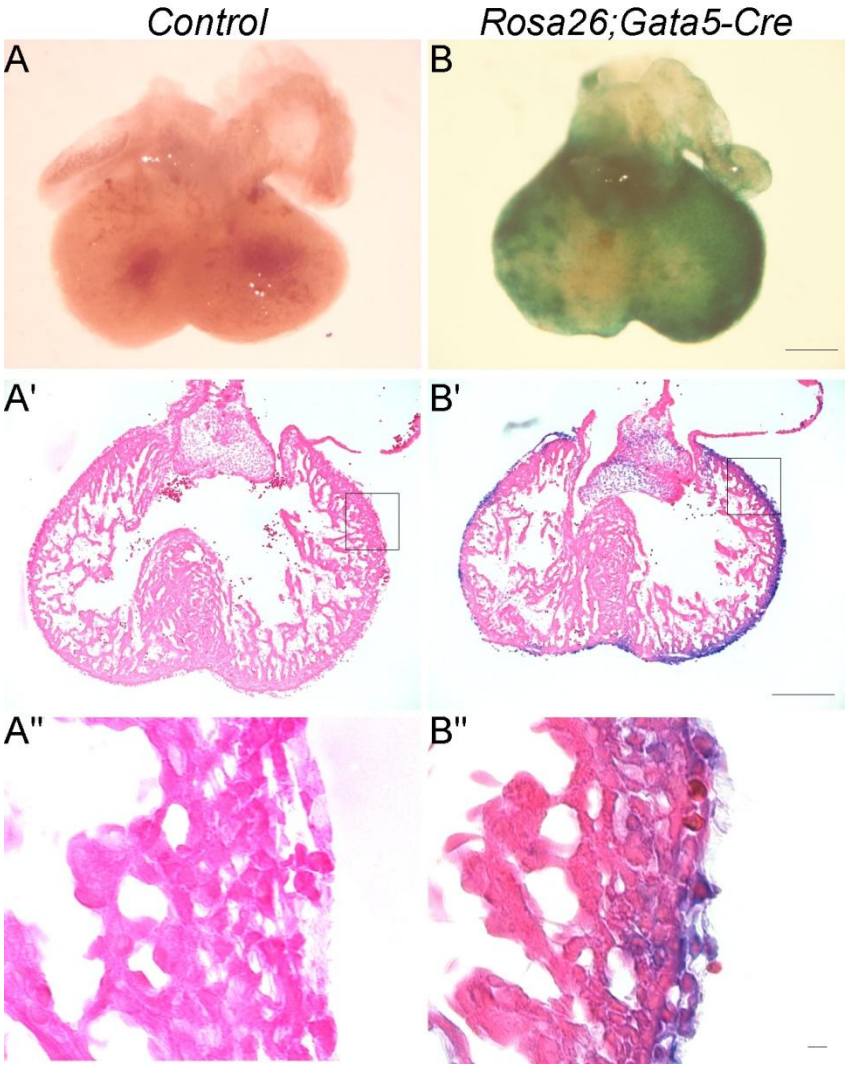


Figure 1.

β-galactosidase staining of control hearts at E13.5. (A-B) Whole mount β-galactosidase staining for (A) control (*Rosa26*) and (B) *Rosa26;Gata5-Cre* at E13.5. (A') Coronal cross section of control without *Cre* and (B') coronal section of *Rosa26;Gata5-Cre*. (A''-B'') Magnified inset from black squares in (A'-B'). Scale bars: (A-B) and (A'-B') 500μm, (A''-B'') 10μm.

Figure 2.

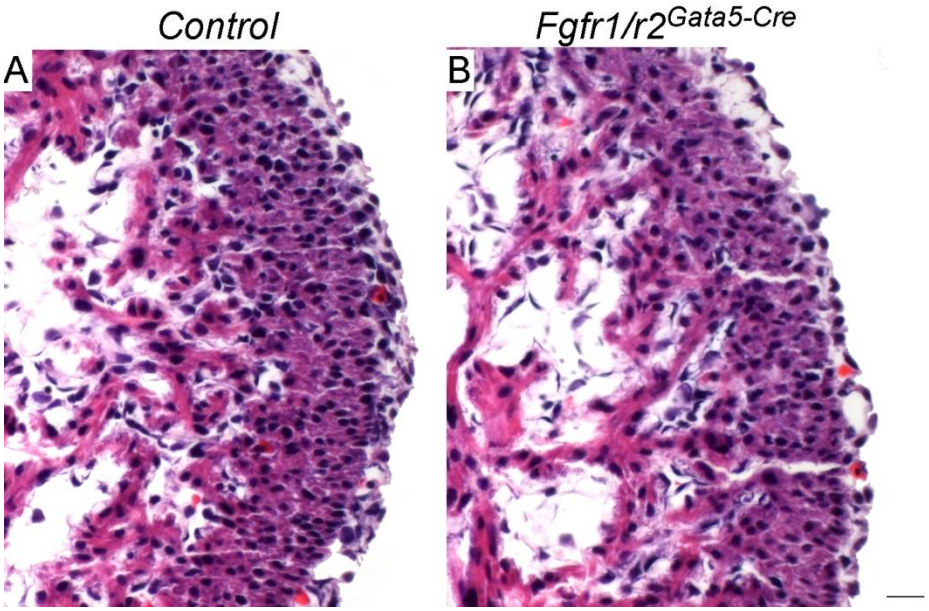


Figure 2.

H&E staining. (A) Coronal cross section of control heart stained with hematoxylin and eosin at E13.5. (B) Coronal cross section of *Fgfr1/r2*^{Gata5Ccre} heart stained with hematoxylin and eosin at E13.5. Scale bar: 20μm.

Figure 3.

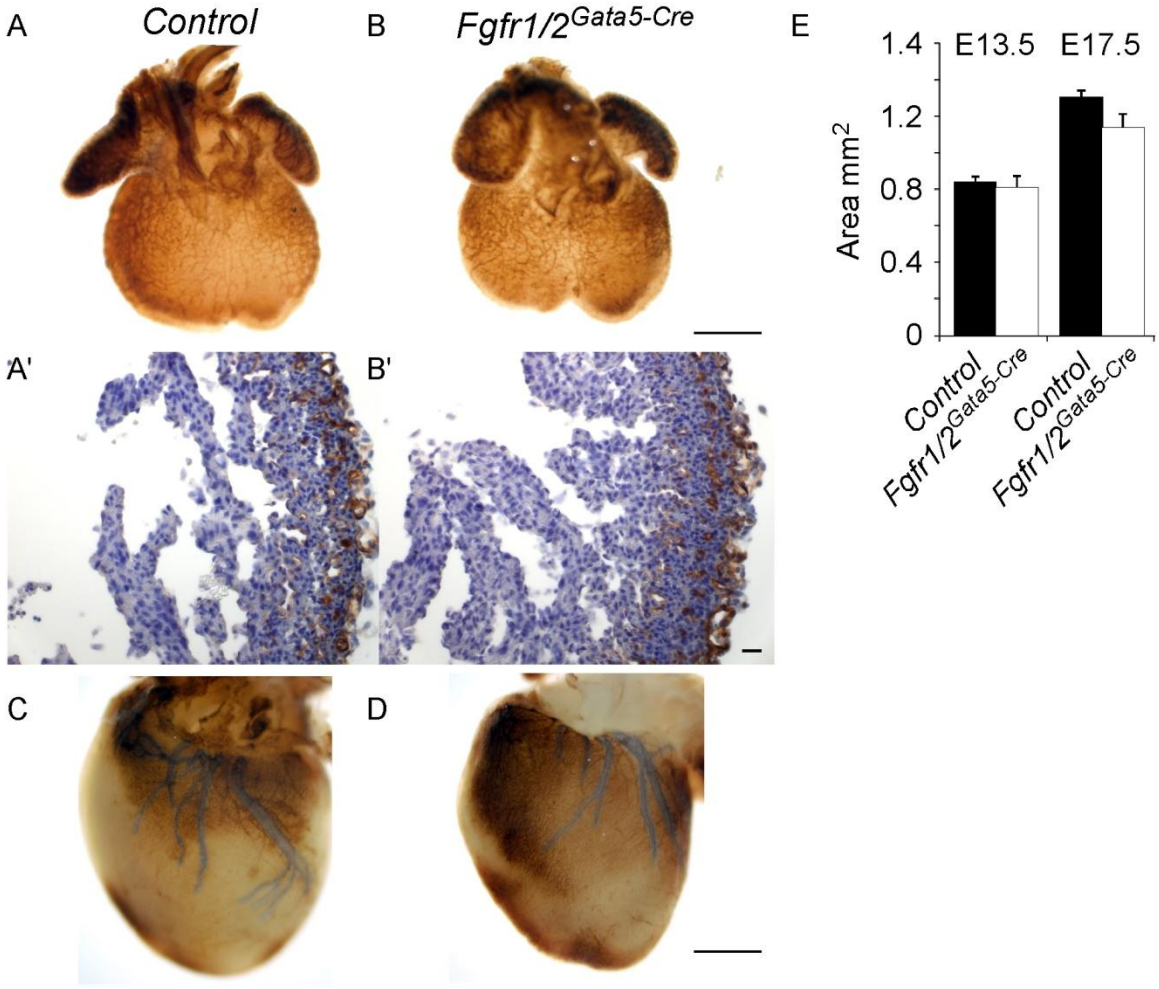

A
Control
B
Fgfr1/2Gata5-Cre
E
A'
B'
C
D
1.4
1.2
0.8
0.4
0
E13.5
E17.5
Area mm2
Control
Fgfr1/2Gata5-Cre
Control
Fgfr1/2Gata5-Cre

Figure 3.

Pecam immunostaining. (A-B) Whole mount Pecam staining of control (A) and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ (B) at E13.5. (A'-B') Cross-section of whole mount Pecam stained hearts at E13.5 control. (C-D) Pecam staining of control (C) and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ (D) at E17.5. Main coronary vessels are highlighter in gray in (C-D). (E) Quantification of the area of the heart at E13.5 (controls, n=5, *Fgfr1/r2*$^{Gata5\text{-}Cre}$, n=4*)* and E17.5 (*controls*, n=3, *Fgfr1/r2*$^{Gata5\text{-}Cre}$, n=3). Scale bar (A-B) and (C-D) 500μm, (A'-B') 20 μm.

Figure 4.

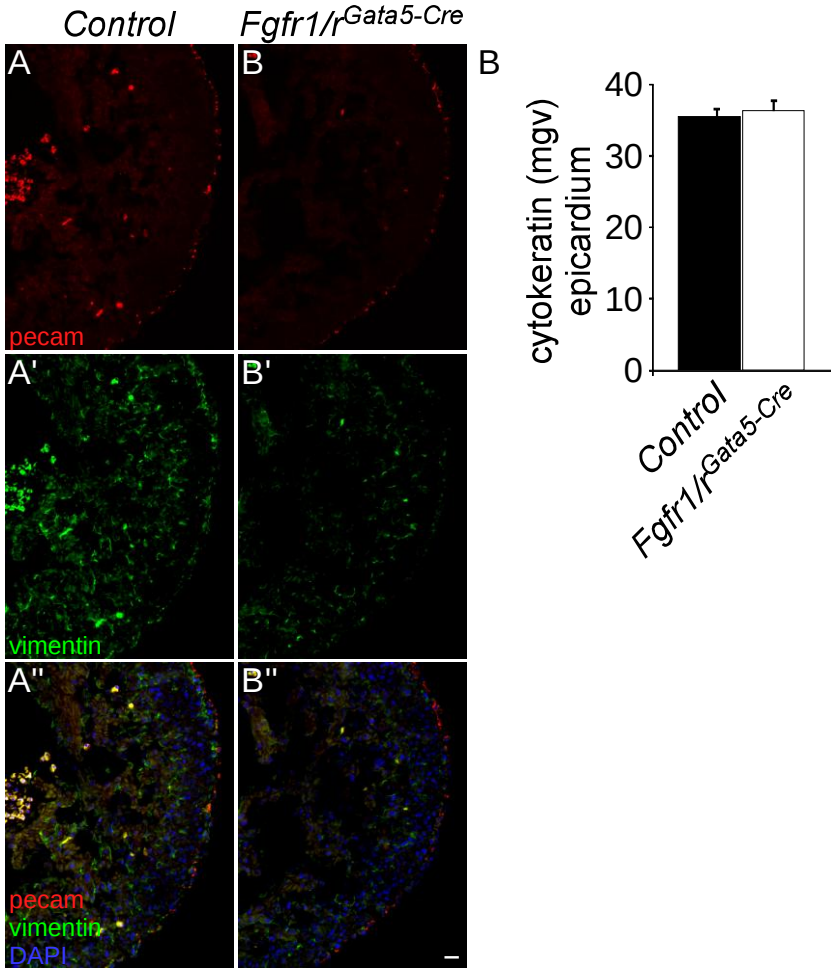
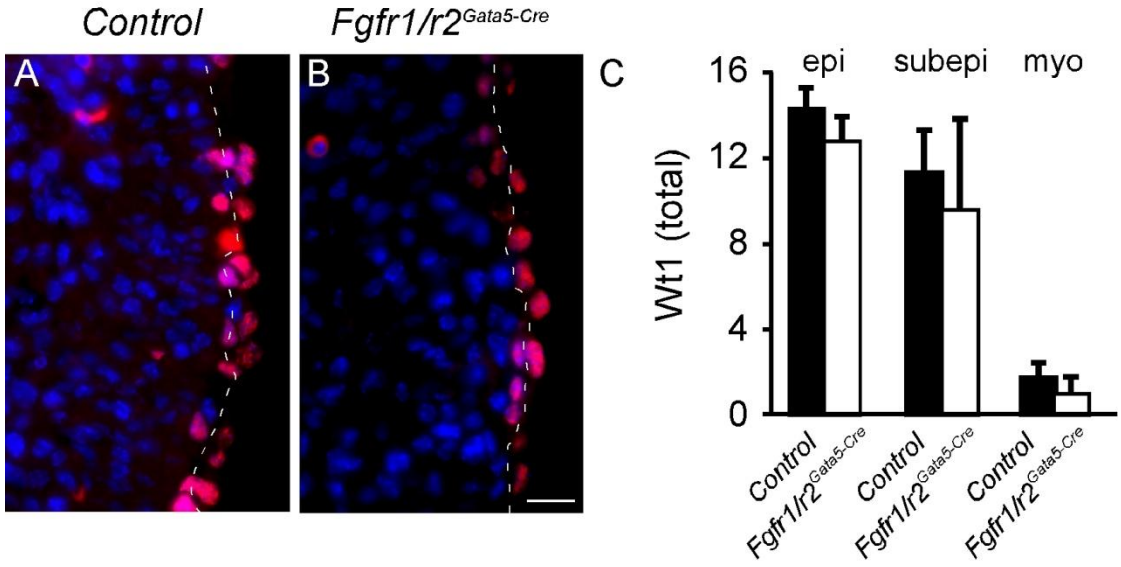

Control
Fgfr1/r Gata5-Cre
A
B
pecam
A'
B'
vimentin
A''
B''
pecam
vimentin
DAPI
B
cytokeratin (mgv)
epicardium
40
30
20
10
0
Control
Fgfr1/r Gata5-Cre

Figure 4.

Immunofluorescence of cytokeratine and vimentin. (A-B) Cytokeratin (red) staining in controls (A) and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ (B). (A'-B'') Vimentin (green) staining in controls (A') and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ (B'). (A''-B'') Merge with DAPI (blue) staining in controls (A'') and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ (B''). (C) Quantification of cytokeratin average fluorescence *controls*, n=9, *Fgfr1/r2*$^{Gata5\text{-}Cre}$, n=6. Dapi (blue). Scale bar: 20μm.

Figure 5.


Control
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A
B
C
epi
subepi
myo
Wt1 (total)
16
12
8
4
0
Control
Fgfr1/r2Gata5-Cre
Control
Fgfr1/r2Gata5-Cre
Control
Fgfr1/r2Gata5-Cre

Figure 5.

Wt1 immunofluorescence. (A-B) Wt1 (red) immunostaining for control (A, n=9) and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ (B, n=5). White dashed line delimits the boundary between epicardium and myocardium. (C) Quantification of total Wt1 in epicardium (epi), subepicardium (subepi), and myocardium (myo) of control and *Fgfr1/r2*$^{Gata5\text{-}Cre}$ at E13.5. DAPI (blue). Scale bar: (A-B) 20μm.