REGULATION OF CARDIAC FIBROBLAST AND CORONARY VASCULAR SMOOTH MUSCLE DEVELOPMENT BY PLATELET DERIVED GROWTH FACTOR RECEPTORS

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To my friends and family, especially my parents Mike and Mary Smith

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by

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Coronary vascular smooth muscle cells (cVSMC) and cardiac fibroblasts are essential for coronary artery development and are important mediators of myocardial pathogenesis. These cells form when a subset of epicardial cells undergoes an epithelial-to-mesenchymal transition (EMT) and migrates into the myocardium. The pathways and mechanisms regulating epicardial derived cell (EPDC) development remain largely unknown. Using mice with epicardial specific deletions of the PDGF receptors, I discovered that these receptors control EPDC fate and epicardial EMT. I demonstrated that each receptor was required for development of a unique epicardial derivative, PDGFRα for cardiac fibroblasts and PDGFRβ for cVSMC. I also found that deletion of both PDGF receptors led to a complete loss of EPDCs caused by a failure in cell exit from the epicardium. This defect resulted from decreased expression of genes involved in the EMT process and continued epithelial gene expression. Finally, I showed that Sox9, an SRY-related transcription factor, is a critical downstream mediator of PDGF signaling. This body of work establishes PDGF signaling as a key EMT regulatory pathway and suggests a novel role for Sox9 in regulating EPDC development.

Table of Contents

List of Publications

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List of Figures

List of Abbreviations

PDGF	Platelet derived growth factor
EPDC	Epicardial derived cell
cVSMC	Coronary vascular smooth muscle cell
EMT	Epithelial to mesenchymal transition
Myo	myocardium
Epi	Epicardium
LV	Left ventricle
RV	Right ventricle
EKO	Epicardial knockout
pm	papillary muscle
SEM	Subepicardial mesenchyme
E	Embryonic Day
R26R	Rosa 26 reporter
Gata5-Cre	Epicardial cre line
WT1iCre	Tamoxifen inducible epicardial cre line
TAB	Thoracic aortic banding
PI3K	Phosphatidylinositol 3-kinase
β-Gal	β-Galactosidase
GFP	Green fluorescent protein
tdT	tandem dimer Tomato
YFP	Yellow fluorescent protein
HW/BW	Heart weight to body weight ratio
HW/TL	Heart weight to tibial length ratio
F2/F2	Mice unable to signal through PI3K downstream of PDGFRβ
F5/F5	Mice unable to signal through PI3K, RasGAP, SHP-2, and PLCγ downstream of PDGFRβ
F7/F7	Mice unable to signal through Src, Grb2, PI3K, RasGAP, SHP-2, and PLCγ downstream of PDGFRβ

Chapter I

Introduction

The Platelet derived growth factor (PDGF) receptors have been thought to be important for development of many mesenchymal cell populations (Andrae et al. 2008), but their role in coronary vascular development has not been extensively studied. This body of work focuses on the role of PDGF receptor signaling in the development of cardiac fibroblasts and coronary vascular smooth muscle cells.

Cardiovascular Disease

Cardiovascular disease is the number one cause of death for men and women in the United States (Roger et al. 2011). More than one in three Americans has some form of cardiovascular disease, including high blood pressure, coronary artery disease (CAD), heart failure, stroke and congenital cardiovascular defects (Roger et al. 2011). There are many risk factors that predispose individuals to heart disease, including: smoking, hypertension, high cholesterol, diabetes, and obesity (Roger et al. 2011). Prevention of this disease lies in reducing these risk factors. However, patients cannot change their genetic predisposition and may develop symptoms despite maintaining a healthy lifestyle.

While there are many treatments designed to combat heart disease, it is a progressive disease that if not adequately controlled, can cause several complications, including myocardial infarction, stroke, aneurysm, sudden cardiac arrest, and heart failure. Of these, heart failure is the most common and accounts for 34% of all cardiovascular deaths (Roger et al. 2011). Current treatments and therapies are inadequate for long-term patient survival and treat only symptoms and not the underlying pathology causing the chronic disease.

One way to treat the adverse chronic affects of heart failure is to repair the damaged myocardium. Cardiac regeneration has been well documented in two vertebrate species, newt

and zebrafish, where cardiac injury initiates dedifferentiation and proliferation of cardiomyocytes (Oberpriller and Oberpriller 1974; Kikuchi et al. 2010). However, whether or not regeneration occurs in the mammalian heart has been controversial. A couple of recent studies have shown that there is myocyte turnover in the mammalian heart, but it is likely insufficient to compensate for the widespread loss of cardiomyocytes seen in myocardial infarctions (Drenckhahn et al. 2008; Bergmann et al. 2009). These new studies suggest there is either a cardiomyocyte precursor population or a population of differentiated cardiomyocytes with proliferation potential. However, there is no clear consensus on how to identify these cells and it is unclear if they can be used on a scale necessary to improve cardiac function. An alternative strategy to using resident cardiac stem cells has been to use stem cells derived from extracardiac sources, such as bone marrow stem cells and mesenchymal stem cells to try and regenerate the myocardium (reviewed in (Hansson et al. 2009)). This approach has had varying levels of success and while there appears to be a modest increase in cardiac function, it is not clear this is due to cardiac regeneration (Hansson et al. 2009).

Regardless of the mechanism used to regenerate the myocardium, there are many physiological barriers to overcome that occur during heart failure, including fibrosis and insufficient blood vessel generation, in order for these therapies to be successful. It is clear that non-cardiomyocyte populations in the heart, such as coronary vascular smooth muscle cells (cVSMC) and cardiac fibroblasts, are causing many of these barriers.

Under non-pathological conditions, cVSMC function to provide support to blood vessels and regulate blood flow in the myocardium. Cardiac disease, such as coronary artery disease, damages or blocks the coronary arteries. This prevents blood from reaching the myocardium, leaving the heart without oxygen and nutrients needed to function, and causing a myocardial

infarction. Without new blood vessel formation into the damaged region, any attempt to regenerate the myocardium will be unsuccessful because nutrients needed to sustain the cell based therapies will not be present. Therefore, understanding the origins and signaling pathways that regulate cVSMC formation is essential in providing a niche suitable for myocardial regeneration. In chapter II, I will discuss one signaling pathway, PDGFRβ, which is a key factor in regulating cVSMC development.

The cardiac fibroblast main functions to maintain extracellular matrix homeostasis and is the major non-myocyte in the heart (Nag 1980). The ECM is essential for structure, connectivity, and mechanical signaling in the heart, and fibroblasts are believed to secrete and organize the majority of the cardiac ECM; the major components are type I and type III collagens(Chapman and Eghbali 1990; Weber et al. 1994)(Baudino et al. 2006). Although important to the normal architecture of the heart's blood vessels and myocardium, these cells are better known for the adverse effects they cause during pathological heart conditions. Congestive heart failure, hypertension, and myocardial infarction are known cardiac insults that can lead to cardiac fibrosis (Brown et al. 2005). This reactive process initially creates stability within the myocardial wall, but eventually leads to reduced contractility, electrical conductivity, and oxygen availability, as well as, myocyte disorganization, and subsequent ventricular dysfunction(Brown et al. 2005). The contribution of cardiac fibroblasts to heart disease is well established, but few studies have aimed to examine the signaling pathways required for development and function of this cell population in vivo. I will discuss a crucial factor, PDGFRα, in cardiac fibroblast formation in chapter III.

Epicardial Development

Two of the non-cardiomyocyte lineages in the heart, cardiac fibroblasts and coronary vascular smooth muscle cells (cVSMC), are derived from the outermost layer of the heart, the epicardium (Mikawa et al. 1992). The development of the epicardium is a multi-step process involving cell proliferation, epithelial to mesenchymal transition (EMT), and mesenchymal cell fate specification (Mikawa et al. 1992; Mikawa and Gourdie 1996; Dettman et al. 1998; Gittenberger-de Groot et al. 2000; Perez-Pomares et al. 2002) (Summarized in Figure 1.1). Disruption of any of these processes leads to defects in formation of epicardial derived cardiac fibroblasts and cVSMC (Gittenberger-de Groot et al. 2000; Merki et al. 2005; Wagner et al. 2005). In addition to being a key component of coronary vascular development, recent studies have found that in the adult the epicardium retains the ability to generate smooth muscle cells (Smart et al. 2007; van Tuyn et al. 2007). However, while the developmental origin of epicardial derived cells (EPDC) is clearly established, the mechanisms and signaling pathways involved in regulating their formation are not well understood (Figure 1.1). Therefore, identification of signals controlling EPDC development, could lead to the ability to manipulate the adult epicardium and enhance revascularization in an injured heart.

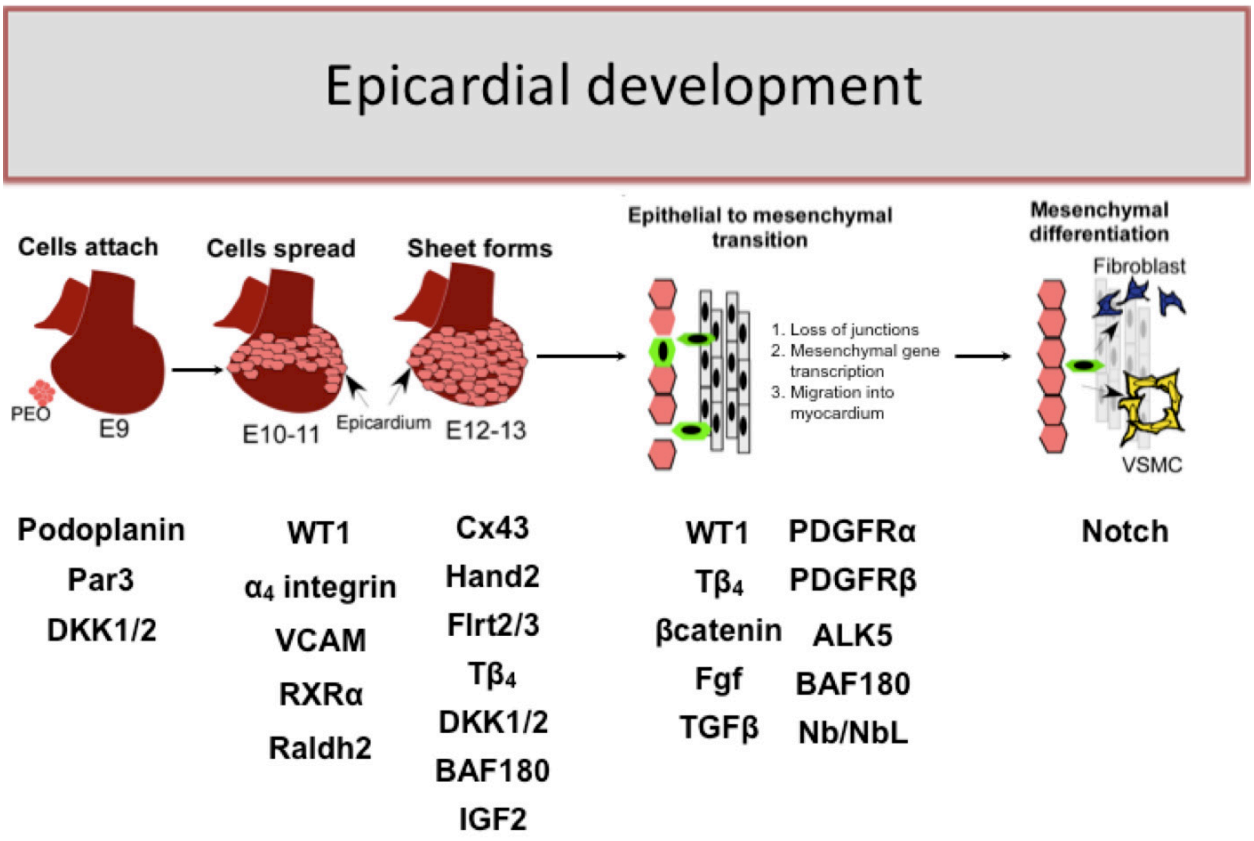

Figure 1.1 Epicardial development
Illustration of the different stages of epicardial and epicardial derived cell development. Signaling pathways currently known to affect these different stages are listed.

The epicardium is derived from an extracardiac source as a transient structure in the embryo, called the proepicardial organ (PEO). It is a cluster of cells that bud from the septum transversum around E9.5 in the mouse (Komiyama et al. 1987; Mikawa et al. 1992; Olivey et al. 2004). These cells then form villi or cysts and attach to the dorsal aspect of the developing myocardium to begin forming the epicardium (Komiyama et al. 1987). Currently, there are very few molecules that are known to be involved in PEO specification or formation (Figure 1.1). Only the Wnt signaling pathway has been implicated in PEO specification. Loss of two Wnt inhibitors, DKK1/2, leads to a hyperplastic PEO, thought to be caused by an over specification of proepicardial progenitors (Phillips et al. 2010). However, the authors did not rule out an increase in progenitor expansion due to proliferation.

Two other proteins also show defects in PEO formation, a mucin-like transmembrane glycoprotein, podoplanin (Mahtab et al. 2008), and a component of the apical cell-polarization machinery, Par3 (Hirose et al. 2006). Mutations in both of these components do not affect PEO specification, but do not form a continuous epicardium. For example, Par3 mutant animals fail to generate cell cysts (or villus structures) that will eventually generate the epicardium (Hirose et al. 2006). One problem with these studies is that they lack expression of these genes in all cells and therefore cannot rule out secondary effects caused by the absence of these genes in other tissues.

Epicardial spreading and sheet formation begins after the proepicardial cysts or villi attach to the dorsal aspect of the myocardium (E10.5-E11.5). Upon this contact, there are many signaling pathways and molecules important for proper epicardial formation, but several seem to impinge on epicardial interactions with the extracellular matrix (ECM) or myocardial cells. Disruption in alpha 4 integrin expression, or its ligand, VCAM1, results in formation of a patchy

and sometimes absent epicardium (Kwee et al. 1995; Yang et al. 1995). Alteration of fibronectin interacting proteins, Flrt2/3, can also lead to defects in epicardial spreading (Muller et al. 2011). Recently, two transcription factors, Wilms Tumor 1 (WT1) (Kirschner et al. 2006) and Hand2 (Barnes et al. 2011) have also reported similar defects. Interestingly, it was discovered that alpha 4 integrin is downregulated in WT1 mutants (Kirschner et al. 2006) and is significantly upregulated in Hand2 mutants (Barnes et al. 2011). These studies clearly indicate that ECM/cell interactions are crucial for epicardial formation.

At E12.5 in the mouse, after proepicardial extension and epicardial sheet formation,, a subset of epicardial cells undergo an epithelial to mesenchymal transition and migrate into the myocardium (Viragh and Challice 1981). The epicardial EMT is one of the least well-studied stages of epicardial development, but a few signaling pathways have been implicated in this process. Both FGF and TGFβ signaling has been shown to stimulate EMT in the chicken PEO (Morabito et al. 2001). TGFβ has been more extensively studied, especially in the mouse. Recently, it was shown that $TGF\beta_1$ and $TGF\beta_2$ could induce epicardial EMT in primary mouse epicardial cultures and also cause VSMC differentiation (Compton et al. 2006). Additionally, a crucial role for TGFβ signaling in epicardial EMT was uncovered when the type I ALK5 receptor (required for TGFβ family signaling) was deleted. The authors observed decreased epicardial cell migration, a failure to change cellular morphology, and an inability to form EPDC (Sridurongrit et al. 2008). Interestingly, while they interpret their results to represent a generic EMT phenotype, some molecular data is inconsistent with this observation. First, expression of WT1 is reduced in the mutants, suggesting there might be an earlier defect in epicardial development or a potential dysregulation of the mesothelial nature of the epicardium (Martinez-Estrada et al. 2010). Second, there is reduced N-cadherin and ZO-1 expression, usually markers

of the epithelial nature of the epicardium, consistent with a potentially disrupted epicardial identity. Therefore, further investigation is needed to truly determine if this is solely an epicardial EMT defect.

The final stage of epicardial development is mesenchymal differentiation into the cardiac fibroblast and cVSMC lineages. Several proteins have been implicated in the development of coronary vascular smooth muscle cells (cVSMC) from the epicardium, including the ones discussed above (Lu et al. 2001; Merki et al. 2005; Wagner et al. 2005; Hirose et al. 2006; Compton et al. 2007; Zamora et al. 2007; Mahtab et al. 2008; Zhou et al. 2009). However, the Notch signaling pathway is the only pathway that disrupts differentiation of EPDC populations without affecting earlier developmental stages. Loss of Rbpj, a Notch signaling partner, or the Notch1 receptor, leads to the failure to generate cVSMC (del Monte et al. 2011; Grieskamp et al. 2011). Additionally, overexpression of the notch intracellular domain (NICD) resulted in ectopic cVSMC formation in the epicardium (Grieskamp et al. 2011).

While many proteins are important for cVSMC formation, nothing is known about signals involved in cardiac fibroblast development. They are proposed to be essential for normal cardiac function, and their role in matrix deposition during cardiac injury is well established, but our knowledge about the development of this population is lacking, mostly because disruption of epicardially-expressed genes often results mutants that do not survive long enough to determine the impact on the formation of EPDCs (Lu et al. 2001; Merki et al. 2005; Wagner et al. 2005; Hirose et al. 2006; Compton et al. 2007; Zamora et al. 2007; Mahtab et al. 2008; Zhou et al. 2009). Additionally, there are no good markers specific for the cardiac fibroblast lineage and many that are used also label the cVSMC population. To fully understand the underlying pathological conditions occurring during heart failure, we need to first understand the signals in

generating cardiac fibroblasts. I will cover the role of PDGF signaling in the formation of cVSMC (chapter II) and cardiac fibroblasts (chapter III).

Epithelial-to-Mesenchymal Transition

Epithelial-to-mesenchymal transition (EMT) is a biological process that occurs when a polarized epithelial cell with tight and adherens junctions undergoes multiple changes that enable it to adopt a mesenchymal cell phenotype, including enhanced migratory capacity and invasiveness (Hay 1995). EMT has been classified into three different types: Type 1 (Developmental), Type 2 (Pathological), and Type 3 (Cancer metastasis) (Kalluri and Weinberg 2009). These three classes encompass all known types of EMT that occur during development and in adulthood, but there are many different mechanisms governing each type.

Several developmental processes depend on EMT (Type 1) for proper embryonic development. One of the earliest EMT process occurs during gastrulation and is essential for the formation of the three germ layers and all mesodermal derived tissues, including all types of muscle (cardiac, skeletal, and smooth)(Nakaya and Sheng 2008). It was originally described in the formation of the chick primitive streak and is one of the first examples of developmental EMT (Trelstad et al. 1967; Greenburg and Hay 1982). A second, well-studied embryonic EMT occurs when the neuroectoderm generates a migratory mesenchymal population called the neural crest cell (Duband and Thiery 1982). These cells will eventually generate part of the cranial skeleton, the peripheral nervous system, melanocytes, and connective tissue throughout many organs, including the cardiac outflow tract (Le Douarin and Kalcheim 1999). Both of these developmental processes define the canonical EMT program initiated to generate mesenchymal cells in the embryo.

The type 2 EMT process is associated with organ fibrosis and tissue regeneration. It occurs when an organ is damaged and various growth factors and inflammatory cytokines are released, triggering an EMT process in the surrounding epithelial structures. For example, during chronic kidney disease, chronic damage causes epithelial cells in the renal tubules to undergo morphological and cellular changes to generate an activated myofibroblast and begin secreting large amounts of extracellular matrix leading to renal scarring (fibrosis) leading to an even further decrease in renal function (Burns and Thomas 2010). The same processes have also been reported to occur in the lung, heart and intestine (Thiery et al. 2009).

The last class, Type 3 EMT, is linked with cancer progression and metastasis. When epithelial cancers progress to late stages, they acquire the ability to degrade their basement membrane and invade into the surrounding tissue, both characteristics of mesenchymal cells. These changes have been of extreme interest in cancer research and many of these studies have revealed that EMT is at least one mechanism that can lead to this malignant phenotype (Thiery et al. 2009). However, secondary metastatic tumors are mainly composed of only the epithelial component from the original tumors, suggesting there might be additional mechanisms governing epithelial metastasis (Tarin et al. 2005). A recent publication has suggested that the EMT that occurs during cancer metastasis actually generates a multipotent mesenchymal cell with stem-like properties capable of regenerating epithelial components (Mani et al. 2008; Morel et al. 2008). This might explain the lack of identifiable mesenchymal components of metastatic tumors and the identification of a “cancer stem cell”. Interestingly, this process has been suggested to occur during other pathological conditions, such as fibrodysplasia ossificans progressiva (FOP) (Medici et al. 2010). However, more research is necessary to determine if the processes occurs in normal development and other EMT processes.

Mechanistically, EMT can be divided into three events that can occur sequentially or simultaneously. First, there are signals, such as growth factors or metabolites, which activate the epithelium to begin the mesenchymal transition. Second, these inductive events trigger gene expression changes that require downregulation of epithelial genes (Ecadherin, ZO-1) with the subsequent upgregulation of mesenchymal genes (Vimentin, Fibronectin). Third, the cells must migrate or delaminate from the epithelial layer into the surrounding tissue. While there are common mechanisms and pathways involved during each stage, there is not one universal EMT program. It is highly diverse depending on the organism, cell type, and environment.

Gastrulation and neural crest delamination (Type 1 EMT) use similar signaling pathways to initiate the mesenchymal transformations. Most notably, canonical Wnt signaling is absolutely required for gastrulation and is important for inducing neural crest formation (Liu et al. 1999; Villanueva et al. 2002). However, FGF and BMP/TGFβ signaling are also necessary for EMT competence and maintenance by modulating Wnt signaling and inducing activation of other factors (Liem et al. 1995; Ciruna and Rossant 2001; Villanueva et al. 2002). Similar pathways have also been implicated in type 2 and 3 EMT processes, but appear to be much more diverse as additional growth factors are capable of inducing EMT in these pathological conditions, including PDGF, EGF, and HGF (Kalluri and Weinberg 2009). The role of PDGF signaling in EMT processes will be discussed later and in chapters II and III.

Prior to receiving induction signals, the epithelium expresses tight and adherens junctional proteins essential for epithelial integrity and function, such as Ecadherin and ZO-1 (Hartsock and Nelson 2008). However, to detach from the epithelial sheet, cells must downregulate these proteins (Cano et al. 2000). The inductive signals regulate this process through post-translational modification of junctional machinery and transcriptional repression

and occur in all types of EMT (Stemmler 2008). Studies of Ecadherin regulation have revealed that direct phosphorylation by CKII can enhance endocytosis and dissolution of the adherens junctions leading to decreased cellular adhesion (Dupre-Crochet et al. 2007). Other components of the junctional complex, including those that interact with the actin cytoskeleton, such as the catenin family (α, β, γ) can also be phosphorylated leading to disruption of the cadherin-catenin-cytoskeletal complex and loss of adhesion (Aberle et al. 1996; Ozawa and Kemler 1998; Roura et al. 1999)

Immediate regulation of the junctional complex is not sufficient to maintain the EMT process and long-term repression is mediated by inhibiting their transcription (Moreno-Bueno et al. 2008). This is mediated by many diverse transcription factors, including βcatenin, Snai1/2, Zeb1/2, Twist, E2a, and Sox9 (Cheung et al. 2005; Thiery and Sleeman 2006; Thiery et al. 2009). These transcription factors repress epithelial genes by either directly binding or inhibiting transcription by recruiting repressor complexes (Herranz et al. 2008) or indirectly through additional transcription cascades (Thiery et al. 2009). These transcription factors are induced by the same pathways initiating the EMT process (FGF, BMP, TGFβ, and Wnt) and are absolutely required to undergo different developmental EMT processes. For example, deletion of Snai1 in mice results in gastrulation failure embryonic lethality around E8.5 (Carver et al. 2001). However, these transcription factors are not always required for specifying mesenchymal cell fate (Carver et al. 2001). Additionally, while each factor is capable of repressing the epithelial phenotype, they have differing abilities to further continue EMT and it there are likely additional factors needed to undergo a complete EMT process.

The subsequent upregulation of mesenchymal genes has been much less studied and only a few transcription factors have been described that control this process. FoxC2 is the only

factor identified that can specifically affect mesenchymal genes, such as vimentin and αSMA with little impact on epithelial genes (Mani et al. 2007). The paucity of factors that regulate mesenchymal genes indicates much more research is needed to understand the complete EMT process. Currently, identification of genes regulating EMT is discovered by overexpressing the protein of interest and assaying for morphological changes indicative of EMT. However, using this method, factors regulating only mesenchymal genes could be missed. In addition, it is possible that mesenchymal gene induction is actually caused by inhibition of repressors, which would also be missed by this technique. Therefore, a new approach is necessary and possibly examination of the reverse process, mesenchymal to epithelial transition (MET), will reveal these additional factors.

The final EMT event occurs when the newly formed mesenchymal cells deliaminate and migrate away from the epithelium. This fundamental event relies not only on the loss of junctions and cell polarity, but also requires changes in cell shape. Two different processes are involved in changing cellular morphology. First, actin must be redistributed in the cell to provide the scaffold for cellular migration. This is a very dynamic process and relies on many of the signaling pathways already mentioned and impinges on a core set of proteins regulating actin polymerization, Arp2/3, WASP, and cortactin (Mullins 2000; Ren et al. 2009). This machinery is utilized to generate small protrusions called lamellipodia, filopodia, and or invadopodia that penetrate the basement membrane and explore the surrounding environment interacting with extracellular matrix and other growth factors that reinforce their formation and stability (Yilmaz and Christofori 2009). These structures will eventually drive the directed cell migration away from the epithelial cells and into the surrounding stroma (Yilmaz and Christofori 2009).

Migration and delamination also rely on mobilizing the cell to use the remodeling cytoskeleton and the Rho family of small GTPases play a critical role in regulating changes that control cell shape and motility (Thiery et al. 2009). In neural crest cells disruption of the Rho/Rock pathway causes defects in cell shape due to the inability to phosphorylate and activate myosin light chain kinase, a component of the cellular motor machinery (Berndt et al. 2008). Their gene expression and activity is dynamically regulated and is intimately linked with the transcriptional regulators of EMT. Loss of either RhoV or Rac1 leads to impairment of EMT by reducing expression of Snai2, Sox9, or Twist (Broders-Bondon et al. 2007; Guemar et al. 2007).

Not all EMT processes can be strictly classified into the above categories. During development, there are several populations of cells that are generated by a non-canonical EMT process. For example, smooth muscle cells that comprise the gut and coronary vasculature arise from a unique layer of epithelial cells called the serosal mesothelium (Mikawa et al. 1992; Wilm et al. 2005). This unique cell population possesses properties of both an epithelium and mesenchymal cells. It expresses both tight and adherens junctions as well as maintains an apical-basal polarity, characteristics of epithelial cells (Perez-Pomares et al. 2002; Wu et al. 2010). In addition, it also expresses markers of mesenchymal cells, such as vimentin (Morabito et al. 2002). This dual nature makes study and molecular characterization of the mesothelial EMT difficult, but it is clear that it undergoes the morphological changes of EMT to generate different vasculogenic cell types, including smooth muscle and fibroblasts during development and disease. Discovering and understanding signals and pathways that regulate the mesothelial EMT and direct cell fate will likely provide insights into disease processes, such as cardiac fibrosis. I will discuss the role of PDGF receptors in the EMT process of the epicardium and their role in generating cVSMC and cardiac fibroblasts in the following chapters.

Platelet Derived Growth Factors

Platelet derived growth factors (PDGF) were originally identified as potent mitogens for vascular smooth muscle cells (VSMC)(Ross et al. 1974). The PDGF ligands exert their function by causing dimerization and activation of the PDGF receptors (Heldin and Westermark 1999). Ligand binding leads to phosphorylation of these receptor tyrosine kinases on tyrosine residues in their cytoplasmic domains. This, in turn, results in activation of a multitude of intracellular signaling cascades (Heldin and Westermark 1999). The outcomes of these signaling events are diverse and include proliferation, migration, matrix deposition, survival, and epithelial to mesenchymal transition (Betsholtz et al. 2001). In vivo experiments suggest that the type of response elicited by PDGF receptor signaling depends on the cell population receiving the signal.

The PDGF family consists of two receptor genes, *PDGFRα* and *PDGFRβ*, and four ligand genes, *PDGFA, B, C,* and *D*. The ligands exist as disulfide-linked polypeptides, and all are homodimers with the exception of the PDGFAB heterodimer (Fredriksson et al. 2004). The PDGF receptors can function as either homo- or heterodimers, and each of the ligands binds the two receptors with different affinities. PDGFAA and PDGFCC exclusively bind and activate PDGFRαα(Claesson-Welsh et al. 1989; Matsui et al. 1989; Li et al. 2000), while PDGFDD preferentially activates PDGFRββ(Bergsten et al. 2001; LaRochelle et al. 2001). PDGFBB can bind and activate either receptor homodimer (PDGFRαα or PDGFRββ), and PDGFBB and PDGFAB activate the heterodimer (PDGFRαβ)(Hammacher et al. 1989; Kanakaraj et al. 1991).

The PDGF ligands and receptors are expressed in a broad range of tissues throughout the developing embryo. In the embryonic heart, all four ligands are present. PDGFA (Kang et al. 2008) is expressed in the epicardium, PDGFB in endothelial cells (Hellstrom et al. 1999; Van Den Akker et al. 2005), PDGFC in cardiomyocytes (Ding et al. 2000; Aase et al. 2002), and

PDGFD in the epicardium/myocardium (Ponten et al. 2005). Both PDGF receptors are also expressed in different populations of the heart, including neural crest cells and the epicardium (Schatteman et al. 1995; Van Den Akker et al. 2005; Richarte et al. 2007). Recent studies have also shown that PDGF signaling in the adult heart can lead to pathological changes. Transgenic overexpression of PDGFRα or PDGFRβ specific ligands (PDGFC, PDGFD, respectively) in the myocardium induced extensive collagen deposition and cardiac fibroblast proliferation followed by cardiac hypertrophy (Ponten et al. 2003; Ponten et al. 2005). Another study recently showed that mice treated with inhibitory antibodies against PDGFRα and PDGFRβ displayed decreased collagen deposition after myocardial infarction and decreased vessel angiogenesis, respectively (Zymek et al. 2006). These data highly suggest a role for PDGF receptors in cardiac development and function.

Roles of PDGF signaling in development have been extensively studied using numerous knockout, knock-in, and transgenic animals. These studies have revealed many tissues and cells are dependent on proper PDGF signaling in order to develop properly (Andrae et al. 2008). Neural crest cells and their derivatives are one of the most affected tissues when PDGF signaling is disrupted (reviewed in (Smith and Tallquist 2010)). Loss of PDGF receptor signaling, predominantly PDGFRα, by deletion of the receptors or cognate ligands leads to craniofacial abnormalities (cleft palate and cranial bones), persistent truncus ateriosus (PTA), ventricular septal defects (VSD), aortic arch abnormalities and melanocyte defects (Smith and Tallquist 2010). While loss of PDGFRβ compounds the neural crest cell defects, it is also required for VSMC development throughout the embryo (Hellstrom et al. 1999). The mechanisms behind the embryonic defects remain elusive but proliferation, migration, matrix deposition, survival, and

epithelial to mesenchymal transition (EMT) have all been proposed processes regulated by PDGF signaling (Betsholtz et al. 2001) (and this report).

There are only a few examples that link PDGF signaling and EMT. One of the early associations is during cancer progression where a transient EMT leads to metastasis. PDGF signaling has been shown to induce morphological and molecular changes associated with EMT during prostate (Kong et al. 2008) and liver (Fischer et al. 2007) cancer by regulating cell motility and survival pathways. Another pathological process linking PDGF and EMT occurs during renal fibrosis, where inhibition of PDGF signaling led to a decrease in extracellular matrix deposition and generation of fewer myofibroblasts from renal epithelial cells (Ostendorf et al. 2006). PDGF has also been implicated in developmental EMT processes, specifically in the generation of smooth muscle cells from the chick proepicardium through a RhoA/SRF dependent pathway (Lu et al. 2001). However, how PDGF specifically contributes to these changes is still unclear.

In the following chapters, I will address the role of PDGF receptors during epicardial, cVSMC and cardiac fibroblast development. Specifically, in chapter II, I will illustrate the requirement for PDGFRβ in cVSMC formation and in chapter III, I will show PDGFRα is required for cardiac fibroblast development. Finally, in chapter III I will demonstrate that the mechanism of PDGF signaling is to regulate epicardial EMT.

Chapter II

Platelet derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations

Abstract

The epicardium plays an essential role in coronary artery formation and myocardial development, but signals controlling the development and differentiation of this tissue are not well understood. To investigate the role of platelet derived growth factor receptor β (*PDGFRβ*) in development of epicardial-derived vascular smooth muscle cells (VSMC), we examined *PDGFRβ*$^{-/-}$ and *PDGFRβ* epicardial-mutant hearts. We found that *PDGFRβ*$^{-/-}$ hearts failed to form dominant coronary vessels on the ventral heart surface, had a thinned myocardium, and completely lacked coronary VSMC (cVSMC). This constellation of defects was consistent with a primary defect in the epicardium. To verify that these defects were specific to epicardial derivatives, we generated mice with an epicardial deletion of PDGFRβ that resulted in reduced cVSMC distal to the aorta. The regional absence of cVSMC suggested that cVSMC could arise from two sources, epicardial and non-epicardial, and that both were dependent on PDGFRβ. In the absence of PDGFRβ signaling, epicardial cells adopted an irregular actin cytoskeleton leading to aberrant migration of epicardial cells into the myocardium in vivo. In addition, PDGF receptor stimulation promoted epicardial cell migration, and PDGFRβ-driven phosphoinositide 3' kinase (PI3K) signaling was critical for this process. Our data demonstrate that PDGFRβ is required for the formation of two distinct cVSMC populations and that loss of PDGFRβ-PI3K signaling disrupts epicardial cell migration.

Introduction

Vascular smooth muscle cells (VSMC) arise from a diverse range of tissues including neural crest, somites, splanchnic mesoderm, gut mesothelium, and epicardium (reviewed by Majesky (Majesky 2007)). Coronary VSMC (cVSMC) are derived from the embryonic epicardium (Mikawa and Gourdie 1996; Verberne et al. 1998). While several genes have been identified that are essential for the formation, attachment, and spreading of the epicardium, few genes have been identified that are essential during epithelial to mesenchymal transition (EMT) and subsequent differentiation into cVSMC and cardiac fibroblasts.

Platelet derived growth factor receptor (PDGFR) tyrosine kinases are one family of signaling proteins that are potentially involved in epicardial cell function. Analyses in the mouse have shown that PDGFRβ signaling promotes proliferation and migration of VSMC in multiple vascular beds including the heart (Leveen et al. 1994; Soriano 1994; Lindahl et al. 1997; Lindahl et al. 1998; Hellstrom et al. 1999). Therefore, we investigated the function of PDGFRβ signaling during epicardial development. We have examined *PDGFRβ*$^{-/-}$, epicardial-specific *PDGFRβ* mutant, and *PDGFRβ* signaling deficient embryos. We discovered that epicardial deletion resulted in the absence of cVSMC distal to the aorta and that PDGFRβ signaling through PI3K was required for proper cytoskeletal organization in epicardial cells. Our results designate PDGF receptor signaling as another growth factor system involved in epicardial development.

Results

Loss of PDGFRβ results in coronary blood vessel and myocardial defects

Although PDGFRβ signaling is considered mitogenic for VSMC, only select populations of VSMC are affected by the loss of PDGFRβ or PDGFB (Hellstrom et al. 1999). Because cVSMC arise from a unique mesothelial origin, we wanted to determine how PDGFRβ signaling

regulates this cell population. The *Gata5CreTg* mouse line expresses Cre recombinase in the proepicardial organ (PEO; the embryonic source of epicardial cells) and the epicardium (Merki et al. 2005). Crossing this transgenic line to mice with a *PDGFRβ loxP*-flanked allele, generated animals lacking PDGFRβ in the epicardium (*PDGFRβ*^{EKO}). Using mice that possessed either a *PDGFRβ* null allele or this epicardial deletion of *PDGFRβ*, we examined coronary vessel formation.

We began our analysis by investigating the myocardial thickness in these PDGFRβ deficient hearts. In comparison to the myocardial defects reported in *PDGFRβ*$^{-/-}$ hearts (Van den Akker et al. 2008) and data not shown, the myocardial compact zone in *PDGFRβ*^{EKO} hearts was relatively normal. *PDGFRβ*^{EKO} compact zones at E18.5 were 88 $\pm$ 4% the thickness of controls (n=4 controls and n=4 *PDGFRβ*^{EKO}). Therefore, the myocardial defects observed in PDGFRβ$^{-/-}$ hearts may be secondary to systemic vascular defects rather than epicardial loss of PDGFRβ as the epicardial mutant hearts had minimal myocardial disruption.

We next examined coronary artery development in these hearts. At E14.5, hearts of all genotypes possessed a capillary plexus, but *PDGFRβ*$^{-/-}$ and *PDGFRβ*^{EKO} hearts possessed abnormal clusters of endothelial cells (Figure 2.1A). In *PDGFRβ*$^{-/-}$ hearts these defects persisted and dominant coronary vessels failed to form on the ventral surface. In contrast, at E17.5 endothelial vessel formation in *PDGFRβ*^{EKO} hearts was similar to wild type. These results indicated that coronary vessel disruption occurs in PDGFRβ-deficient hearts, but in the case of the epicardial deletion, vessel remodeling is later recovered.

To determine if the endothelial defects were accompanied by similar VSMC defects, we generated PDGFRβ deficient animals that possessed the *XlacZ4* transgene. *XlacZ4* mice express

a nuclear-localized β-galactosidase protein in VSMC (Tidhar et al. 2001), including those derived from the epicardium (unpublished observation CS and MT, 2007). In wild type hearts, a few β-galactosidase^{+} cells were observed as early as E14.5. A majority of cells were present in close proximity to nascent coronary vessels, while single cells were found scattered on the surface of and within the heart ventricles (Figure 2.1A and data not shown). In comparison, *PDGFRβ*$^{-/-}$ and *PDGFRβ*^{EKO} hearts contained no β-galactosidase^{+} cells (Figure 2.1A). At E17.5 VSMC were present in wild type hearts both along the major coronary arteries as well as scattered along smaller vessels within the ventricles. Remarkably, E17.5 *PDGFRβ*$^{-/-}$ hearts did not possess β-galactosidase^{+} cells along the heart surface or within the ventricles (Figure 2.1A and data not shown), although a few β-galactosidase^{+} cells were observed in the aortic arch. In contrast, epicardial loss of PDGFRβ caused loss of a majority of cVSMC, but β-galactosidase^{+} cells were still present along the vessels descending from the aortic root (Figure 2.1A).

To confirm that PDGFRβ deficient hearts lacked cVSMC, we stained for additional VSMC markers, smooth muscle myosin heavy chain (smMHC) and α smooth muscle actin (αSMA). Similar to the results we observed using the *XlacZ4* transgenic line, we detected very few smMHC^{+} or αSMA^{+} cells in PDGFRβ$^{-/-}$ hearts (Figure 2.1B), while *PDGFRβ*^{EKO} hearts lacked these cells in the apex of the heart (data not shown). Therefore, the requirement for PDGFRβ in the formation of cVSMC is different than the requirement for VSMC in other tissues that have been investigated. The complete loss of this VSMC population in the *PDGFRβ*$^{-/-}$ heart demonstrates a greater dependence on PDGFRβ signaling in cVSMC development and suggests that PDGFRβ may function beyond the role of a mitogen or chemotactic factor.

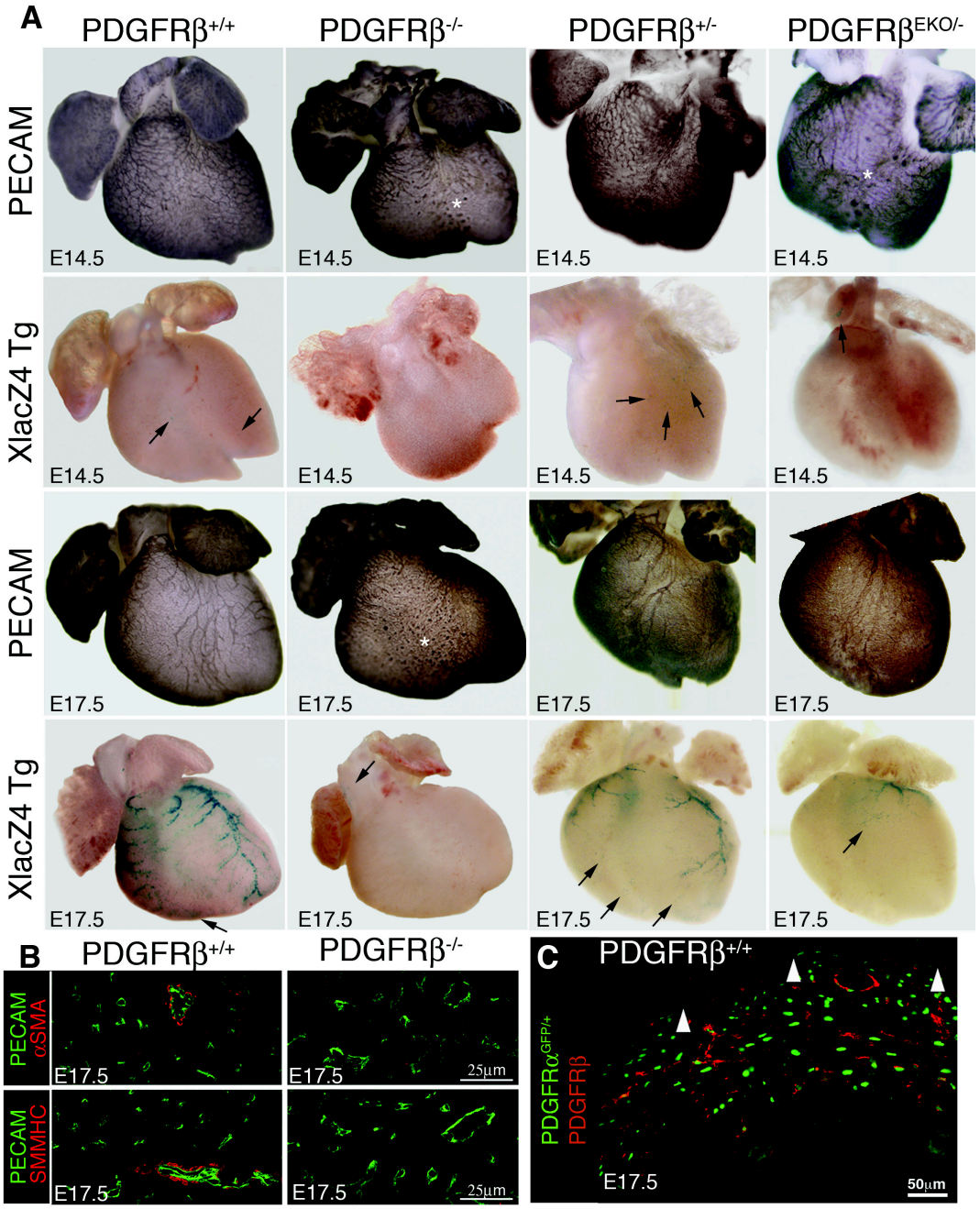

Figure 2.1. PDGFRβ expression is required for proper coronary artery development.
(A) Whole mount hearts stained for PECAM (endothelial cell) immunohistochemistry or β-galactosidase activity of *XlacZ4* transgene. Asterisks indicate sinusoidal PECAM staining. Arrows indicate scattered β-galactosidase^{+} cells. (B) Representative confocal images of sections stained for αSMA and smMHC. (C) Lateral, left ventricle wall of *PDGFRα*$^{GFP/+}$ heart stained with PDGFRβ antibody. White arrowheads indicate the edge of the epicardium.
Expression of PDGF receptors during heart development

Expression of PDGF receptors during development

To identify when PDGFRβ was expressed in the heart, we examined wild type embryos at various stages of coronary vascular development. Consistent with chick expression studies (Van Den Akker et al. 2005), PDGFRβ was expressed as early as E9.5 in the proepicardium (data not shown). At E13.5, we observed PDGFRβ in the cells of the epicardium (Figure 2.2A). At E15.5 PDGFRβ expression remained in the epicardium but was also observed in cells surrounding vessels within the myocardium. By E17.5, PDGFRβ expression was reduced in the epicardium, and the majority of positive cells were presumably VSMC adjacent to endothelial vessels.

Recent reports have also demonstrated that PDGFRα is expressed by epicardial cells (Kang et al. 2008). Using an allele of the *PDGFRα* that expresses a nuclear localized green fluorescent protein from the *PDGFRα* locus (Hamilton et al. 2003), we investigated the spatial and temporal expression of this receptor (Figure 2.2B). Similar to PDGFRβ, PDGFRα is expressed by epicardial cells. PDGFRα continues to be expressed by epicardial cells after they have migrated into the myocardium. At E17.5 very few cells co-express both receptors within the myocardium (Figure 2.1C).

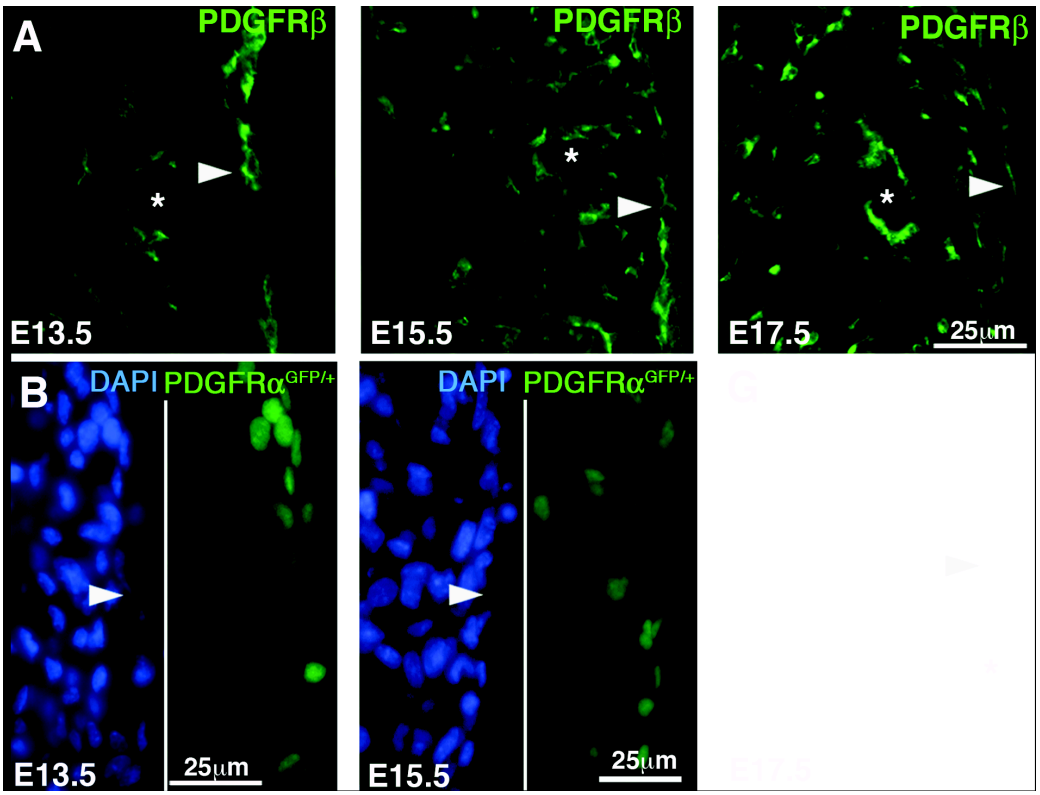

Figure 2.2. PDGFRα and PDGFRβ are expressed in the epicardium
(A) Control hearts stained for PDGFRβ. Asterisks indicate larger blood vessels. (B) *PDGFRα*$^{GFP/+}$ heart. White arrowheads indicate the edge of the epicardium.

Non-epicardial source for cVSMC

We next investigated the source of the persisting cVSMC present in *PDGFRβ*^{EKO} hearts. From the whole mount images, we found that cVSMC were present at the aortic root. We then quantified the number of cVSMC and found that *PDGFRβ*^{EKO} hearts contained similar numbers of cVSMC in the base of the heart but exhibited a significant reduction of these cells within the heart apex (Figure 2.3A-B).

This region-specific loss of cVSMC suggested that these cVSMC arise from an origin separate from the epicardium or that recombination of the PDGFRβ locus was inefficient or too late. To test these possibilities, we examined PDGFRβ expression by generating E11.5 epicardial cells. Real-time PCR showed that expression of PDGFRβ transcripts was significantly reduced (Figure 2.4A). We also examined PDGFRβ protein expression in the epicardium at E12.5 and found that PDGFRβ was absent from the epicardium (podoplanin^{+} cells (Mahtab et al. 2008)) of *PDGFRβ*^{EKO} hearts including cells surrounding the conotruncal region (Figure 2.4B-C). At E13.5 we found that PDGFβ was absent from all epicardial cells but was still present in other smooth muscle populations and cardiac valve primordial (Figure 2.4D-E). We next determined the cell populations within the heart that have had Cre activity using ROSA26 reporter mice. As shown in Figure 2.4F, Cre recombination could be detected throughout the epicardium by E12.5, including the epicardium surrounding the conotruncal region and atria. Finally, to determine if an independent epicardial Cre-deletion strain yielded similar results, we used *WT1*^{CreGFP} mice. In this mouse line Cre is expressed very early during epicardial development and has been used to trace epicardial derived cells (Zhou et al. 2008). Using the ROSA26 reporter to follow Cre activity, we observed that a substantial number of these cVSMC were not epicardial derivatives (Figure 2.4G-H). Thus, it appears that there are

two distinct cell populations for generating coronary artery VSMC, and PDGFRβ function is required in both populations.

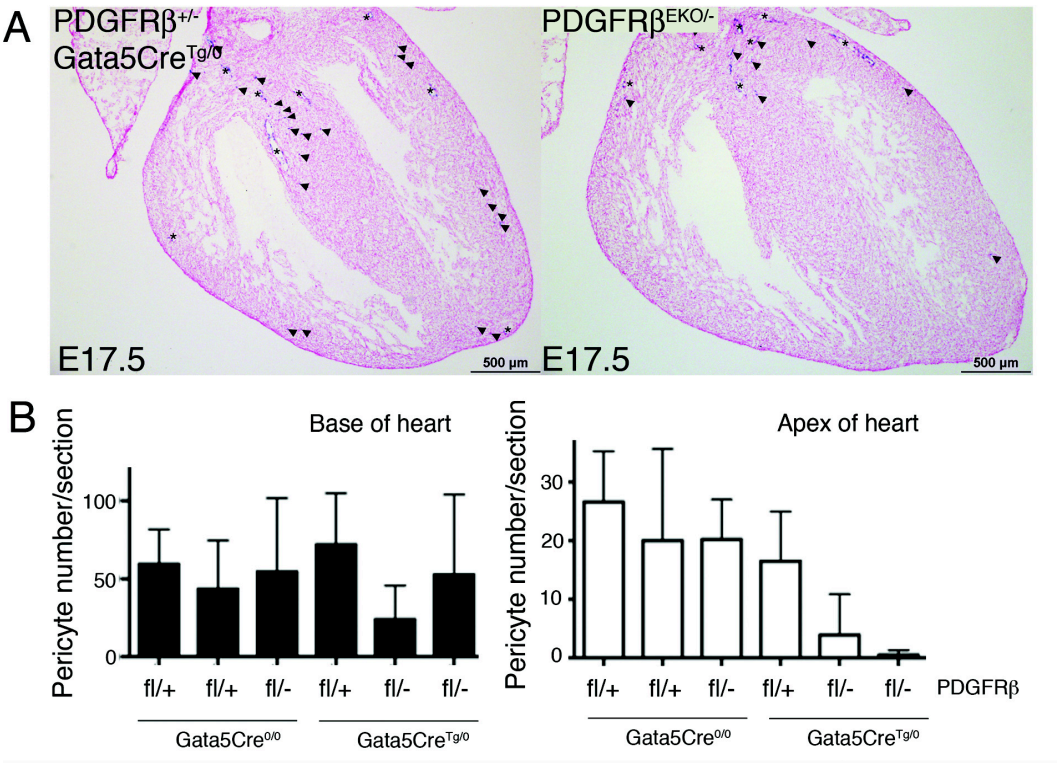

Figure 2.3. Some proximal coronary VSMC are not epicardial derivatives
(A) Sections of E17.5 hearts demonstrate a lack of VSMC in the apex of *PDGFRβ*$^{EKO/-}$ hearts compared to control hearts. VSMC were present in the coronary root in both samples. (B) Quantification of pericyte positive cells in base and apex of heart at E17.5 from indicated genotypes. Base was considered the upper half of the ventricles while apex was the lower half of the ventricles. A minimum of ten sections in the mid coronal plane of the heart were used for quantification of each data point. fl=*loxP* flanked allele.

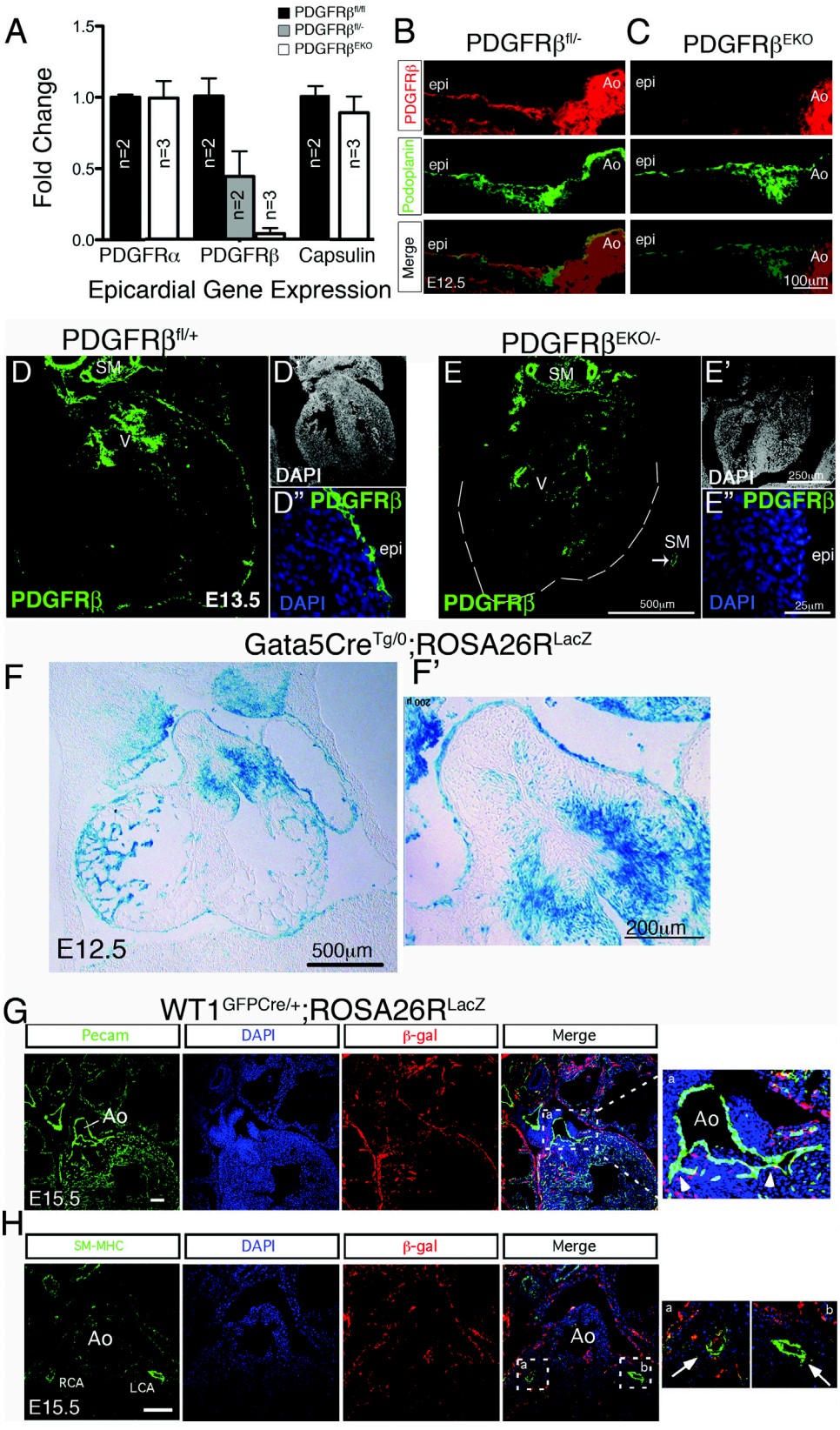

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Figure 2.4. Loss of PDGFRβ and non-epicardial-derived cVSMC surrounding the ostia
(A) Real-time PCR results for gene expression of the indicated transcripts. (B-E) Loss of PDGFRβ expression in epicardial cells at (B-C) E12.5 (right ventricle next to aorta) and (D-E) E13.5 hearts. Podoplanin was used to identify epicardial cells. Body wall vessels (arrow), valves (v) and smooth muscle (sm) of aortic region. (D', E') DAPI view of (D and E) and (D", E") closeup view of epicardium of lateral ventricl. (F) Cre activity in E12.5 heart using *ROSA26R*^{LacZ} reporter mice. The entire epicardium has Cre activity by E12.5, including the epicardium surrounding the atria and the contruncal region. (G, H) *WT1*^{GFPCre} is not expressed in a majority of the cVSMC present in the coronary ostia. (G) E15.5 *WT1*^{GFPCre}*;ROSA26R*^{LacZ} hearts were costained for PECAM and β-galactosidase expression to detect endothelial cells and WT1 expressing cells, respectively. (a') Closeup of coronary ostia (boxed area in G). White arrowheads indicate that very few β-galactosidase positive cells are present surrounding the coronary ostia. (H) E15.5 *WT1*^{GFPCre}*;ROSA26R*^{LacZ} hearts were costained for SMMHC and β-galactosidase. (a, b) Close-up of boxed areas in H (right and left coronary vessels, respectively). White arrows indicate that very few cells coexpress β-galactosidase and SMMHC. Ao, aorta; RCA, right coronary artery; and LCA, left coronary artery.

Epicardium and epicardial derivative formation in the PDGFRβ$^{-/-}$ hearts

Because we observed a complete absence of epicardial-derived cVSMC we determined if the epicardium developed normally in our mutant hearts. To identify the proepicardium and epicardium we used a mouse line that expresses β-galactosidase from the *capsulin* locus (*capsulin*$^{LacZ/+}$) (Lu et al. 2000), which is expressed by proepicardial and epicardial cells. Figure 2.5A,B, demonstrates that at E9.5 both wild type and *PDGFRβ*$^{-/-}$ embryos formed a proepicardium and have clusters of cells that have attached to the dorsal aspect of the heart. From E13.5- E15.5, capsulin^{+} epicardial cells have spread over the surface of the heart (Figure 2.5C-H). Although the *PDGFRβ*$^{-/-}$; *capsulin*$^{LacZ+}$ epicardial cell pattern appeared less uniform and exhibited small gaps between cells compared to the controls, we still concluded that epicardial cell attachment and spreading over the myocardium occurred in the absence of PDGFRβ signaling.

After epicardial cells attach to and spread over the heart, a subset undergoes EMT and subsequently migrate into the myocardium. Using β-galactosidase expression from *capsulin*$^{LacZ/+}$ mice to trace epicardial cell presence in the myocardium, we found that *PDGFRβ*$^{-/-}$ hearts had reduced numbers of β-galactosidase^{+} cells within the myocardium at both E14.5 and E15.5 (Figure 2.6A-B). To verify this reduction of epicardial cell derivatives in the myocardium, we used an independent marker for undifferentiated epicardial cells, Wilms tumor 1 (WT1) (Moore et al. 1999; Zamora et al. 2007). We observed a marked decrease in WT1^{+} cells in the myocardium of *PDGFRβ*$^{-/-}$ and PDGFRβ^{EKO} hearts compared to wild type controls, although WT1 expressing cells are clearly found in the epicardium (Figure 2.6C-H). The reduction in epicardial-derived cells within the myocardium could be caused by either reduced proliferation, increased cell death, or decreased migration into the heart. We analyzed *PDGFRβ*$^{-/-}$ hearts for

proliferation and apoptosis and found no difference between control and *PDGFRβ*$^{-/-}$ hearts for either of these parameters at E13.5 and E14.5 (Figure 2.7, Table 2.1, and data not shown). Taken together these data suggest that the reduction of epicardial cells within the myocardium was caused by a failure of epicardial cells to exit the epicardium.

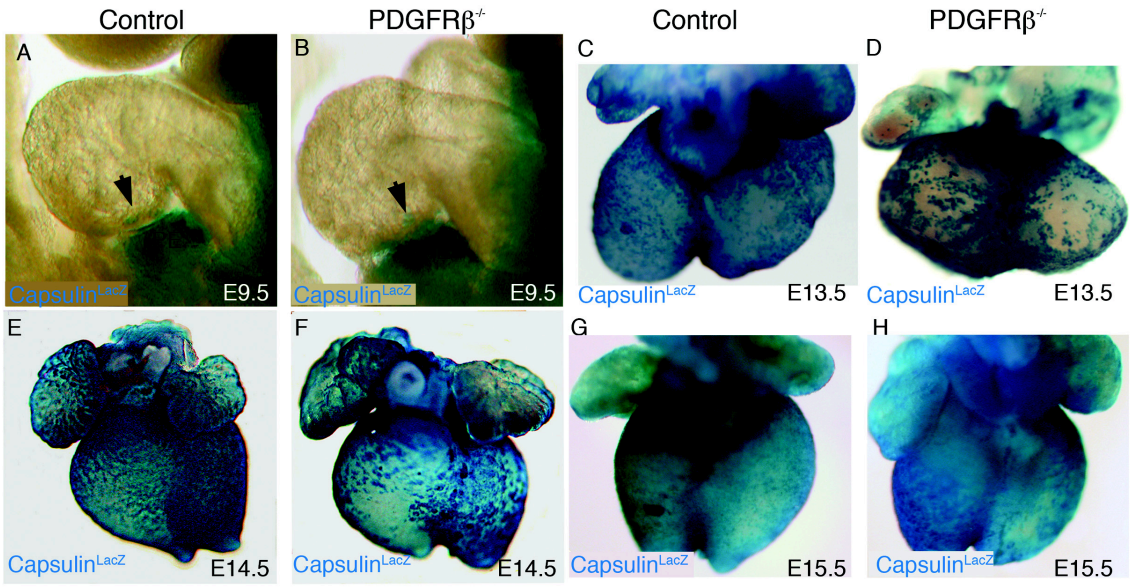

Figure 2.5. Epicardial cell tracing using *Capsulin*^{LacZ}
Hearts of the indicated genotypes and ages were stained in whole mount for β-galactosidase activity. Arrowheads point to proepicardial cells that have attached to the heart. PE, proepicardium.

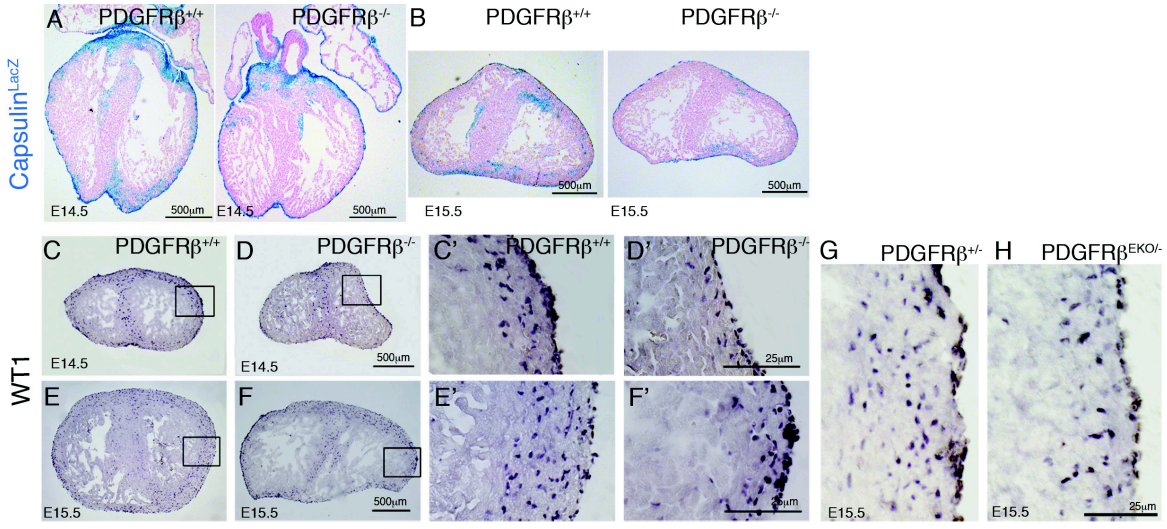

Figure 2.6. Decreased presence of epicardial derivatives in *PDGFRβ*$^{-/-}$ hearts
(A, B) Cells of the epicardium were identified using *capsulin*$^{lacZ/+}$ driven-expression of β-galactosidase (blue). (C-H) Sections stained for WT1 of E14.5 (C,D) and E15.5 (E-H) hearts. Figures in C'-F' are higher magnification views of boxed areas in C-F.

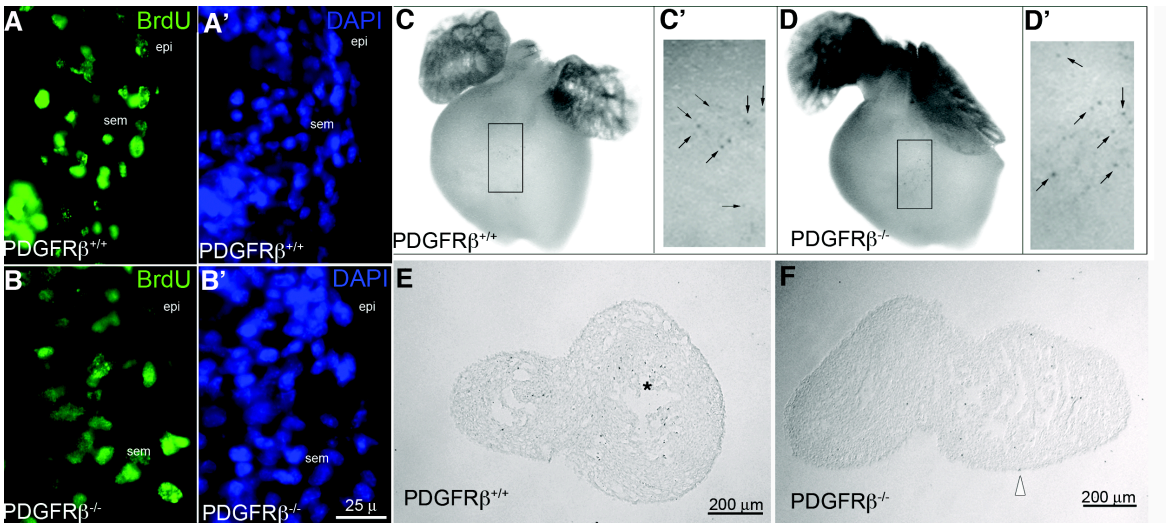

Figure 2.7. Proliferation and apoptosis are unaffected in *PDGFR* $\beta^{-/-}$ hearts
(A, B) BrdU incorporation in E14.5 hearts. (A', B') DAPI was used to visualize nuclei. For quantification see Stable I. (C-F) Apoptosis was examined by performing TUNEL on E14.5 hearts. (C, D) Whole mount hearts. (C', D') Boxed region in C and D were magnified. Arrows point to apoptotic nuclei. Representative of 3 hearts of each genotype. No statistical difference was observed between mutant and wild type. (E, F) Independent E14.5 hearts were sectioned and subjected to TUNEL. White arrowhead indicates single $TUNEL^{+}$ epicardial cell. Asterisk indicates red blood cell background staining was observed in wild type sample. Representative of two hearts of each genotype at two time points each, E13.5 and E14.5.

Table 2.1. Regional proliferation index of control and mutant hearts

	E13.5		E14.5	
Genotype	PDGFRβ$^{+/+}$ (4)	PDGFRβ$^{-/-}$ (5)	PDGFRβ$^{+/+}$ (3)	PDGFRβ$^{-/-}$ (3)
Epicardium	16 ± 3%	15 ± 2%	16 ± 3%	15 ± 3%
Intramyo. mes.	27 ± 0.8%	26 ± 1%	25 ± 1%	28 ± 0.1%

BrdU incorporation of epicardium and intramyocardial mesenchyme was quantified. A minimum of three 40X fields of view of the left, lateral ventricle from each heart was quantified. Number in parentheses is the number of individual hearts that were examined. The regions were identified by their location as follows: epicardium-single layer cells on the surface of the heart and intramyocardial mesenchyme-three-four cell layers below the epicardium. Proliferation index = BrdU^{+} nuclei/total nuclei X 100. Intramyo. mes.= intramyocardial mesenchyme.

Because failure of epicardial cell migration into the myocardium could also be a result of EMT disruption, we examined the ability of *PDGFRβ*$^{-/-}$ epicardial cells to upregulate transcription of a mesenchymal marker, vimentin (Perez-Pomares et al. 1997). At E13.5 abundant vimentin^{+} cells were observed in the myocardium of control hearts (Figure 2.8A). At E14.5 vimentin-expressing cells progressed further into the myocardium (Figure 2.8C), and at E15.5 the vimentin^{+} cells were distributed throughout the myocardium (Figure 2.8E). In contrast, fewer vimentin^{+} cells were observed within the myocardium of *PDGFRβ*$^{-/-}$ hearts at each developmental stage, even though vimentin was expressed in *PDGFRβ*$^{-/-}$ epicardium at each stage (Figure 2.8C,D,F). Quantification of epicardial derived mesenchyme demonstrated a 52% reduction in vimentin^{+} cells within the heart, similar to what we observe with WT1 staining (%vimentin^{+} area excluding epicardium: control 11.39% ± 2.56 (n=5) and *PDGFRβ*$^{-/-}$ 5.88% ± 1.44 (n=5); $p<0.005$). In addition, there appears to be more cells in the *PDGFRβ*$^{-/-}$ epicardium that retain vimentin expression at E14.5 and E15.5 than in wild type epicardium. This leads to the possibility that although the cells initiate the transcription program for EMT, they lack the appropriate signals for migration. All results thus far are consistent with a role for PDGFRβ signaling in instructing epicardial cell invasion into the myocardium.

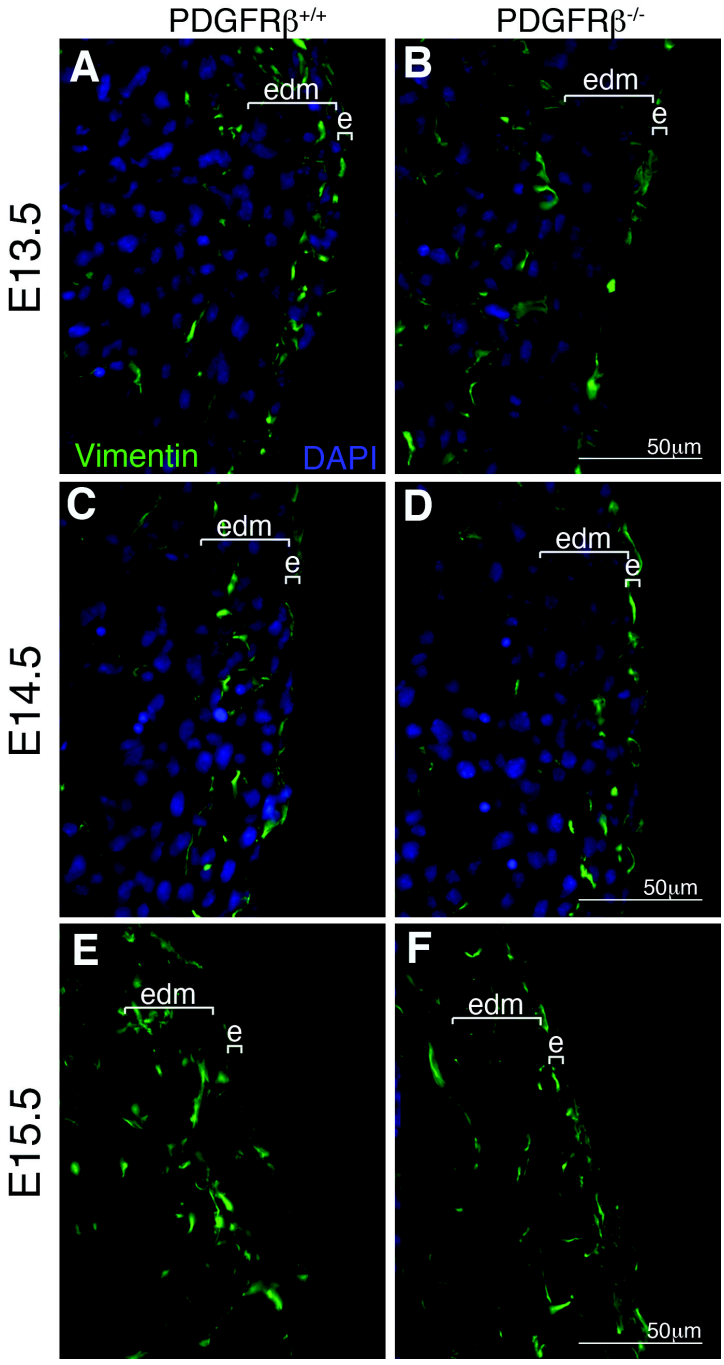

Figure 2.8. *PDGFRβ*$^{-/-}$ hearts express the mesenchymal marker, vimentin
(A-F) Vimentin expression was assessed. e; epicardium, and edm; epicardial derived mesenchyme.

A role for PDGFRβ in epicardial migration

The reduction in capsulin$^+$ and WT1$^+$ cells within the myocardium of PDGFRβ-deficient hearts caused us to investigate epicardial cell migration in greater detail. To identify the signaling pathway responsible for epicardial cell migration, we examined hearts of mice that fail to activate specific signaling pathways downstream of the PDGFRβ alongside PDGFRβ$^{-/-}$ and PDGFRβ^{EKO} hearts. We first quantified the number of WT1$^+$ cells in the myocardium at E15.5. We found that both *PDGFRβ*$^{-/-}$ and *PDGFRβ*^{EKO} hearts possessed roughly half the WT1$^+$ cells of littermate controls in both the left and right ventricles of the heart (Figure 2.9A). We next investigated WT1$^+$ cell migration in mice bearing signaling point mutants of PDGFRβ. Previous analyses have shown that cVSMC development occurs less efficiently in these animals (Tallquist et al. 2003). The PDGFRβ mutant receptors lacked PDGFRβ-induced PI3K (*PDGFRβ*$^{F2/F2}$); PI3K, RasGAP, Shp2, and PLCγ (*PDGFRβ*$^{F5/F5}$); or Src, Grb2, PI3K, RasGAP, Shp2 and PLCγ (*PDGFRβ*$^{F7/F7}$) pathways. All three PDGFRβ mutant strains exhibited a 40-50% reduction in the number of WT1$^+$ cells within the myocardium (Figure 2.9A). Because PI3K is the only signaling pathway disrupted in all three mutant strains, we infer that the PI3K pathway may be the essential and predominant signaling pathway for initiating PDGFRβ-driven epicardial cell migration into the heart. This observation agrees with the known role of PI3K signaling in actin reorganization and cell migration (Sasaki and Firtel 2006).

To further evaluate epicardial cell responses to PDGF ligand stimulation, we used a wound closure assay. Monolayers of epicardial cells were generated from E11.5 hearts. A scratch was made to form a wound, and we assessed the ability of the cells to migrate into the wound (Figure 2.9B-E). We saw that stimulation with PDGFBB and PDGFDD enhanced the rate of wound closure in wild type cultures when compared to unstimulated cultures, but had no

effect on PDGFRβ$^{-/-}$ culture wound closure (Table 2.2). The migration of wild type epicardial cells was similar to the migration induced by FGF, which also has a demonstrated role in migration of epicardial cells (Morabito et al. 2001).

To directly determine if PDGFRβ stimulation could accelerate the number of cells entering the myocardium, we used an ex vivo system of migration. It has been demonstrated previously that the epicardial surface of hearts can be specifically labeled with the intravital, fluorescent dye, carboxyfluorescein (CCFSE), to follow the epicardial cells as they undergo EMT (Morabito et al. 2001) (Figure 2.9F-I). We cultured hearts from *PDGFRβ*$^{-/-}$ and wild type embryos in media containing either PDGFBB or serum. When wild type hearts were cultured in 10% FBS, dye-labeled cells were observed in the myocardium (Figure 2.9F). In contrast, when *PDGFRβ*$^{-/-}$ hearts were cultured under the same conditions, few cells were observed entering the heart (Figure 2.9G). These data suggested that in the absence of PDGFRβ signaling epicardial cells migrated inefficiently. When wild type hearts were stimulated with PDGFBB, numerous labeled cells migrated into the myocardium compared to hearts stimulated by vehicle alone (Figure 2.9H,J). These results demonstrated that *PDGFRβ* is necessary and sufficient for epicardial cell migration.

To quantify the effect of PDGFRβ signaling in epicardial cell migration and examine migration in the signaling point mutants, we performed a similar ex vivo assay using adenoviral GFP transduction of the epicardium (Compton et al. 2006). When wild type hearts were cultured in the presence of PDGFBB, GFP^{+} cells that migrated from the epicardium were observed in the myocardium. The response was similar to known migratory factors, TGFβ1 and bFGF. However, when *PDGFRβ (F2/F2, F5/F5, F7/F7)* mutant hearts were cultured with PDGFBB, a significant reduction of GFP^{+} cells within the myocardium was observed. Additionally, wild

type hearts treated with a PI3K inhibitor (LY294002) and stimulated with PDGFBB showed a reduction in migration similar to PDGFRβ$^{F2/F2}$ signaling mutants (Figure 2.9J). Consistent with our previous data, these data suggest the PI3K pathway is essential for PDGFRβ-driven epicardial cell migration and is the dominant signaling pathway involved in this response.

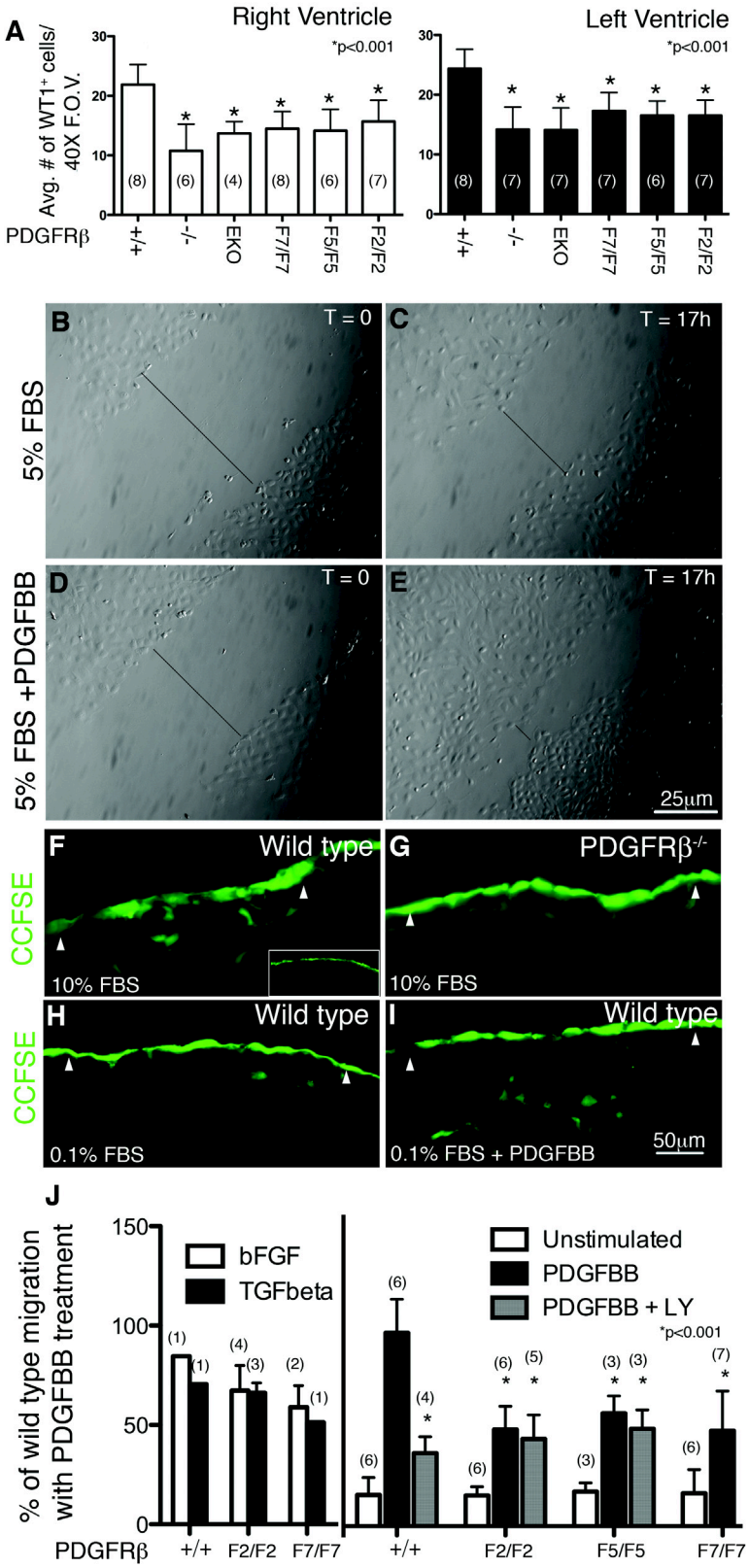

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Figure 2.9. PDGFRβ stimulation induces epicardial cell migration
(A) Quantification of $WT1^+$ cells in right and left lateral ventricles of E15.5 hearts from the indicated genotypes. Numbers in parentheses indicate the number of independent hearts quantified. All mutant alleles exhibited a reduction in $WT1^+$ cells within the myocardium compared to those present in wild type myocardium. (One- way ANOVA, *$p<0.001$). F2/F2-PI3K binding mutant, F5/F5-PI3K, SHP2, PLCγ, RasGAP binding mutant, F7/F7-PI3K, SHP2, PLCγ, RasGAP, Grb2, Src binding mutant. (B-E) Primary epicardial cell migration. Cell location was assessed at (B, D) time 0 and at (C, E) 17 hours. Black lines run between the edges of the wound in each epicardial culture. See Stable II for quantification. (F-J) Migration of epicardial cells by CCFSE dye tracing or adenoviral GFP. Inset in F shows a time zero E13.5 heart labeled with CCFSE. Arrowheads mark the epicardial boundary. J. Quantification of adeno-viral GFP-labeled epicardial cells that have migrated into the myocardium. Numbers in parentheses indicate the number of hearts assayed. LY=LY294002. All samples were compared to wild type cell migration. (Two-way ANOVA, *$p<0.001$)

Table 2.2. Wound closure

% Closure	Basal media	Basal media + PDGFBB	Basal media + PDGFDD	Basal media + FGF2
Control	57.4% ± 12.6 (10)	81.8% ± 6.8 (3)	100% (1)	90.9% ± 15.7 (3)
PDGFRβ$^{-/-}$	-	-	47.7% ±7.6 (3)	100% (1)

% closure was calculated by dividing the difference between the distance at time zero and the distance at 17 hours by the distance at time zero. Dash indicates that treatment was not performed. Number in parentheses indicates the number of independent epicardial cultures that were examined.

Cellular morphology and cytoskeletal organization in PDGFRβ$^{-/-}$ epicardial cells

Because proper organization of the cytoskeleton is essential for cell migration we examined actin localization in epicardial cells. Using phalloidin staining we imaged actin organization of hearts in whole mount and in sections of E14.5 PDGFRβ$^{+/+}$ and PDGFRβ$^{-/-}$ hearts (Figure 2.10A-D). In wild type hearts, actin was consistently localized to the basal surface of the epicardial cells (Figure 2.10C). However, *PDGFRβ*$^{-/-}$ and *PDGFRβ*$^{EKO/-}$ hearts showed an increase in cells that exhibited a subcortical actin distribution (Figure 2.10 C-E). Consistent with a role for PI3K in actin localization, *PDGFRβ*$^{F2/F2}$ hearts also demonstrated an increase in cortical actin localization (Figure 2.10E).

We examined the ultrastructure of epicardial cells at E14.5 (Figure 2.10F-G) and E13.5 (data not shown). TEM revealed that epicardial cells in *PDGFRβ*$^{-/-}$ hearts lacked the epithelial morphology exhibited by wild type epicardial cells. Occasionally, a few rounded cells were observed in wild type hearts but these were rare in occurrence compared to the number of those observed in *PDGFRβ*$^{-/-}$. These results further support a role for PDGFRβ in promoting the cellular processes involved in actin reorganization and migration of epicardial cells.

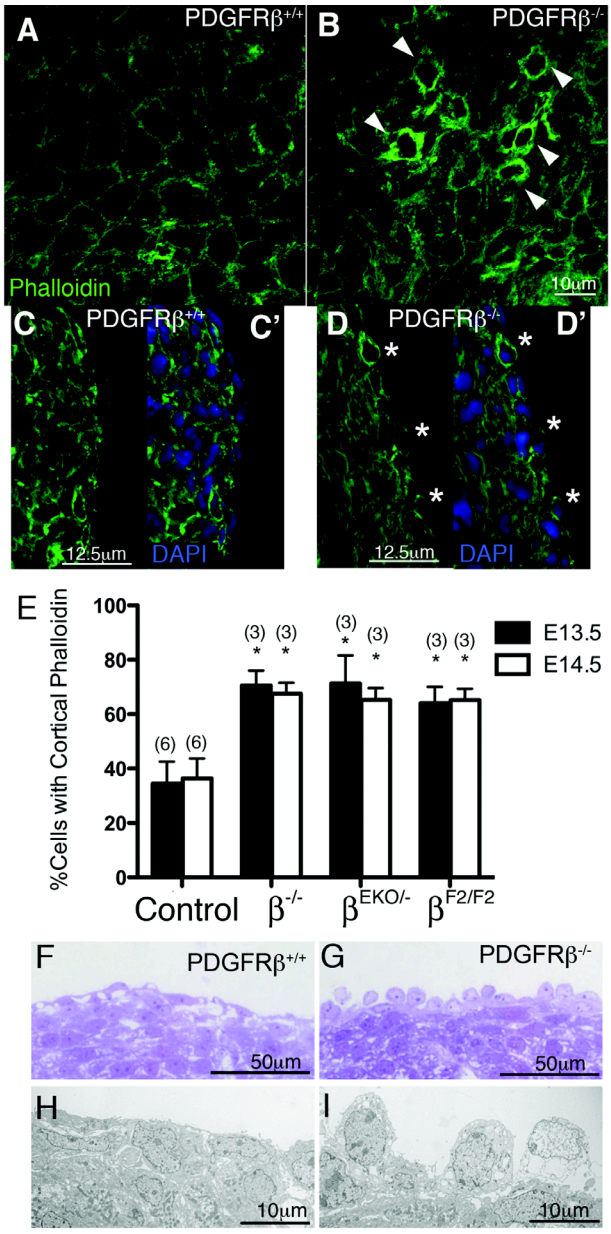

Figure 2.10. Aberrant actin organization and epicardial cell migration in PDGFRβ signaling mutant hearts

(A-D) Actin localization in control and *PDGFRβ* mutant hearts using fluorescent-phalloidin. (A,B) Confocal imaging of whole mount actin localization of E14.5 (A) $PDGFR\beta^{+/+}$ and (B) $PDGFR\beta^{-/-}$ hearts. Arrowheads indicate cells with cortical actin distribution. (C, D) Confocal imaging of E14.5 sectioned hearts. (C) $PDGFR\beta^{+/+}$, (D) $PDGFR\beta^{-/-}$ heart. Figures indicated by the prime have DAPI staining to permit identification of epicardial cells on the exterior of the heart. Asterisks indicate cells that have cortical actin localization. (E) Quantification of cells possessing cortical actin at the specified ages in the indicated genotype compared to controls at the same age. (Two-way ANOVA *$p<0.001$) (F, G) Thick sections of E14.5 hearts processed for TEM and imaged on a light microscope. (H,I) Representative TEM images of thin sections.

PI3K signaling is required for PDGFRβ-dependent cortactin localization

We next examined the phosphorylation status of proteins downstream in the PI3K pathway (AKT, p70S6K, GSK3β) and involved in actin polymerization (cortactin) in LY294002-treated and PDGFRβ$^{F2/F2}$ epicardial cultures. PDGFDD stimulation of wild type epicardial cells induced phosphorylation of AKT, GSK3β, p70S6K, cortactin and ERK1/2 (Figure 2.11A), but when PI3K signaling was disrupted, phosphorylation of all of these proteins was significantly reduced or absent, with the exception of ERK1/2. Residual AKT and p70S6K activation was observed in PDGFRβ$^{F2/F2}$ epicardial cells. This was likely due to the fact that epicardial cells express both PDGFRα and PDGFRβ, and PDGFDD weakly induces PDGFRα/β heterodimers (LaRochelle et al. 2001).

Because activation of many PI3K downstream components is associated with actin polymerization, we next evaluated the ability of epicardial cells to form lamellipodia and localize proteins to these cellular areas (Figure 2.11B). Stimulation with PDGFDD increased cortactin localization to lamellipodia in wild type cells. However, PDGFRβ stimulation was unable to direct cortactin to lamellipodia in PDGFRβ$^{EKO/-}$ or LY294002-treated cells. These data suggest PI3K is required downstream of PDGF signaling for activating proteins involved in actin reorganization and localizing cortactin to the leading edge of a migrating cell.

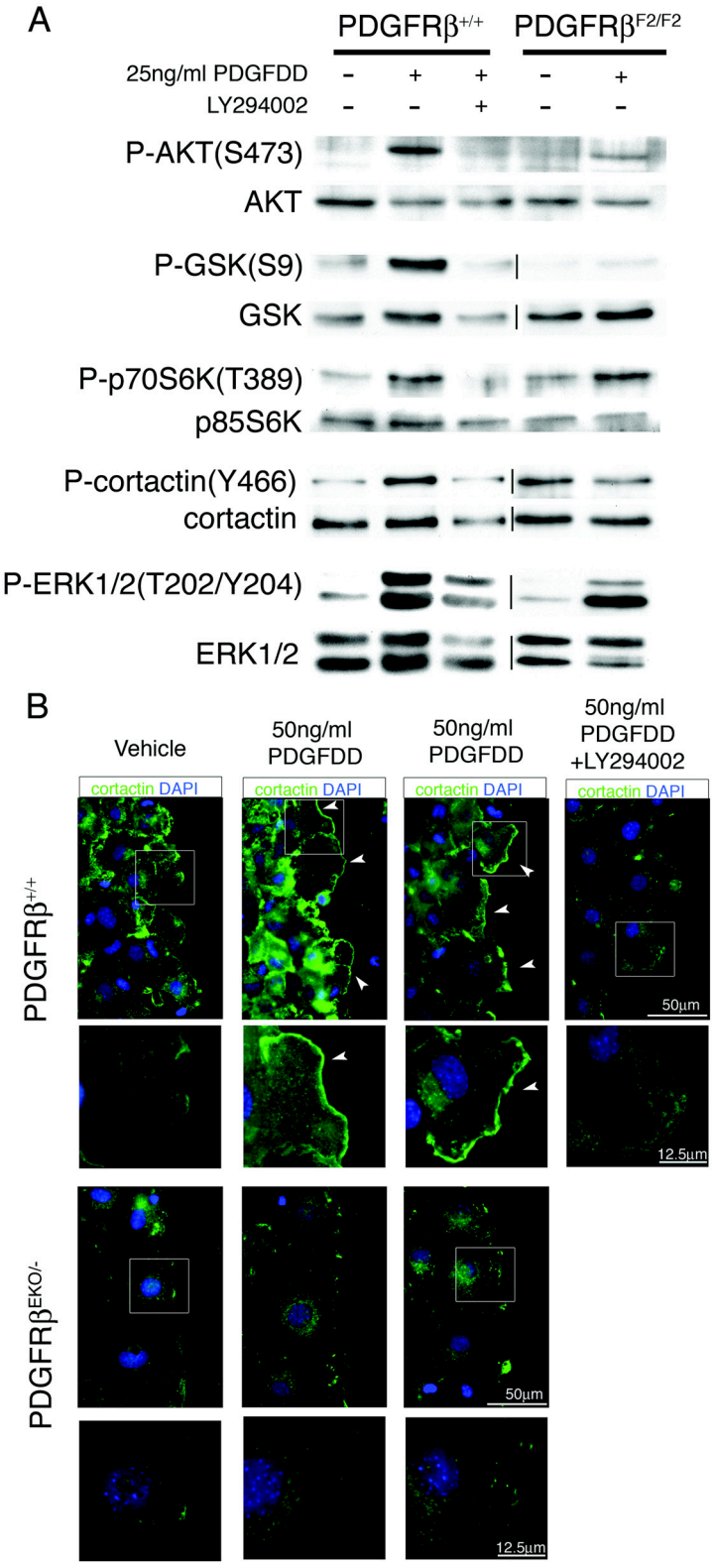

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Figure 2.11. Disruption of PDGF signaling results in failure to activate and localize cortactin
(A) Western blot analysis of primary epicardial cell lysates from the indicated genotypes and treatments were probed for phosphorylated (top panel) and total protein (bottom panel). (B) Cortactin localization on wounded epicardial cells. Two representative images of PDGFDD treated samples are shown to demonstrate reproducibility. Arrowheads indicate cortactin localization to lamellipodia.

Discussion

The epicardium retains stem cell-like properties and when given the appropriate cues, it can differentiate into multiple cardiac cell types (Smart et al. 2007; van Tuyn et al. 2007; Cai et al. 2008). In a zebrafish cardiac injury model, the epicardium is an essential component of the regeneration process (Lepilina et al. 2006). In addition to being a source of cardiac (Cai et al. 2008; Zhou et al. 2008) and vascular cells (Mikawa and Gourdie 1996; Gittenberger-de Groot et al. 1998), the epicardium also secretes growth factors essential for myocardial development (Chen et al. 2002; Lavine et al. 2005). Therefore, a better understanding of the epicardium during development may help define the signals essential for reactivation of these differentiation processes to improve outcomes of human heart disease.

We show that PDGFRβ provides essential cues for efficient epicardial migration, cVSMC formation, and coronary vessel maturation. Previous expression studies have demonstrated ligands and receptors within the epicardium foreshadowing a requirement for PDGF signal transduction (Ponten et al. 2005; Van Den Akker et al. 2005). Explant studies in rat and chick proepicardial and epicardial cells have demonstrated that stimulation with PDGF ligands leads to filamentous actin formation and expression of smooth muscle cell markers (Landerholm et al. 1999; Wada et al. 2003). We now provide in vivo illustration of the role for PDGFRβ in epicardial function and show that disruption of this signaling impacts more than just VSMC proliferation. The heart is the first tissue to demonstrate an absolute requirement for PDGFRβ signaling to promote VSMC differentiation. In most other tissues VSMC differentiation occurs but expansion is disrupted in the absence of PDGFRβ (Hellstrom et al. 1999).

It is an established fact that VSMC are a heterogeneous population and that they come from a vast range of embryonic origins. In the chick, lineage-tracing analysis has demonstrated that the majority of cVSMC are derived from the proepicardium (Mikawa and Gourdie 1996; Dettman et al. 1998; Perez-Pomares et al. 1998). Consistent with recent lineage tracing studies (Cai et al. 2008; Zhou et al. 2008), we have shown that in the mouse, a majority of VSMC also arise from the epicardium. However, one question concerning the heterogeneity of cVSMC is the origin of the residual cells that are present around the coronary arteries in PDGFRβ epicardial mutant hearts. From the current experiments it is difficult to determine if these cVSMC arise ectopically due to the absence of epicardially-derived VSMC, or if they are a normal subpopulation of cells contributing to the coronary arteries. Both neural crest-derived cells and cells from the secondary heart field can contribute to VSMC in the outflow tract and the coronary arteries (Jiang et al. 2000; Verzi et al. 2005; Sun et al. 2007). The possibility that cVSMC are heterogeneous in origin is an important consideration because the two cell populations are likely to express different genes and respond differently under pathological conditions or in response to drug treatment.

Based on our analysis of the formation of the coronary vessels in PDGFRβ mutant hearts, we propose that PDGFRβ signaling can indirectly help shape the mature coronary vasculature. The observation that the coronary arteries defects in *PDGFRβ*^{EKO} but not PDGFRβ$^{-/-}$ hearts improve over time suggests that cVSMC may be involved in the coronary vessel remodeling process. We have shown that cVSMC formation was completely disrupted, while epicardial cell migration was reduced but not abrogated in *PDGFRβ*$^{-/-}$ hearts. The most likely explanation for this observation is that presence of PDGFRα can compensate for loss of PDGFRβ in the epicardium but not cVSMC. These two receptors signal through very similar pathways and can

bind some but not all of the same ligands (Fredriksson et al. 2004). We have recently shown that PDGF receptor function in neural crest cells is also partially redundant, therefore ligand availability may be a key factor in determining the contribution of each receptor. The exquisite expression of the PDGFDD ligand in the epicardium (Ponten et al. 2005), which predominantly activates PDGFRβ (Bergsten et al. 2001), could favor signaling through the PDGFRβ. Therefore, it is possible that PDGFDD may provide an autocrine signal that induces cytoskeletal rearrangements necessary for EMT. Recently, PDGFDD overexpression has been demonstrated to induce an EMT-like transformation in prostate cancer cells (Kong et al. 2008).

Our current data cannot determine the temporal requirement for PDGFRβ signaling. The migration defect we observe may predominantly affect only the cells destined to become cVSMC. In this scenario, cVSMC progenitors might not reach their final destination to receive the differentiation cues from myocardium or endothelium. In support of this possibility, clonal analysis of VSMC in the chick has shown that specification of VSMC occurs prior to the formation of the epicardium and that some VSMC markers are expressed in the proepicardium (Mikawa and Gourdie 1996). Because the requirement for PDGFRβ signaling in many tissues is to promote VSMC proliferation (Lindahl et al. 1997; Lindahl et al. 1998), another way to explain our results is that PDGFRβ may be required at two stages: within the epicardium for migration and within the epicardial-derived mesenchyme to promote VSMC differentiation or expansion.

A role for PDGFRβ in directing cytoskeletal rearrangements via PI3K has been established in multiple cell types (reviewed by Heldin (Heldin et al. 1998)). Here, we have demonstrated that PDGF stimulation of PI3K is also required for cortactin localization to lamellipodia. The failure of cortactin localization is likely due to the loss of PI3K-induced Rac

activation (Head et al. 2003). The epicardial cells are incapable of controlling directed actin filament growth, and directed migration into the myocardium is inefficient.

In conclusion, we have demonstrated a novel role for the PDGFRβ in epicardial development in vivo and identified PI3K signaling as one of the pathways associated with this process. In the absence of this signaling, the epicardium fails to adopt a motile phenotype leading to a reduction in cVSMC and abnormal coronary vessels. Overall, these findings suggest that PDGF signaling is acting to promote epicardial migration and that modulation of PDGF receptor signaling should be considered when exploring options for therapeutic applications of epicardial-derived cells.

Materials and methods

Experimental animals

Wild type, $PDGFR\alpha^{GFP}$ (Hamilton et al. 2003), *PDGFRβ* null allele (Soriano 1994), $PDGFR\beta^{fl}$ (Richarte et al. 2007), PDGFRβ signaling mutants (Tallquist et al. 2003), $GataCre^{Tg/0}$ (Merki et al. 2005), $capsulin^{LacZ/+}$ (Lu et al. 2000), $WT1^{GFPCre/+}$ (Zhou et al. 2008), and *XlacZ4* transgenic (Tidhar et al. 2001) mice were maintained on a mixed C57Bl/6 X 129SV background. Where indicated, $GataCre^{Tg/0}$; $PDGFR\beta^{+/-}$ males were used to generate epicardial deletion of PDGFRβ ($PDGFR\beta^{EKO/-}$). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center and performed according to the Guide for the Care and Use of Laboratory Animals published by the NIH. Dr. Eric Olson kindly provided $capsulin^{LacZ/+}$ mice, and Dr. Pilar Ruiz-Lozano kindly provided $GataCre^{Tg/0}$ mice.

Whole Mount Immunohistochemistry

Whole mount PECAM staining was performed as previously described (Hogan 1994) with the following adaptations. Briefly, hearts were fixed overnight in 4:1 Methanol:DMSO at 4° C, bleached for 5-10 hours in 4:1:1 Methanol:DMSO:H_2O_2 at 4° C, and then washed for 20 minutes in 1:1 Methanol:PBS. Hearts were then blocked for 2 hours in PBSMT (2% milk, 0.5% TritonX-100 in PBS) and incubated with PECAM (1:200; BD Biosciences) for 4 hours-overnight. Hearts were washed five times for a total of eight hours in PBSMT, followed by 2 hours in 1:200 biotinylated anti-rat (Vectashield anti rat kit). One overnight and 5 additional 1 hour washes in PBT (0.5% TritonX-100 in PBS) were performed. PECAM antibody was detected using the Vectastain Rat ABC kit and developed with DAB (Vector Labs). For phalloidin staining, samples were fixed for 2 hours in 4% PFA. Hearts were permeabilized in 0.5% TritonX-100 for 1.5 hours, then blocked with 1% BSA/ 0.5%TritonX-100 for 1 hour, and incubated with phalloidin 488 (1:200; Molecular Probes) for 2 hours.

Paraffin and frozen section immunohistochemistry

For frozen sectioning, hearts were isolated from the embryo and fixed for 2 hours in 4% PFA, put in 30% sucrose overnight at 4°C and embedded in OCT. The following primary antibodies were used: PDGFRβ (1:200, eBioscience); PECAM (1:200, BD Pharmingen); αSMA (1:250, Sigma); smMHC (1:250, BTI); phalloidin 488 (1:200, Molecular Probes); vimentin (1:500 Sigma). WT1 (1:100, DAKO) hearts were paraffin embedded, sectioned, and microwave antigen retrieval was accomplished using 10 mM Tris, 1 mM EDTA, pH 9.0 at 98ºC for 15 minutes in the EZ Retriever (Biogenex). Sections were then blocked for 30 minutes, stained with antibody for 2 hours, and visualized by Vectastain ABC and DAB kits (Vector

laboratories). For the WT1 quantification, images were taken under 40X magnification of transverse sections throught the heart, and a box of 324 x 792 pixels was drawn in Photoshop CS2 to ensure same area of tissue was quantified. WT1 positive cells within the lateral wall of either the right or left ventricle were quantified (epicardial cells were excluded). The width of the box included all WT1 positive cells present within the myocardium. Four-5 nonconsecutive sections from each heart were quantified. Compact zone thickness of PDGFRβ$^{EKO/-}$ hearts was measured in Image J. The percent thickness of mutant compact zone hearts was calculated by dividing the mutant thickness by the thickness of littermate control hearts and then multiplying by 100. Four hearts were measured for each genotype.

Real-time PCR analysis

For real-time analysis, primary epicardial cell cultures were isolated as mentioned above. Five days after explant was removed, RNA was isolated using Trizol (Invitrogen) following manufactures protocol. 1μg of RNA was used to generate cDNA using PowerScript Reverse Transcriptase (Clontech) and random hexamers. Gene expression was analyzed using standard real-time PCR methods using SYBR Green master mix on an ABI7000 instrument (Applied Biosystems). Each sample was done in triplicate. Primers used for PDGFRα and PDGFRβ were described previously (French et al. 2008). Sequence used for Capsulin primers were: (For) 5'-agctacatcgctcacttaag-3' and (Rev) 5'-ctcgcacctccaaggtcagg-3'

Proliferation index

Pregnant females were injected with BrdU (10 μg/gram of body weight, Sigma) two hours before embryo isolation. Hearts were fixed, frozen embedded, and sectioned. Antigen

retrieval was carried out either by processing for 30 minutes in 2 N HCl at 37°C and then neutralizing for 5 minutes in 0.1 M NaBorate pH 8.5 or by performing microwave antigen retrieval as described above. Sections were then blocked for 45 minutes, and stained for 2 hours with BrdU antibody (1:50; Becton Dickinson). Three 40x fields of view of the lateral ventricles were used per sample to determine the proliferation index. Proliferation index was calculated by dividing the number of BrdU positive cells within a given region by the number of DAPI stained nuclei and multiplying by 100. Three hearts of each genotype were compared at each embryonic stage.

TEM

Hearts were isolated from the embryo and fixed in 2.5% glutaraldehyde in 0.1M Cacodylate buffer. Hearts were routinely processed for TEM.

Primary epicardial cultures and wound closure assay

Primary epicardial cells were isolated from E11.5 or E12.5 hearts from which the conotruncal region had been removed similar to previous reports (Zamora et al. 2007). Hearts were plated on rat tail collagen (0.001mg/ml, Roche) coated dishes in 15% FBS. Hearts were removed two days after explant. The epicardial identity of these cultures was demonstrated by localization of β-catenin at the cell membrane and cobblestone morphology. Neural crest contribution to the cultures was ruled out using Wnt1Cre-ROSA26 lineage tracing (Jiang et al. 2000) and could also be easily identified by their mesenchymal morphology.

For immunocytochemistry, epicardial cells were starved in 0.1% FBS containing media overnight prior to the scratch and stimulated with PDGFDD (50ng/ml, R&D Systems) for 30

min. Inhibition of PI3K was achieved by preincubation of cells with LY294002 (5μM, Cell Signaling) for 1 hour. Cells were fixed in 4% Paraformaldehyde for 20min and blocked in 1.5% normal serum in PBT (0.5% TritonX-100) for 30 min. Subsequently, cells were incubated with an anti-cortactin (1:750, Upstate) antibody for 1h then an anti-mouse secondary (Molecular Probes).

For the wound closure assay, hearts were plated on rat tail collagen (0.001mg/ml, Roche) coated glass bottom dishes (Matek Corp.) in 15% FBS. Four days after heart removal media was changed to 5% or 10% FBS with or without growth factor and a scratch was made with P10 tip. Either PDGFBB (20ng/ml; R&D Systems), PDGFDD (20ng/ml; R&D Systems) or FGF4 (20ng/ml; R&D Systems) were added to the cultures. Imaging was performed at 0 hours and 17 hours and wound closure was determined on bright field images in PhotoshopCS2. Similar results were obtained with 5% and 10% FBS.

For western blotting, primary epicardial cultures were generated as above. Five days after heart removal, cultures were starved overnight in 0.1% serum. Cultures that were treated for PI3K inhibition were incubated with LY294002 (5μM, Cell Signaling) for 1 hour prior to stimulation. Cultures were stimulated with PDGFDD (25ng/ml, R&D Systems) for 10 minutes. Protein concentration was determined using the Bradford reagent (Bio-Rad) and equal amounts were run on SDS-PAGE and transferred onto nitrocellulose membranes following standard protocols. Membranes were incubated with 1% BSA/0.05% Tween-20 in TBS for 30 minutes, with 1:1000 primary antibody overnight at 4ºC, and secondary antibody for 1h. Results were visualized using ECL (Amersham Bioscieces). The following antibodies were used: AKT/P-AKT(S473) (Cell Signaling), GSK3β/P-GSK3β(S9) (Cell Signaling), P-P70/P85 S6K(T389)

(Cell Signaling), Cortactin (Upstate), P-Cortactin(Y466) (Invitrogen), Erk1/2(Upstate), and P-ERK1/2(T202/Y204) (Cell Signaling).

CCFSE

E13.5 hearts were isolated from the indicated strains. Hearts were incubated with 24 μM CCFSE (Molecular Probes) in 0.1% FBS for 1 hour. Hearts were rinsed 3 times in PBS and then cultured in either 10% FBS or 0.1%FBS with indicated ligand concentration for 48 hours. Hearts were fixed in 4% PFA and embedded in OCT. Ten-micron thick frozen sections were incubated in 50mM NH_3Cl in TBS for 1 hour to quench the autofluorescence. Slides were mounted in 1:1 glycerol:PBS.

Migration

E12.5 hearts were isolated from indicated genotypes and incubated with GFP-expressing adenovirus. Hearts were cultured in 10% FBS 1:1 DMEM:M199 supplemented with bFGF (2ng/ml, Sigma) for 48h with vehicle, 50ng/ml PDGFBB, 50ng/ml PDGFBB + LY294002 (2μM, Cell Signaling), hTGFβ1 (20ng/ml, R&D Systems), or bFGF (25ng/ml, Sigma). Hearts were isolated, fixed, and frozen embedded as performed in the CCFSE experiments. Ten-micron sections were DAPI stained and mounted in 1:1 PBS glycerol. Migration was quantified by counting the number of GFP-positive cells beneath the epicardium in a 40x field of view from five non-consecutive sections. Percentage of migrated cells was determined by dividing the average number of GFP-positive cells for each sample by the average of wild type GFP-positive cells that had migrated in response to PDGFBB stimulation and multiplying by 100.

Imaging

Imaging was performed as follows: confocal imaging (LSM510META (grant NIH 1-S10-RR019406-01)), fluorescent imaging (Zeiss Axiovert 200 with a Hamamatsu ORCA-ER camera), color imaging (Zeiss Axiovert 200 with an Olympus DP71 camera), and whole mount imaging (Nikon SMZ1000 with a Nikon Coolpix digital camera). TEM images were taken on a 1200 EX Electron Microscope.

Chapter III

Epicardial Derived Cell Epithelial-to-Mesenchymal Transition and Fate Specification require PDGF signaling

Abstract

Vascular smooth muscle cells (VSMC) and cardiac fibroblasts are important mediators of myocardial pathogenesis, and arise when a subset of epicardial cells undergoes epithelial-to-mesenchymal transition (EMT). We show that PDGF signaling controlled the process of EMT and that the loss of both receptors lead to defects in both EMT gene expression and failure to form mesenchymal derivatives. We identified Sox9 as a downstream mediator of PDGF-driven EMT. PDGF receptor-deficient epicardial cells have reduced levels of Sox9, and overexpression of Sox9 restored EMT in mutant epicardial cells. The loss of epicardial EMT results in hearts that lack epicardial-derived cardiac fibroblasts and VSMC. We further demonstrate that loss of only PDGFRα, leads to failure of cardiac fibroblast development. This establishes PDGF signaling as a key EMT signaling pathway and suggests that after EMT each PDGF receptor independently has a distinct function in the two epicardial derived cell populations, cardiac fibroblasts and VSMC.

Introduction

Cardiac disease is the leading cause of death in the industrial world. While recent stem cell therapies have attempted to regenerate myocardium, there are still many physiological barriers to overcome, including fibrosis, inflammation, and insufficient blood vessel generation. Induction of cardiomyocyte regeneration is one proposed way to improve cardiac function, but it is clear that the non-cardiomyocyte populations in the heart also contribute to the repair process. Non-cardiomyocyte lineages (endothelial cells, vascular smooth muscle cells, and cardiac fibroblasts) are essential for blood vessel formation and matrix organization, and an understanding of the developmental signals that shape these cells may provide insights into disease pathogenesis and better heart injury therapies.

Coronary vascular smooth muscle cells (cVSMC) and cardiac fibroblasts develop from the epicardium in a multi-step process involving cell proliferation, epithelial-to-mesenchymal transition (EMT), and mesenchymal cell fate specification (Winter and Gittenberger-de Groot 2007). Several proteins have been implicated in the development of cVSMC from the epicardium (Olivey and Svensson 2010), but less is known about the epicardial derived cardiac fibroblast population. It is proposed that cardiac fibroblasts are essential for normal cardiac function, and their role in matrix deposition during cardiac injury is well established. Yet, signaling pathways regulating their development are poorly understood.

Platelet derived growth factor (PDGF) receptor tyrosine kinases are important for embryonic development and play essential roles in the forming vasculature (Andrae et al. 2008). Previously, we identified a role for PDGFRβ as an important factor regulating epicardial derived cVSMC development (Mellgren et al. 2008). We and others observed PDGFRα in the epicardium (Kang et al. 2008; Bax et al. 2009); however, no data exists regarding the fate of

epicardial derived cells (EPDCs) when PDGFRα is disrupted. The receptors are co-expressed in the epicardium until E13.5, but after this time point receptor expression becomes mutually exclusive. These initial findings led us to investigate the role of PDGFRα individually and combined with PDGFRβ during EPDC formation.

Using cre/loxP recombination, we generated animals that lacked PDGFRα, PDGFRβ, or both PDGF receptors in the epicardium. Epicardial deletion of both PDGF receptors resulted in failure of epicardial EMT and EPDC formation. Loss of PDGF signaling led to reduced Sox9 expression, and when Sox9 expression was restored in mutant hearts, the EMT defect was rescued. Interestingly, mutants lacking only one of the PDGF receptor genes exhibited a lineage specific requirement for each individual receptor. Loss of *PDGFRα* resulted in a deficit in cardiac fibroblast formation, while cVSMC development was unperturbed. Conversely, PDGFRβ was required for cVSMC development (Mellgren et al. 2008) but not cardiac fibroblast development. Combined, our data demonstrate a novel role for PDGF receptors in epicardial EMT and EPDC development.

Results

Loss of both PDGF receptors causes defects in epicardial cell migration

Both PDGF receptors, α and β, are expressed by the epicardium, and loss of PDGFRβ alone causes a reduction in cVSMC (Mellgren et al. 2008). To investigate a combined role for PDGF receptor signaling, we deleted PDGF receptors from epicardial cells using mice expressing the *Gata5Cre* transgene (Merki et al. 2005). $PDGFR^{EKO}$ (EKO – epicardial knockout) hearts had regions of epicardial detachment and hemorrhaging (Figure 3.1A). The detachment progressed temporally from the dorsal to ventral heart surface but resolved by birth

(data not shown). Despite this phenotype, the epicardium expressed multiple, established epicardial markers (Figure 3.2A-B). Because PDGF receptor signaling has been associated with proliferation and survival (Hoch and Soriano 2003), we examined the epicardium and EPDCs for BrdU incorporation and cleaved caspase-3 activation. *PDGFR*^{EKO} mutant values were similar to those obtained in controls (Figure 3.2C-D), suggesting that loss of PDGF receptor signaling does not affect cellular proliferation or survival of epicardial cells in vivo.

Because epicardial formation was unaffected in *PDGFR*^{EKO} mutants, we next assayed hearts for epicardial cell entry into the myocardium. To trace epicardium lacking PDGF receptors, we induced cre-mediated recombination in epicardial cells just prior to EMT (E12.5) using a tamoxifen inducible *WT1*^{iCre} allele (Zhou et al. 2008) and analyzed migration at E14.5. Using R26R^{YFP} reporter activity to follow the epicardial cells, we observed a loss of EPDCs when PDGF receptors were absent (Figure 3.1B-C). Similar results were obtained when using markers of undifferentiated EPDCs, WT1 (Moore et al. 1999; Zamora et al. 2007) or mesenchymal cells, vimentin (Perez-Pomares et al. 1997). Both markers showed a severe reduction in the region immediately underlying the epicardium in *PDGFR*^{EKO} hearts (Figure3.3 and data not shown). Note that vimentin is a broad mesenchymal marker that is also expressed by non-epicardial derived coronary endothelial cells (Red-Horse et al. 2010). Additionally, in an ex vivo migration assay (Mellgren et al. 2008), fewer cells exited the epicardium in *PDGFR*^{EKO} mutant hearts even when stimulated with EMT-inducing growth factors, hTGFβ_1 (Compton et al. 2006) and bFGF (Morabito et al. 2001) (Figure 3.1D).

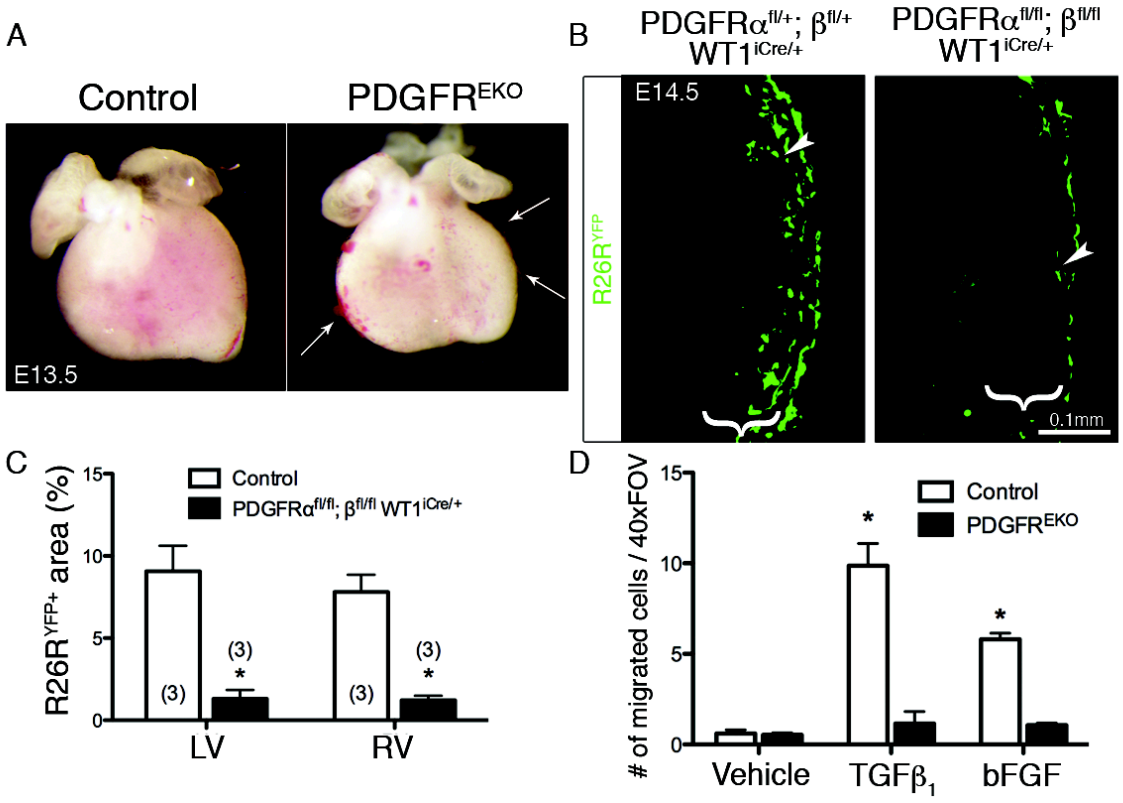

Figure 3.1. *PDGFR^{EKO}* epicardial cells fail to migrate into the myocardium
(A) Whole mount images showing regions of epicardial detachment and hemorrhaging (Arrows). (B) R26R^{YFP} IHC was used to examine epicardial cell migration into subepicardial mesenchyme (brackets) from indicated genotypes induced with tamoxifen at E12.5. Arrowheads point to migrated cells within the subepicardial mesenchyme. (C) Quantification of the R26R^{YFP} fluorescent area in (B). N values are indicated in parentheses. (*) $p<0.005$ (D) Quantification of GFP^{+} cells within myocardium of E12.5 hearts transduced with an adenovirus expressing GFP and stimulated with hTGFβ_1 or bFGF (n=3 for each genotype/condition). Data are represented as mean ± SD. (*) $p<0.001$ (compared to vehicle treated control) (LV – left ventricle, RV – right ventricle, EKO – epicardial knockout)

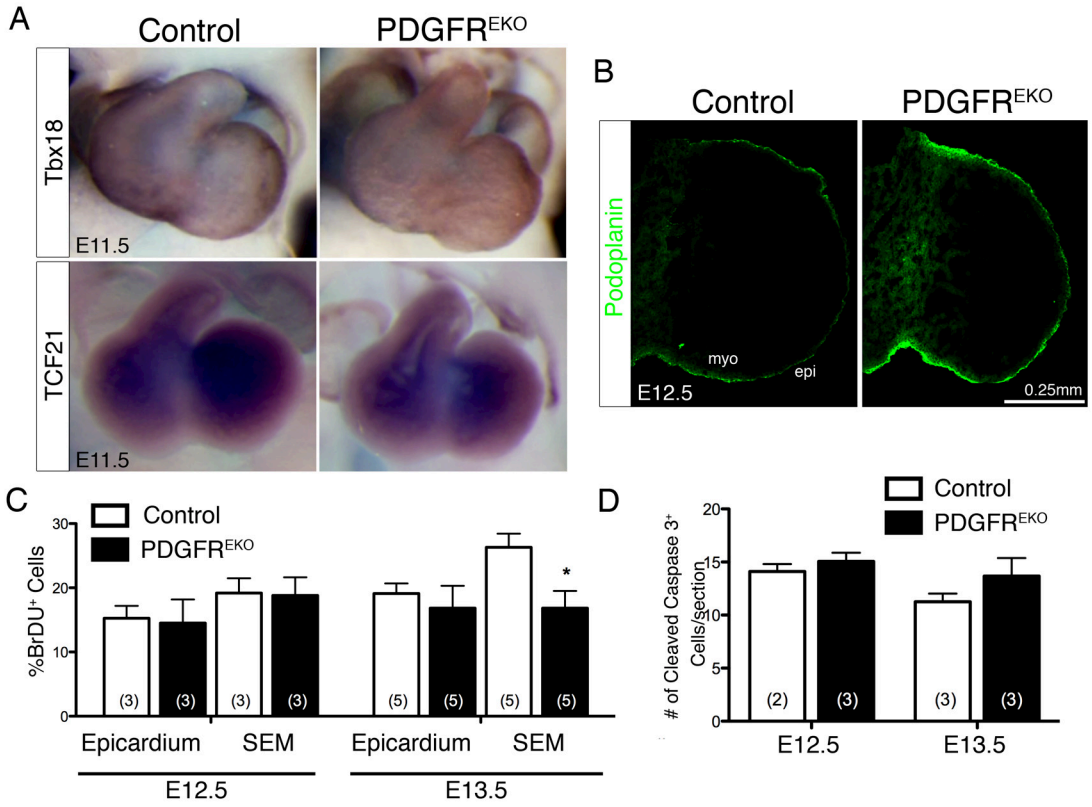

Figure 3.2. Epicardial specification, proliferation and apoptosis
(A) Whole mount in situ hybridization for epicardial transcription factors as indicated. (B) IHC for Podoplanin, a protein expressed by developing epicardial cells. Myo-myocardium and epi-epicardium. (C-D) Quantification of BrdU incorporation and activated (cleaved) caspase 3 staining. (*) $p<0.001$ (SEM – subepicardial mesenchyme)

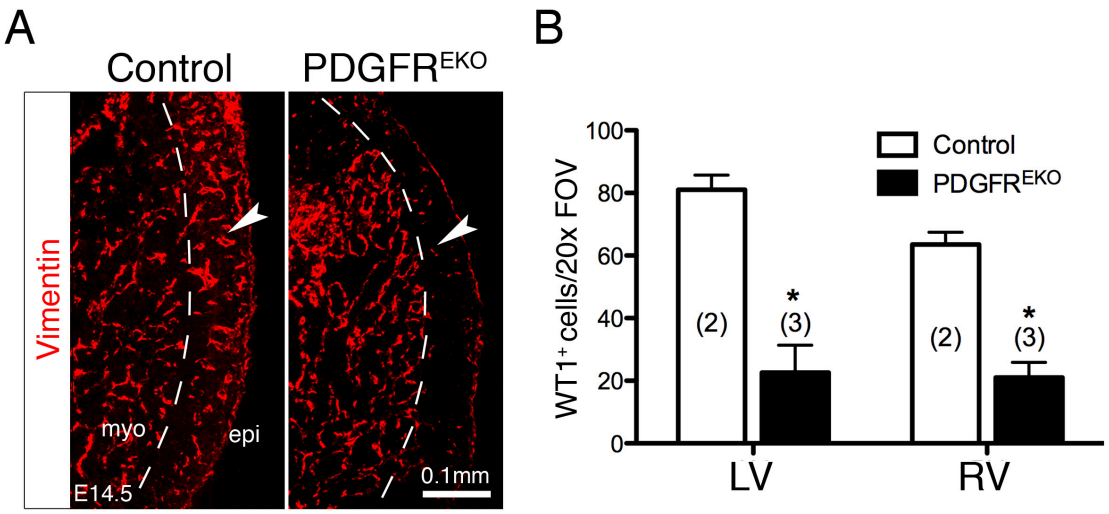

Figure 3.3. *PDGFR^{EKO}* epicardial cells fail to migrate
(A) Vimentin IHC was used to examine epicardial cell migration into subepicardial mesenchyme. Note that endothelial cells are also vimentin positive. Arrowheads point to subepicardial mesenchyme. Dashed lines delineate border between myocardium and subepicardial zone. myo-myocardium and epi- epicardium. (B) E15.5 hearts were stained for WT1 and quantified for $WT1^+$ cells/FOV within the myocardium. N values are indicated in parentheses. (*) $p<0.001$

PDGF receptor signaling is required for epicardial cell EMT

We surmised that loss of epicardial cell migration was caused by a defect in epicardial EMT. We first examined $PDGFR^{EKO}$ embryonic hearts for expression of transcriptional inducers of EMT, *Snail* (Veltmaat et al. 2000), *Slug* (Savagner et al. 1997), and *Sox9* (Cheung et al. 2005; Sakai et al. 2006). Interestingly, while epicardial genes, such as *WT1*, *Tbx18*, were unchanged, we consistently observed a significant reduction in *Snail*, *Slug*, and *Sox9* transcript levels (Figure 3.4A).

EMT involves a complex series of events including the loss of epithelial morphology and the acquisition of mesenchymal actin filaments. Therefore, we examined EMT induction in primary epicardial cultures using a cocktail of growth factors. We compared cell morphology by bright field imaging and immunostaining for adherens junctions and filamentous actin organization (βcatenin and phalloidin, respectively). While control cultures lost their epithelial characteristics (junctional βcatenin) and gained mesenchymal cell morphology (cytoplasmic actin stress fibers), $PDGFR^{EKO}$ mutant cultures remained epithelial, illustrating a failure to initiate EMT (Figure 3.4B, Figure3.5A).

EMT is also associated with changes in gene expression. To evaluate additional EMT markers we performed qPCR analysis. Initial experiments revealed that primary cultures undergo EMT, but many of the genes commonly used to assess EMT were not significantly altered in the stimulated epicardial cultures. For example, we observed no changes in *E-cadherin*, *ZO-1*, *αSMA* and *vimentin* expression (data not shown). Therefore, to generate an EMT profile specific for primary epicardial cultures, we performed gene expression analysis on E12.5 cultures treated with vehicle or $hTGF\beta_1$ (10ng/ml), PDGFBB (20ng/ml), and bFGF (25ng/ml), all three being growth factors known to stimulate EMT (Thiery et al. 2009) (GEO

Series GSE27181). From these data, we generated a list of candidate genes and verified a subset that correlated with a change from an epithelial to a mesenchymal phenotype. Two epithelial markers that were consistently down-regulated upon EMT induction were *Krt14* (Chamulitrat et al. 2003; Ke et al. 2008) and *BVES* (Wada et al. 2001). We also identified a group of mesenchymal genes that were induced upon EMT induction. This list included *Calponin* (Compton et al. 2006), *Snail* (Murray et al. 2006), *Sox9* (Sakai et al. 2006), *Cdh6* (Inoue et al. 2008), *Col7a1* (Vindevoghel et al. 1998), *MMP10* (Wilkins-Port and Higgins 2007), and *OPG* (Vidal et al. 1998; Sakata et al. 1999; Corallini et al. 2009).

Having established these gene sets, we then investigated their expression during the EMT response in mutant cultures. For every gene examined, we found that expression in the mutant cultures was significantly different from stimulated control cultures (Figure 3.4C-E). Interestingly, vehicle treated mutant cultures consistently exhibited increased levels of the epithelial gene, *Krt14*. These data suggest that a defect in the process of EMT was present in $PDGFR^{EKO}$ epicardial cells.

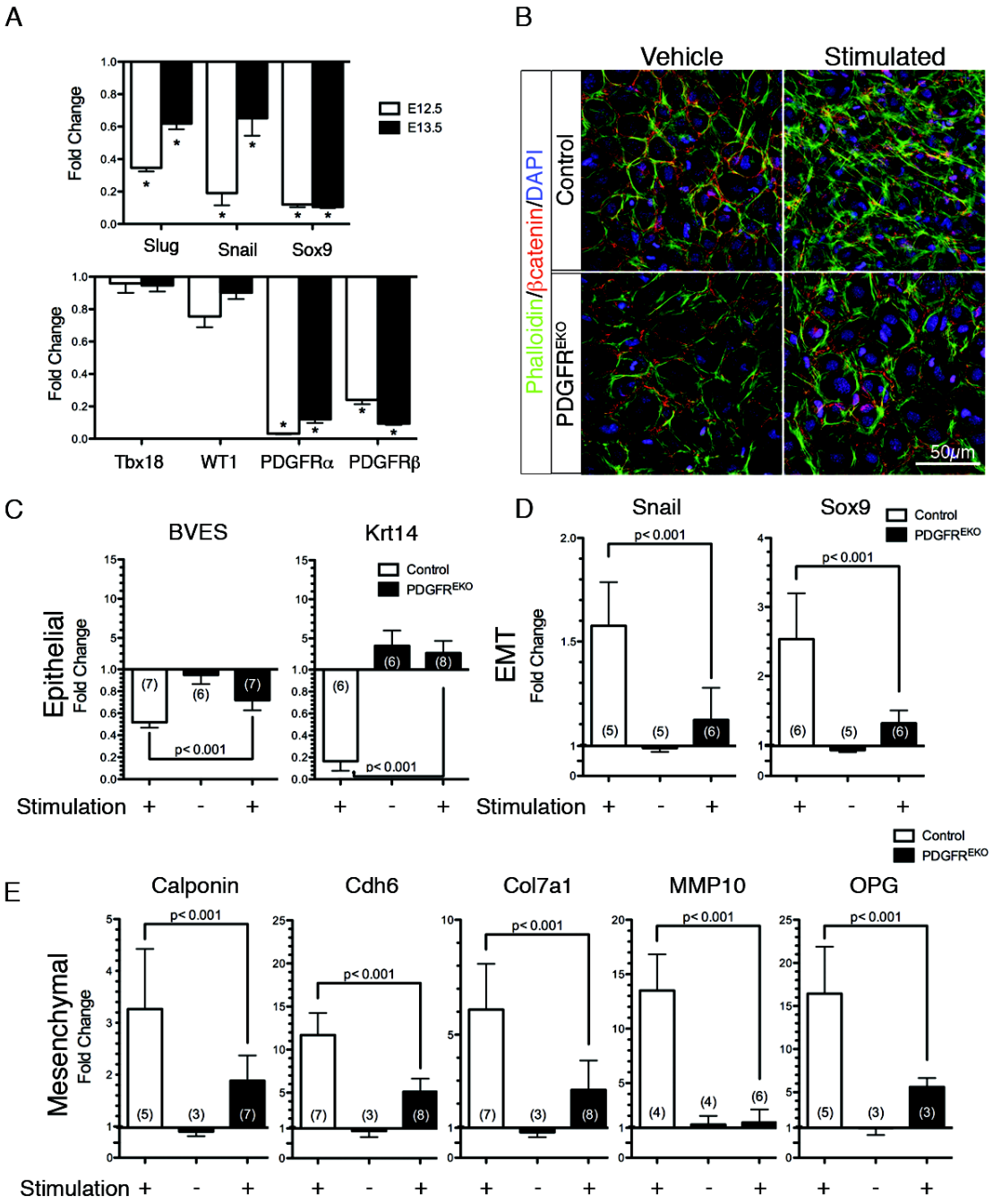

Figure 3.4. *PDGFR^{EKO}* epicardial cells fail to undergo EMT

(A) qPCR analysis of gene expression in *PDGFR^{EKO}* whole hearts (atria and conotruncal regions removed) for transcriptional EMT markers (*Snail*, *Slug*, *Sox9*) and epicardial markers (*Tbx18*, *WT1*, *PDGFRα*, *PDGFRβ*). Data were compared to control littermates and are representative of three independent experiments. (*) p<0.001 (B) βcatenin and phalloidin localization in primary epicardial cultures after 48h of stimulation with hTGFβ$_1$ and PDGFBB. (C-E) qPCR on primary cultures for expression of epithelial, transcriptional, and mesenchymal markers. Data were compared to vehicle treated control epicardial cultures (represented by a baseline of 1.0). Data are represented as mean ± SD. N values are indicated in parentheses.

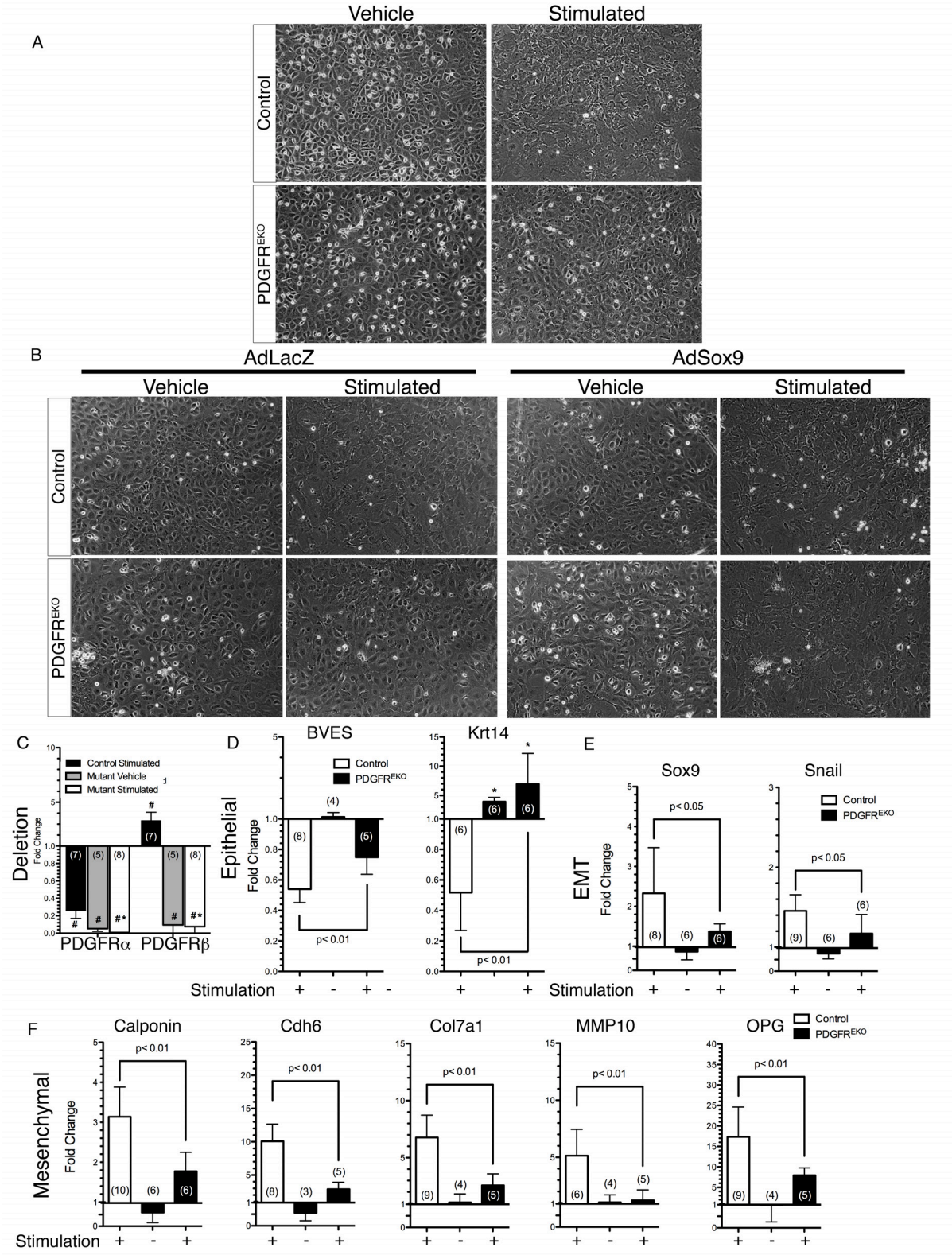

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Figure 3.5. EMT morphology

(A) Brightfield images of control and *PDGFR*^{EKO} epicardial cultures stimulated with hTGFβ$_1$ and PDGFBB. (B) Bright field images of primary epicardial cultures of the indicated genotypes transduced with AdLacZ or AdSox9 in the presence of vehicle or stimulation (hTGFβ$_1$ and PDGFBB). (C) qPCR analysis of primary epicardial cultures showed loss of PDGF receptor expression in the presence of Cre. (*) $p<0.001$ (compared to stimulated control), (#) $p<0.001$ (compared to vehicle control). (D-F) qPCR analysis demonstrated that AdGFP transduction did not alter primary epicardial culture responses to stimulation. Data are represented as mean ± SD. N values are indicated in parentheses.

Expression of Sox9 in *PDGFR^{EKO}* cells rescues the EMT defect

To determine potential genes that mediate PDGF driven EMT, we screened for gene expression differences using microarray data sets from whole hearts and primary epicardial cultures (GEO Series GSE27181). Comparison of control and *PDGFR^{EKO}* data demonstrated that transcripts of an SRY-related family member, *Sox9*, were decreased in mutant E12.5 and E13.5 hearts and in primary epicardial cultures (Figure 3.4A, data not shown).

The correlation of *Sox9* transcript levels with PDGF signaling led us to investigate a role for *Sox9* in PDGF dependent EMT. In primary epicardial cultures, PDGF stimulation resulted in increased *Sox9* expression (Figure 3.6A). We next determined how Sox9 induced expression impacted these cultures. In the absence of stimulation, Sox9 overexpression had little effect on the cultures, regardless of the genotype. However, AdSox9 transduced cultures stimulated with hTGFβ_1 and PDGFBB changed from an epithelial morphology to a mesenchymal morphology (Figure 3.6B, Figure 3.5B). These data suggested that additional signaling pathways were required to initiate a Sox9-mediated EMT in our primary epicardial cell cultures, similar to what has been observed in neural crest cells (Cheung and Briscoe 2003; Sakai et al. 2006), but that Sox9 expression could induce EMT even in *PDGFR^{EKO}* epicardial cells.

To examine Sox9's role in epicardial EMT gene expression, we transduced cultures with AdSox9 (Figure 3.6C-E). These cultures had reduced epithelial (*BVES* and *Krt14*) gene expression, but mesenchymal gene expression remained unchanged. However, similar to the morphological assay, AdSox9 transduced and stimulated (hTGFβ_1 and PDGFBB) cultures had both decreased epithelial gene expression and increased mesenchymal gene expression regardless of genotype (Figure 3.6C-E). Adenoviral transduction alone did not change the gene expression profile of epicardial cells (Figure 3.5D-F).

Because Sox9 expression has not been documented in the epicardium previously, we examined hearts for Sox9 protein. Sox9 was present in a subpopulation of epicardial cells at E13.5 (Figure 3.7A), and a day later, $Sox9^{+}$ cells were present in both the epicardium and subepicardial mesenchyme (Figure 3.7A). Using the $WT1^{iCre}$ and $R26R^{YFP}$ alleles, we confirmed that $Sox9^{+}$ cells are epicardial derived (Figure 3.8A). In contrast to controls, Sox9 expression in $PDGFR^{EKO}$ hearts was significantly reduced at E13.5 and virtually absent at E14.5 (Figure 3.7A-B). We observed a PDGF receptor gene dosage affect on Sox9 expressing cells that correlated with the number of functional PDGF receptor alleles present Figure 3.8B). Individual PDGF receptor epicardial mutants also contained reduced numbers of $Sox9^{+}$ cells (Figure 3.7B), suggesting that signaling from either receptor is involved in Sox9 expression.

To determine if Sox9 could rescue the $PDGFR^{EKO}$ epicardial migration defect, we transduced control and $PDGFR^{EKO}$ hearts with AdGFP and AdSox9 (Figure 3.7C). AdSox9 transduction was able to induce migration of epicardial cells from both control and $PDGFR^{EKO}$ hearts. This result suggested that in whole heart cultures, Sox9 was sufficient for inducing epicardial EMT but not in isolated epicardial cultures. The myocardium may provide additional cues in vivo. Taken together, these results implicate a role for Sox9 in PDGF receptor dependent EMT and demonstrate that Sox9 can partially rescue the EMT defect caused by PDGF receptor deletion in epicardial cells.

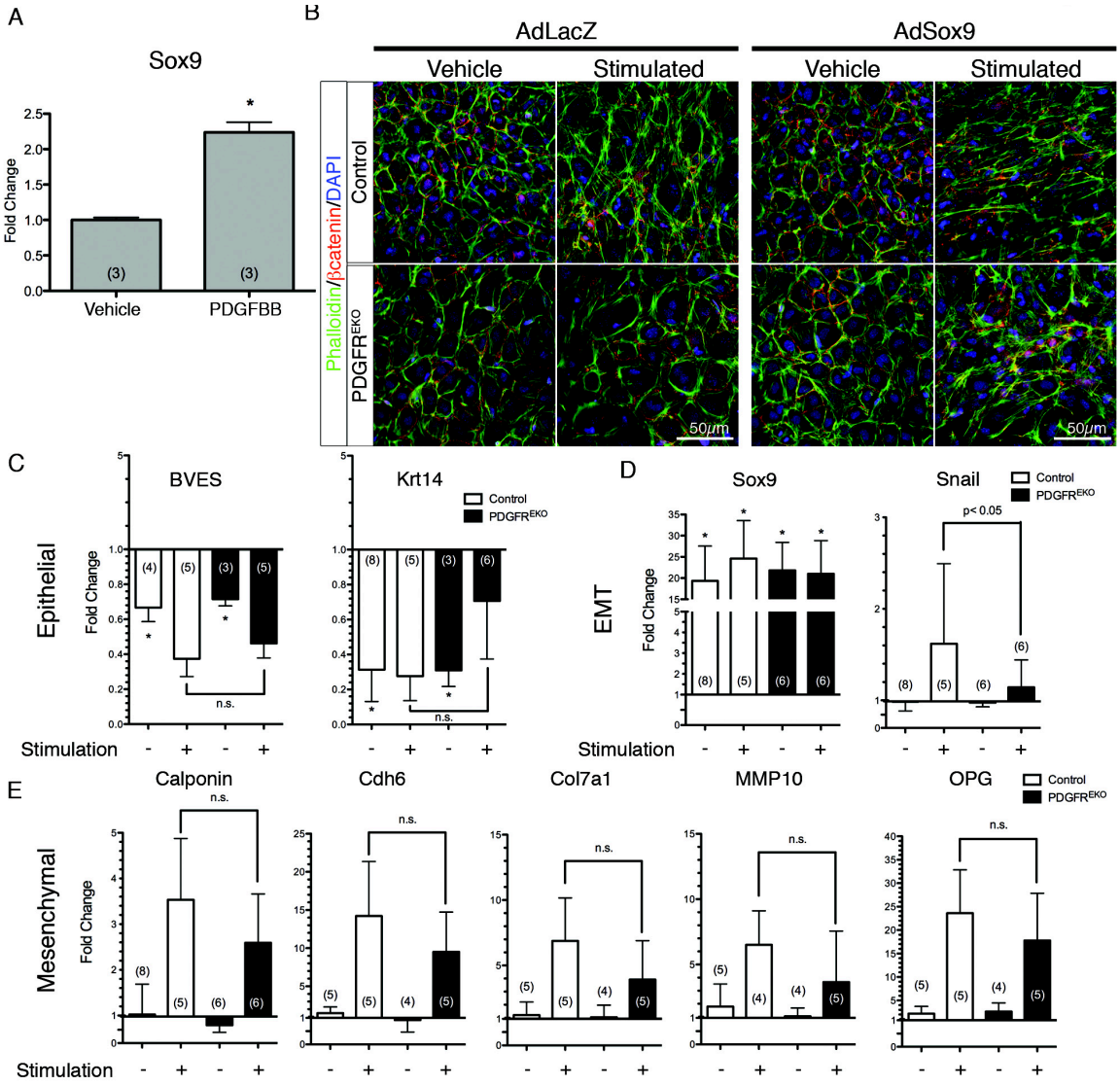

Figure 3.6. Sox9 rescues PDGF receptor mutant phenotypes
(A) qPCR for *Sox9* expression in primary epicardial cultures stimulated with PDGFBB for 24h. (*) p<0.001 (B) βcatenin and phalloidin localization of control and *PDGFR*^{EKO} cultures stimulated with hTGFβ$_1$ and PDGFBB in the presence of adenoviral LacZ or Sox9. (C-E) qPCR analysis of epithelial, mesenchymal, and EMT transcription factors of primary epicardial cultures transduced with adenoviral Sox9. Data were compared to vehicle treated, GFP adenoviral transduced control epicardial cultures (Figure 3.5D-F) (represented as a baseline of 1.0). Data are represented as mean ± SD. N values are indicated in parentheses. (*) p<0.001 (n.s. – no significant difference)

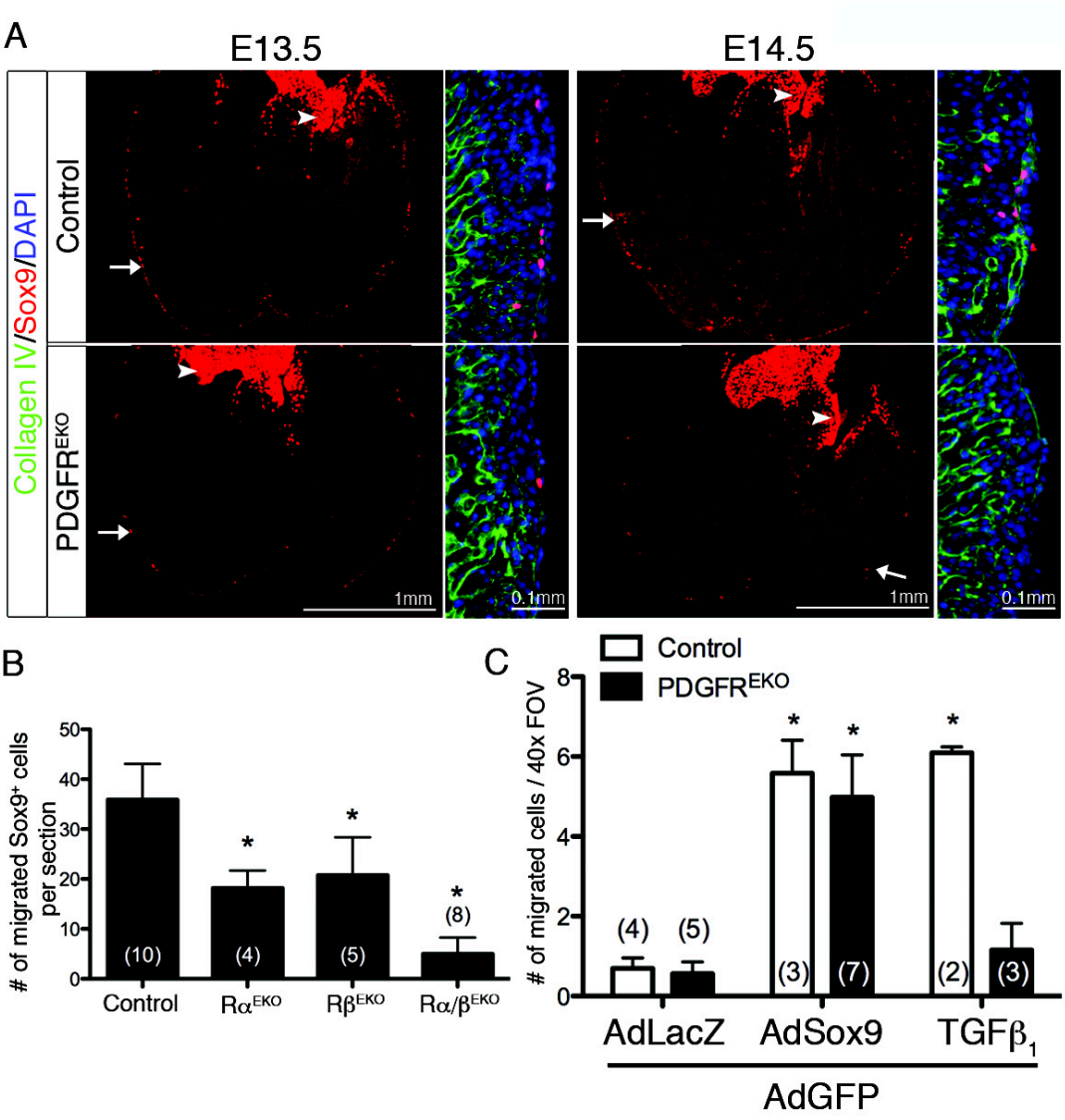

Figure 3.7. Epicardial expression of Sox9
(A) Sox9 expression in hearts of indicated genotypes. Images to the right of each frame are higher magnification of left ventricle. Arrows point to examples of Sox9$^+$ cells. Arrowheads indicate Sox9$^+$ cells in the valves. (B) Quantification of Sox9$^+$ cells within the myocardial ventricular wall at E14.5. Valves, epicardium, and septum were excluded from analysis. (C) Quantification of GFP$^+$ cells within myocardium of E12.5 hearts transduced with indicated viruses and/or stimulation with hTGFβ$_1$. Data are represented as mean ± SD. N values are indicated in parentheses. (*) $p<0.001$ (compared to vehicle treated AdGFP/AdLacZ control)

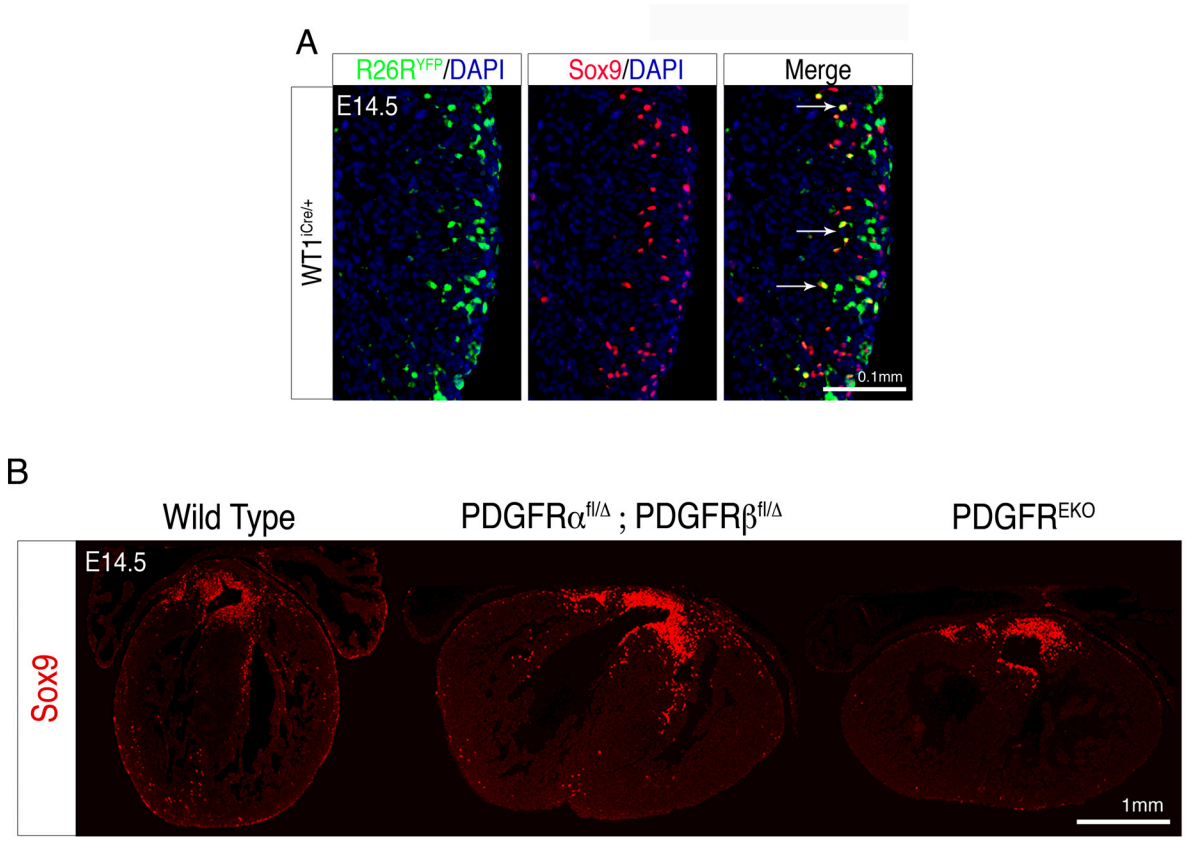

Figure 3.8. Sox9^{+} cells are derived from the epicardium and numbers vary according to genotype
(A) WT1$^{iCre/+}$, R26R^{YFP} lineage traced hearts. Embryos were induced with tamoxifen at E12.5 and costained with Sox9 and GFP antibodies at E14.5. Arrows show overlap of YFP^{+} and Sox9^{+} cells. Note this embryo had less efficient cre induction, as few YFP^{+} cells are present in the epicardium. When Cre induction (at E12.5) is efficient, a high percentage of labeled cells remain in the epicardium (See manuscript). (B) Sox9 expression in the indicated genotypes. We include this image to illustrate that in a wild type (no mutant alleles for the PDGF receptors), we observe even more Sox9^{+} cells than in our controls. Our controls often contained two recombined PDGF receptor alleles (one PDGFRα and one PDGFRβ, designated as Δ) due to Gata5Cre recombination in the male germline.

Loss of PDGFRα leads to an EMT defect in a subpopulation of epicardial cells

Our data suggested that both PDGF receptors are required for EMT, but results from the PDGFRβ epicardial deletion demonstrated a VSMC lineage defect in epicardial development (Mellgren et al. 2008). Therefore, we decided to further investigate the individual role of the PDGF receptors during EPDC development. To obtain a more precise view on PDGF receptor expression overlap, we used flow cytometry. We observed that both receptors were initially co-expressed at early embryonic stages (E13.5) but became mutually exclusive at later stages (E16.5) (Figure 3.9). Using a GFP knock-in allele (Hamilton et al. 2003) to follow cells expressing PDGFRα, GFP expression appeared in most epicardial cells at E13.5 (Figure 3.10A). At E13.5, *PDGFRα*$^{GFP/GFP}$ mutant hearts exhibited epicardial blistering that was milder than *PDGFR*^{EKO} hearts (data not shown). However, this blistering was less severe than that observed in embryos null for a different *PDGFRα* allele (Soriano 1997). The phenotypic differences described for the *PDGFRα* null embryos could be caused by differences in genetic background or by the fact that two of these studies (this report and one by Bax et al (Bax et al. 2010) **)** excluded embryos that had retarded growth from analysis. In regards to the number of GFP^{+} cells in the epicardium, we observed no differences between control and mutant hearts (Figure 3.10A). However, at E17.5, GFP^{+} cells were present within the myocardium of heterozygote hearts, but no GFP expressing cells were observed within the myocardium of *PDGFRα*$^{GFP/GFP}$ hearts. These data suggested that loss of PDGFRα signaling leads to a disruption of a cell population that might arise from the epicardium and is consistent with a recent report showing reduced WT1^{+} cell migration in *PDGFRα*$^{GFP/GFP}$ hearts (Bax et al. 2010).

To determine if the loss of PDGFRα-expressing cells in the myocardium of the null was caused by a failure in PDGFRα dependent cell migration from the epicardium, we deleted

PDGFRα in epicardial cells. *PDGFRα*$^{GFP/EKO}$ hearts showed a reduction of GFP expressing cells within the myocardium, similar to *PDGFRα*$^{GFP/GFP}$ animals (Figure 3.10B). Lineage tracing at E12.5 using an inducible, epicardial specific Cre mouse line (*WT1*^{iCre}) (Zhou et al. 2008) and R26R^{tdT} (Madisen et al. 2010) demonstrated that GFP expressing cells were epicardial derived. In addition, when PDGFRα epicardial function was disrupted, migration of PDGFRα^{GFP} positive cells into the heart was reduced (Figure 3.10C-D).

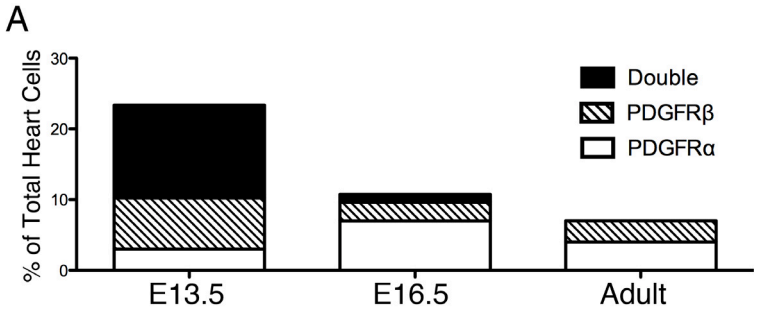

Figure 3.9. PDGF receptor expression during development
(A) Flow cytometric analysis of PDGF receptor expression on cells isolated from embryonic and postnatal hearts with endocardial cushion/valves removal. Antibody detection was used for PDGFRβ while GFP fluorescence was used for PDGFRα expression. (n=1 at E13.5 and n=2 at E16.5 and adult).

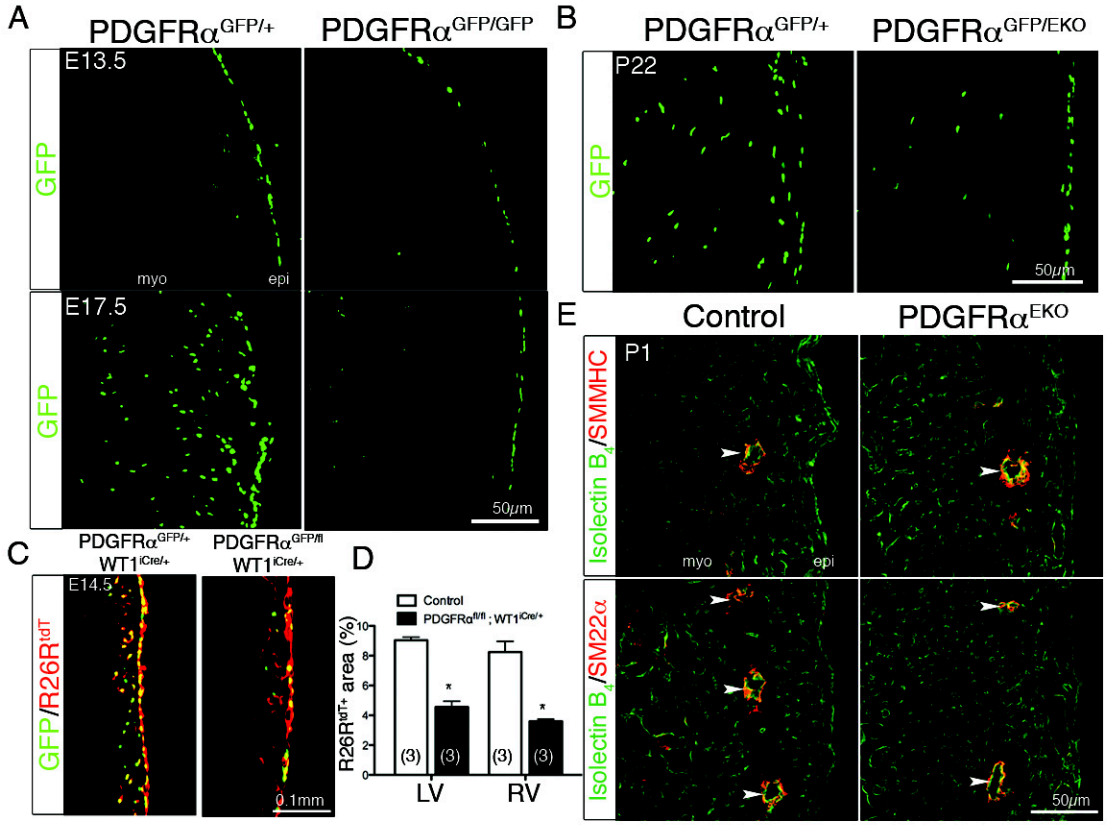

Figure 3.10. PDGFRα epicardial phenotype

(A-B) GFP fluorescence was used to follow PDGFRα expressing cells in the indicated genotypes. (C) Confocal images of $R26R^{tdT}$ and $PDGFR\alpha^{GFP}$ fluorescence of the indicated genotypes induced with tamoxifen at E12.5. (D) Quantification of $R26R^{tdT}$ fluorescent area in (C). (*) $p<0.001$ (E) IHC for coronary endothelial cells (Isolectin B_4) and cVSMC (SMMHC and SM22α). (epi – epicardium, myo – myocardium Arrowheads denote coronary vessels).

***PDGFRα* mutant hearts have a selective loss of cardiac fibroblasts**

We next examined if there was a defect in the formation of epicardial derivatives in the absence of PDGF receptor signaling. Because epicardial EMT was disrupted, we expected aberrant cVSMC and cardiac fibroblast development. Surprisingly, the expected Mendelian ratio of $PDGFR\alpha^{EKO}$, $PDGFR\beta^{EKO}$ and $PDGFR^{EKO}$ mutant animals was recovered at weaning and up to one year after birth. No measurable defects in cardiac size or function were observed (Figure 3.11). Loss of PDGFRβ alone results in an absence of epicardial derived cVSMC, but a secondary population of cVSMC are initially present at the aortic root (Mellgren et al. 2008), which continues to expand as the animals age (data not shown). This rescue may explain why loss of epicardial cVSMC does not lead to lethality. Examination of the endothelial component of the coronary vasculature suggested that patterning of the vessels in the $PDGFR^{EKO}$ hearts was similar to that previously reported for the $PDGFR\beta^{EKO}$ (Mellgren et al. 2008), and that endothelial cell presence within the heart was not disrupted by a lack of EPDCs. We determined the consequences of disrupted epicardial EMT by examining hearts for epicardial derivatives. Staining for cVSMC markers, SM22α, smooth muscle myosin heavy chain (SMMHC), α-smooth muscle actin, and PDGFRβ demonstrated that cVSMC content of $PDGFR\alpha^{EKO}$ hearts was unaffected (Figure 3.10E and data not shown). Because the smooth muscle cell markers that we examined should detect VSMC as well as pericytes, we conclude that loss of PDGFRα does not affect the mural cell lineage. By contrast, $PDGFR^{EKO}$ hearts showed a reduction in all of these markers (data not shown) similar to the loss that we reported in PDGFRβ mutant hearts. These data, in combination with the observed loss of GFP^+ cells, suggested that PDGFRα might be required for the formation of a distinct EPDC population, cardiac fibroblasts.

To determine if PDGFRα was required for cardiac fibroblast formation, we analyzed $R26R^{YFP}$ epicardial lineage tagged hearts for a cardiac fibroblast surface marker, Thy1 (Hudon-David et al. 2007; Ieda et al. 2009; Takeda et al. 2010). Epicardial derived fibroblasts were defined as YFP^+, $Thy1^+$ and $CD31^-$ (Figure 3.12A). Deletion of PDGFRα either individually or in combination with PDGFRβ ($PDGFR\alpha^{EKO}$ and $PDGFR^{EKO}$, respectively) resulted in a loss of epicardial derived cardiac fibroblasts and an absence of YFP^+ cells in $PDGFR^{EKO}$ hearts. Epicardial derived fibroblast numbers in hearts lacking PDGFRβ ($PDGFR\beta^{EKO}$) were similar to controls (Figure 3.12A). Next, we generated primary cardiac fibroblast cultures and traced the epicardial lineage using *Gata5Cre* transgene and a $R26R^{LacZ}$ allele to identify EPDCs. Primary cardiac fibroblasts isolated from $PDGFR^{EKO}$ and $PDGFR\alpha^{EKO}$ hearts had a paucity of epicardial derived cardiac fibroblasts (β-galactosidase$^+$). By contrast, the number of epicardial derived cardiac fibroblasts observed in controls and $PDGFR\beta^{EKO}$ hearts were very similar (Figure 3.12B-C).

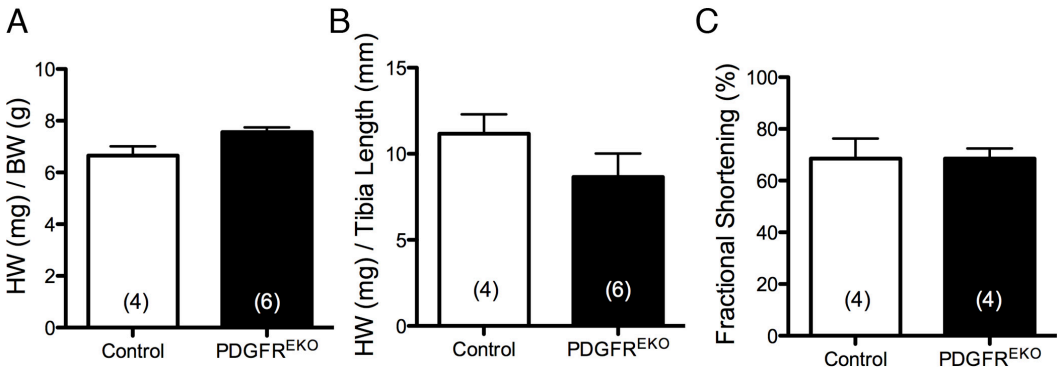

Figure 3.11. Cardiac function in *PDGFR*^{EKO} animals
(A-B) Heart weight / body weight and heart weight / tibia length ratios in 8- to 12-week old mice of the indicated genotypes. (C) Fractional shortening of 8- to 12-week old mice of the indicated genotypes as measured by echocardiography. N values are indicated in parentheses.

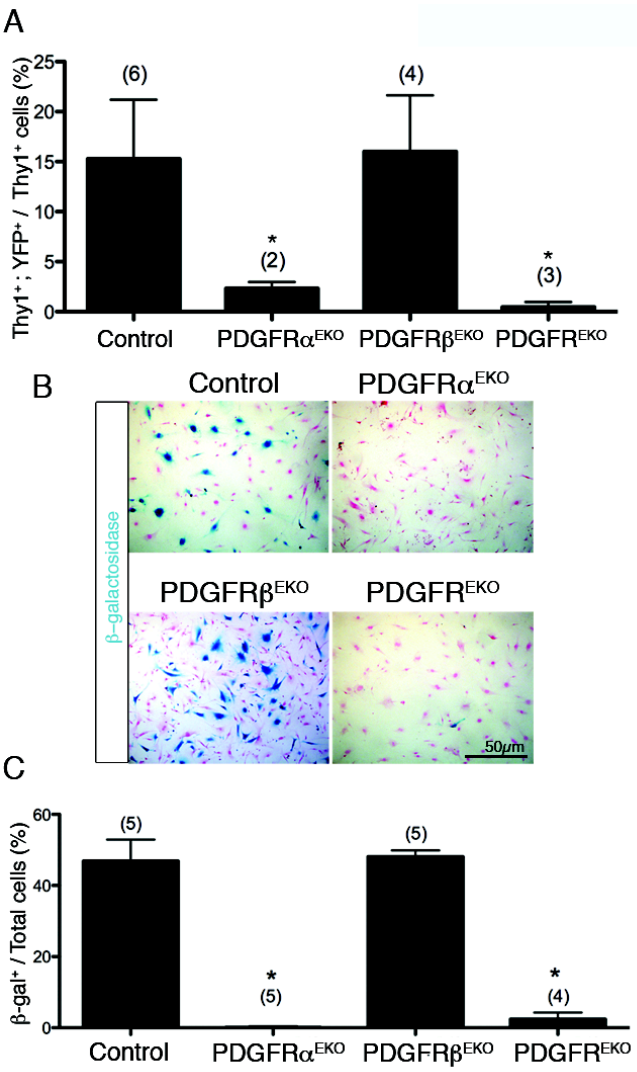

Figure 3.12. PDGFRα is required for epicardial derived cardiac fibroblast formation

(A) Percentage of epicardially-derived cardiac fibroblasts (*Gata5cre*^{Tg}*; R26R*^{YFP}) from P21-P28 *PDGF receptor* mutant hearts. There was a subpopulation of Thy1^{+} CD31^{+} (Hudon-David et al. 2007; Ieda et al. 2009) cells that were excluded from our analysis. (B) β-galactosidase activity (blue) in passage 1 primary cardiac fibroblast cultures using P21-P28 *Gata5cre*^{Tg}*; R26R*^{LacZ} hearts to follow EPDCs. Nuclear fast red was used as a counterstain. (C) Quantification of blue cells in (B). Data are represented as mean ± SD. N values are indicated in parentheses. (*) $p<0.001$ (compared to control)

Because a population of cells grew from *PDGFR*^{EKO} and *PDGFRα*^{EKO} primary fibroblast cultures, we surveyed hearts for overall fibroblast content by detecting transcripts of fibroblast enriched genes. qPCR demonstrated, on average, a 50% reduction in fibroblast gene transcripts in *PDGFR*^{EKO} hearts (Figure 3.13E). We then used *Col1a1* (Figure 3.13A-B) and prolyl-4-hyroxylase β (P4hb) (Kivirikko et al. 1989) (Figure 3.13C-D) to identify individual collagen producing cells. To establish the optimal time point to quantify developing fibroblasts, we examined *Col1a1* and *PDGFRα* expression perinatally. Cells expressing these two genes were evident from E18.5 to P7, but after P7 in situ detection of gene expression appeared to taper off (Figure 3.14A). These data suggested that matrix production by epicardial fibroblasts fell within a very discrete time window. A greater than 50% reduction was observed in the fibroblast population of *PDGFR*^{EKO} at P7 and a reduction in these same fibroblast markers was observed in a *PDGFRα* epicardial mutant at E18.5 (Figure 3.13A-D and Figure 3.14B). These calculations were an over-estimation of remaining fibroblasts as the non-epicardial derived VSMC surrounding the coronary vasculature (Mellgren et al. 2008), the endocardium, and the epicardium also produced collagen (Figure 3.13A). To gain insights into the potential source of the cells in primary cardiac fibroblast cultures, we generated fibroblasts from Tie2Cre;R26R^{LacZ} animals, where all cells of endothelial origin should express β-galactosidase, and found that 21±7% (n=4 cultures) of the prolyl-4 hydroxylase and/or αSMA expressing, adherent cells had an endothelial origin as has been previously suggested (Zeisberg et al. 2007).

To determine if a reduction in fibroblasts resulted in any extracellular matrix (ECM) defects, we examined hearts for levels of periostin (Postn), an extracellular matrix molecule secreted by cardiac fibroblasts (Katsuragi et al. 2004). Periostin expression in *PDGFRα*^{EKO} and *PDGFR*^{EKO} hearts had a marked reduction, while the periostin level in *PDGFRβ*^{EKO} was

unaffected (Figure 3.13F). We also investigated adult mice for generalized defects in matrix deposition. Focusing on perivascular regions, we used Masson Trichrome stain to identify collagen deposition from $PDGFR\alpha^{EKO}$, $PDGFR\beta^{EKO}$, and $PDGFR^{EKO}$ hearts. Deletion of PDGFRα either individually or in combination with PDGFRβ ($PDGFR\alpha^{EKO}$ and $PDGFR^{EKO}$, respectively) led to a reduction in collagen, while mice lacking PDGFRβ ($PDGFR\beta^{EKO}$) in epicardial cells and their derivatives were similar to controls (Figure 3.13G).

These results suggested that cardiac fibroblast development is disrupted in $PDGFR^{EKO}$ and $PDGFR\alpha^{EKO}$ hearts and that these epicardial derived fibroblasts are required for matrix production in the developing heart. In conclusion, our data show a unique role for PDGF signaling in regulating epicardial EMT and fate specification of EPDCs.

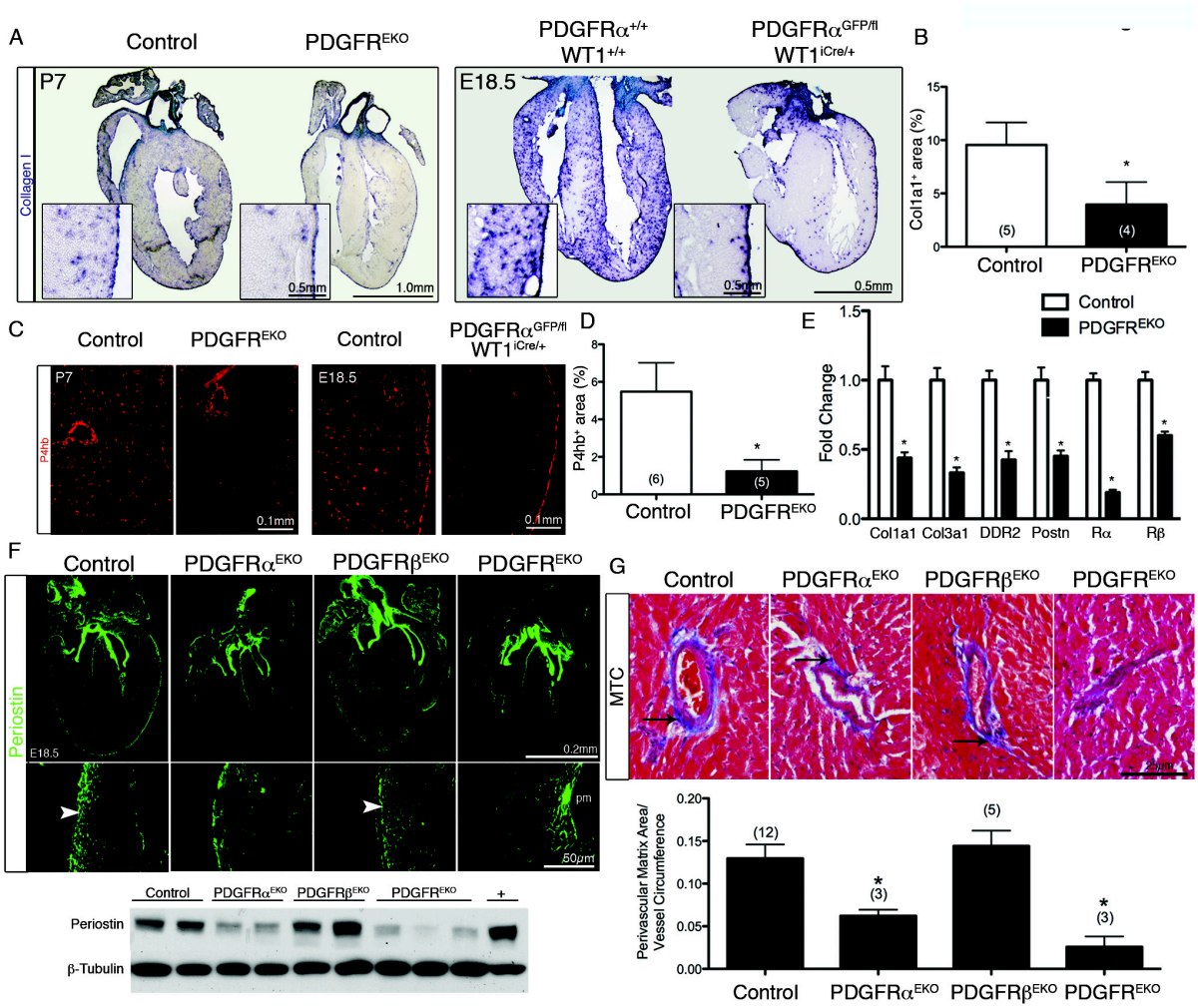

Figure 3.13. PDGFRα is required for cardiac fibroblast development.
(A) *Col1a1* ISH of the indicated genotypes and ages. Insets represent higher magnifications of left ventricle. (B) Quantification of interstitial *col1a1* expression in (A) as described in methods. (*) $p<0.005$ (C) Prolyl-4-hydroxylase β IHC and (D) quantification in the left ventricle of the indicated genotypes. (*) $p<0.001$. (E) qPCR expression levels in the left ventricle of the indicated genotypes (*) $p<0.005$ (F) IHC for periostin in E18.5 hearts (upper panel). (pm - papillary muscle) Arrowheads indicate staining in the ventricular wall. (Lower panel) Western blot for periostin from P1 hearts of indicated genotypes. (G) Masson Trichrome staining of coronary arteries in 8-10 week old adult hearts (upper panel). Perivascular collagen deposition (blue, arrows). (Lower panel) Quantification of perivascular collagen. N values are indicated in parentheses. (*) $p<0.001$ (compared to control)

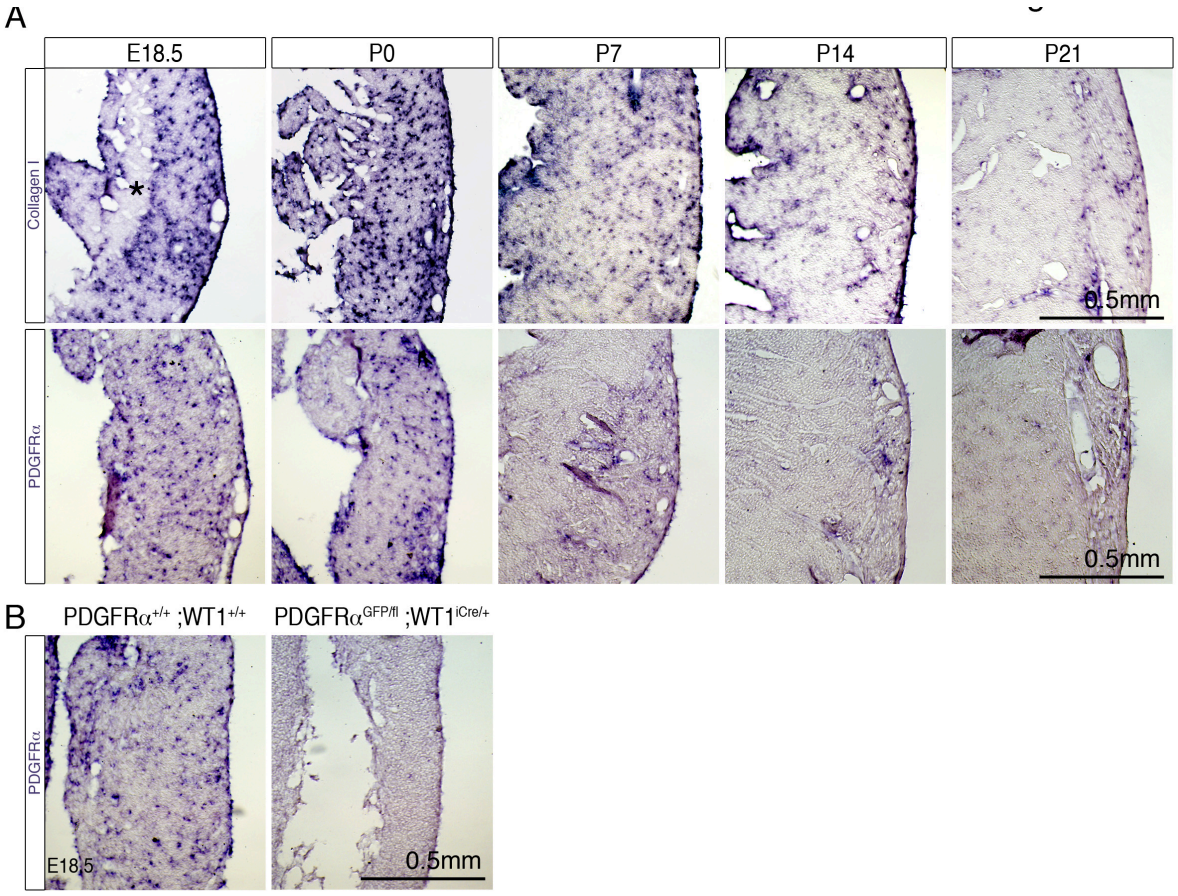

Figure 3.14. Collagen 1a1 expression during postnatal development
(A) *Col1a1* and *PDGFRα* in situ hybridization of left ventricles at the indicated ages. (B) *PDGFRα* in situ hybridization of control and PDGFRα mutant hearts. Timecourse was completed using wild type animals of a mixed background. (*) Indicates lack of staining due to artifact during processing.

Discussion

Since the discovery of the origin of cVSMC and cardiac fibroblasts over 18 years ago, multiple signaling pathways have been identified that affect the formation, attachment, or EMT of the epicardium. However, few genes have been identified that are essential for cardiac fibroblast formation. Here, we have not only identified a unique requirement for PDGF receptor signaling in regulating epicardial EMT and EPDC formation but also have identified an essential role for, PDGFRα, specifically in cardiac fibroblast formation.

Several growth factor signaling pathways have been implicated in the induction of EMT during development and various pathological states, and PDGF has been linked to the EMT process during cancer progression, organ fibrosis (Ostendorf et al. 2006; Fischer et al. 2007; Kong et al. 2008), smooth muscle cell generation from the chicken proepicardium (Lu et al. 2001), and in the regenerating zebrafish heart (Kim et al. 2010). Some suggested mechanisms for PDGF's role in EMT include stabilization of βcatenin (Yang et al. 2006) or induction of transcriptional activators of EMT, such as ZEB1/2 and Snai2 (Kong et al. 2009). However, we do not observe these specific effects in epicardial cells after PDGF stimulation. We have identified the transcription factor, Sox9, as a downstream target of PDGF stimulated EMT. Our data show that Sox9 could rescue the EMT defect seen in PDGF receptor mutants and that Sox9 expression was up-regulated upon PDGF stimulation of epicardial cells. These data are consistent with the known role for Sox9 in avian neural crest cell EMT (Sakai et al. 2006). Sox9 is a member of the SRY related HMG-box family of transcription factors that is important for the development of many tissues and cell types. Cardiovascular defects have been reported in *Sox9* mutants (Bi et al. 2001; Akiyama et al. 2004), but no epicardial phenotype has been described. Our data suggest that Sox9 could be an important component of PDGF receptor signaling during

epicardial EMT, but further investigation is necessary to determine the mechanistic link between PDGF and Sox9.

Our data show that loss of both PDGF receptors led to defective EMT and failure to form any epicardial derivatives. Interestingly, individual deletion of the PDGF receptors also led to reduced epicardial EMT and a loss of only a subpopulation of EPDCs. There are two potential scenarios to explain when PDGF receptor function is required. In the first scenario, epicardial cells are heterogeneous, and each epicardial cell would only give rise to a specific lineage of EPDCs, either VSMC or fibroblast. Here, PDGF signaling might regulate a lineage specific EMT. Consistent with this possibility, experiments using limiting amounts of retrovirus to transduce the proepicardial organ (Mikawa and Gourdie 1996) revealed that tagged cells contributed only to the VSMC lineage. However, there have been no reports suggesting differential gene expression in the epicardium. For example, Tbx18, Tcf21, Raldh2, and both of the PDGF receptors seem to be uniformly expressed in the epicardium prior to EMT. A second possibility for PDGF function is that PDGF signaling by each receptor is redundant in regards to the EMT process, but after EMT, PDGFRα is expressed in fibroblast progenitors, while PDGFRβ is in cVSMC. In this scenario, the most likely role for PDGF receptor signal transduction is expansion and migration of the progenitor population (Betsholtz 2003; Hoch and Soriano 2003). These unanswered questions require further investigation using temporal deletion to identify the window of epicardial fate specification.

The role of cardiac fibroblasts in heart pathogenesis is well appreciated, but the function of these cells during development is poorly understood. It has been proposed that cardiac fibroblasts perform a variety of essential duties during heart formation. These include stimulation of cardiomyocyte proliferation (Ieda et al. 2009), isolation of the ventricular from the

atrial conduction system (Kolditz et al. 2008), distribution of mechanical forces (Krenning et al. 2010), and, of course, deposition and degradation of ECM. Recent estimates are that cardiac fibroblasts comprise about 27% of the cells within the murine heart (Banerjee et al. 2007), but our data demonstrates that these cells are dispensable for heart development. Under non-pathological conditions, mice without epicardial derived fibroblasts lack adventitial collagen, but heart function is normal.

Because many cell populations have been proposed to contribute to fibrosis formation during pathological circumstances, there is the possibility that another source of fibroblasts fills the void. Proposed origins for this substitute fibroblast population include endothelial cells, fibrocytes, monocytes, and mural cells (Krenning et al. 2010). A complete functional substitution by these cells in the absence of epicardial derived fibroblasts is unlikely as mutant hearts that were 8-10 weeks old continued to lack adventitial ECM. Comparison of $PDGFR\alpha^{EKO}$ to $PDGFR^{EKO}$ hearts did suggest a partial rescue of the ECM, presumably by the existing cVSMC, although the levels of matrix never appear to reach wild type levels. While our initial examination suggests no major deficits in mutant animals, further studies are warranted to investigate cardiac homeostasis and other functional parameters such as conduction and stress response.

In summary, we demonstrate that cardiac fibroblast and cVSMC development is mediated by a combined role of the PDGF receptors in controlling epicardial EMT. This process appears to be linked to a lineage specific requirement of the receptors. Specifically, PDGFRα, is essential for cardiac fibroblast development. Finally, we establish a novel role for Sox9 as a critical downstream component of PDGF signaling in regulating epicardial EMT.

Methods

Experimental Animals

Mice were maintained on a mixed C57/Bl6 X 129SV background. The strains in these experiments included *PDGFRα*^{GFP} (Hamilton et al. 2003), *PDGFRα*^{fl} (Tallquist et al. 2003), *PDGFRβ*^{fl} (Richarte et al. 2007; Schmahl et al. 2008), *R26R*^{YFP} (Srinivas et al. 2001), *R26R*^{tdT} (Madisen et al. 2010) (Jackson Labs), *R26R*^{LacZ} (Soriano 1999) *WT1*^{iCre} (Zhou et al. 2008), *Tie2Cre*$^{Tg/0}$ (Kisanuki et al. 2001) and *Gata5Cre*$^{Tg/0}$ (Merki et al. 2005). All animal protocols and experiments were approved by the UTSW IACUC and conformed to NIH guidelines for care and use of laboratory animals. *Gata5Cre* transgenic and *WT1*^{iCre} mice were kindly provided by Dr. Ruiz Pilar-Lozano (Burnham Institute) and Dr. William Pu (Harvard), respectively. *WT1*$^{iCre/+}$ animals were induced with tamoxifen at indicated timepoints. Tamoxifen (MP Biomedicals, 0215673894) was dissolved in sunflower seed oil (Sima, S5007) at 20mg/ml. 0.1mg/g body weight of tamoxifen was administered by oral gavage. Controls used in most experiments were Gata5Cre negative littermates. Animals used for controls in fibroblast isolation(s) were *PDGFRα*$^{fl/+}$ *PDGFRβ*$^{fl/+}$ *R26R*$^{LacZ/+}$ *Gata5Cre*$^{+}$.

EMT

Primary epicardial cultures were stimulated with 10% serum, 1-10ng/ml hTGFβ$_1$ and 20ng/ml PDGFBB for 48 h. For immunocytochemistry, cultures were fixed in 4% paraformaldehyde for 10min. βcatenin (1:500, BD bioscience 610153) and phalloidin (1:200, Invitrogen A12379) were used for staining. For rescue experiments, cultures were treated with adenoviral-GFP, -LacZ (immunocytochemistry) or -Sox9 and treated as specified on the day of explant removal for 48h.

Microarray data can be accessed from the NIH GEO website (http://www.ncbi.nlm.nih.gov/geo/) under the accession number, GSE 27181.

Quantitative PCR

For qPCR analysis, RNA was isolated from three combined hearts for each sample or from two combined cultures for primary epicardial culture analysis using Trizol (Invitrogen). cDNA was generated with 1µg of RNA using SuperScript III (Invitrogen) and random hexamers. Gene expression was analyzed using standard qPCR methods with iTAQ Sybr Green master mix (Bio-Rad) on a CFX96 instrument (Bio-Rad). Each sample was run in triplicate and normalized to cyclophilin. Sequences for primers used in qPCR analyses can be found in Online Table I.

Flow Cytometry

Hearts from E13.5 or E16.5 *PDGFRα*$^{GFP/+}$embryos or 6-week-old adults were dissected and conotruncal/valves were removed. Tissue was digested with 0.25% Trypsin-EDTA (Invitrogen) to generate a single cell suspension. *PDGFRα*$^{GFP/+}$ and *PDGF receptor* mutant adult hearts were digested as described for generating primary cardiac fibroblast cultures. Single cell suspensions were incubated with 1:100 dilution of PDGFRβ-PE (eBioscience, 12-1402-81). For detecting epicardially derived cardiac fibroblasts, single cell suspensions from control and *PDGF receptor* mutant adult hearts were incubated with a 1:100 dilution of Thy1-PE (Invitrogen, MCD9004) and 1:100 CD31-APC (eBioscience, 17-0311-80). All samples were analyzed on a BD FACSCalibur.

Cardiac fibroblast quantification

Masson-trichrome staining was performed on 8-10 week old adult hearts fixed in 10% buffered formalin and paraffin embedded. Hearts were sectioned coronally into 7μm thick sections to generate a four-chamber view and staining was done according to manufacturer's protocol (Thermo scientific, MA). Quantification was conducted by imaging a minimum of 7 different vessels from non-consecutive regions from the lateral ventricles. Collagen deposition was quantified as described elsewhere (Papadopulos et al. 2007) using ImageJ (NIH) and normalized to the vessel circumference.

Collagen 1a1 in situ and P4hb IHC was quantified by measuring the area of positive staining excluding perivascular, endocardial, and epicardial expression using ImageJ (NIH). A minimum of 2 sections per heart was quantified to generate an average for the indicated genotypes.

Cell culture and isolation

Primary epicardial cells were cultured from E12.5 hearts as published previously (Mellgren et al. 2008). Cultures used for immunocytochemistry were plated on glass coverslips coated with 5ug/cm^2 Collagen IV (R&D Systems) and incubated for four days prior to removing explant.

Primary cardiac fibroblasts were isolated from 3-4 week hearts by first removing the atria, aorta, and cardiac valves. The tissue was subsequently minced with forceps and incubated with 0.2U/ml of Blendzyme 3 (Roche) in Earl's buffer (140mM NaCl, 8mM KCl, 1.8mM $CaCl_2$, 0.9mM $MgCl_2$, 25mM HEPES, pH 7.4) for 30min at 37˚C. Cells were washed in PBS and plated overnight in DMEM with 10% serum. Cells were harvested, plated overnight, fixed in 0.2%

glutaraldehyde for 5 min., and stained for β−galactosidase activity. Cells were quantified by imaging a minimum of 3 20x fields of view and counting the number of β−galactosidase^{+} cells/total of cells.

Adenoviral production

AdLacZ and AdGFP were kindly provided by Robert Gerrard (UTSW). AdSox9 was generated from a full-length cDNA (Open Biosystems, 5320371) and cloned into (pAd/CMV/V5-DEST, Invitrogen).

Western Blotting

Protein was isolated from P1 hearts. Positive control protein was generated from differentiated MC3T3-E1 cells (Stock et al. 2003). Western blot membranes were incubated with anti-periostin (1:500, Santa Cruz SC-49480) or anti-β tubulin (1:1000, BD 556321) overnight at 4°C, washed and probed with anti-goat HRP (periostin, 1:1000, sigma) or anti-Mouse IgM (β tubulin, 1:3000, Zymed).

Migration

The ex vivo migration assay was performed as described previously (Mellgren et al. 2008).

Imaging

The following equipment was used for imaging: Fluorescent imaging (Zeis Axiovert 200 with a Hamamatsu ORCA-ER camera), confocal imaging (LSM510META) (grant NIH1-S10-RR019406-01), color imaging (Zeis Axiovert 200 with an Olympus DP71 camera), and whole

mount imaging (Nikon SMZ1000 with an Olympus DP71 camera). Images and figures were edited and created in Photoshop CS4.

Statistics

All statistical calculations were done using Prism 5 (GraphPad). Student t-test, one-way and two-way ANOVA tests were done where appropriate and subsequently analyzed using Tukey and Bonferroni post tests for determining significance.

Proliferation index

Proliferation was measured as described previously with a 1 hour BrdU labeling time (Mellgren et al. 2008).

In situ hybridization

In situ hybridization was done as described previously (Piette et al. 2008). Digoxigenin labeled Tbx18 and Tcf21 probes were synthesized from the plasmids as previously described (Kraus et al. 2001; Lu et al. 2002) and were kind gifts from Sylvia Evans (UCSD) and Ondine Cleaver (UTSW), respectively. Plasmid template for Col1a1 probe generation was a kind gift from Gerard Karsenty (Columbia). PDGFRα was synthesized as previously described (Lindahl et al. 1997).

Chapter IV

Conclusions and Future Directions

My results establish PDGF receptor signaling as a critical determinant of cardiac fibroblast and cVSMC development through controlling epicardial EMT. This process is linked to a lineage specific requirement of the PDGF receptors. Specifically, PDGFRα is essential for cardiac fibroblast development and PDGFRβ is required for cVSMC development. Finally, I showed that a transcription factor, Sox9, is a major component downstream of PDGF signaling that regulates epicardial EMT.

Epicardial fate specification

Since the discovery of the origin of cVSMC and cardiac fibroblasts over 18 years ago, (Mikawa et al. 1992), multiple signaling pathways have been identified that affect the formation, attachment, or EMT of the epicardium. Among these are pathways required for cVSMC generation, including βcatenin (Zamora et al. 2007), TGFBRIII (Compton et al. 2007), Alk5 (Sridurongrit et al. 2008), and WT1 (Moore et al. 1999). However, there are few genes that have been identified that are important for cardiac fibroblast formation. Many loss of function mutations, such as *Postn* (Oka et al. 2007; Shimazaki et al. 2008), *S1P* (Kacimi et al. 2007; Gellings Lowe et al. 2009), and *Klf5* (Takeda et al.), disrupt cardiac fibroblast function, but it is unclear if they directly affect cardiac fibroblast development. Through work I conducted with Amy Mellgren, we have determined that PDGFRβ is another critical factor in regulating cVSMC development. I further identified that that *PDGFRα* is one gene essential for cardiac fibroblast formation. One question that remains from this work is at what stage are EPDC fate determined?

In studying the individual functions of the PDGF receptors, we discovered that loss of each receptor individually only led to reduced epicardial migration. Initially, we

hypothesized that in individual deletions, the other receptor was compensating for the loss, resulting in only a partial migration defect. However, this hypothesis predicts that we would see a reduction in all EPDC populations. We did not observe this; in fact, the loss of each receptor only impacted a specific subpopulation of EPDCs.

There are two potential scenarios to explain these observations, both of which are described in chapter III. Regardless of which scenario occurs, this data challenges the current notion of when EPDC fate is determined. Several growth factor receptors have been implicated in differentiation, but all are thought to work after migration (E15.5) (Olivey and Svensson 2010). However, our data clearly demonstrates that disruption of the EMT (E13.5) process leads to defects in a subpopulation of EPDCs.

To resolve these discrepancies, we must conduct clonal analysis experiments to determine when the cells are specified. Using tamoxifen inducible epicardial cre lines, WT1CreERT2 (Zhou et al. 2008) and TCF21merCremer (Acharya et al. 2011), and recently published fluorescent R26 reporter constructs, confetti and rainbow (Snippert et al. 2010), we can recombine in a subset of cells during different stages of epicardial development. From my data, I would hypothesize that fate specification is occurring during or just prior to EMT. Therefore, I would expect that inducing recombination earlier, at E10.5, would result in both EPDC populations generated from a single cell. By contrast, induction at E12.5/E13.5 I would predict that only one cell population will arise from the same color clone. These experiments are very intriguing and will help determine when the epicardial fate is determined and will provide insight into the therapeutic potential of the epicardium.

PDGF and Sox9 during EMT

Several growth factor signaling pathways have been implicated in the induction of EMT in the epicardium, but this is the first in vivo report illustrating the function of PDGF during EMT, specifically in the epicardium. PDGF has been linked to EMT during prostate (Kong et al. 2008) and liver (Fischer et al. 2007) cancer and during renal fibrosis (Ostendorf et al. 2006). However, these reports only focused on in vitro studies. In addition, how PDGF specifically contributes to these changes is still unclear. Here, I have shown that PDGF receptors are required for epicardial EMT and that a novel transcription factor, Sox9, is a critical downstream component of PDGF signaling in regulating epicardial EMT.

Recently, several mechanisms of PDGF signaling regulating EMT have been proposed. First, regulation of βcatenin stability is crucial during different types of EMT, and one report links PDGF signaling to βcatenin levels by displacing Axin and preventing degradation (Yang et al. 2006). A second possibility is by regulating upstream transcription factors that control epithelial gene expression. Consistent with this mechanism, in prostate cancer cells PDGF signaling can induce known EMT transcriptional initiators, ZEB1/2 and Snai2 by regulating mIR-200b (Kong et al. 2009). However, it is unknown if either mechanism occurs in vivo, and we do not observe these specific effects in the epicardium.

Through gene expression analysis and microarray experiments, I identified Sox9 as a downstream target of PDGF signaling. Sox9 is a member of the SRY related HMG-box family of transcription factors that is important for the development of many tissues and cells types, including cartilage (Bi et al. 1999), testes (Barrionuevo et al. 2006),

cardiac valves (Akiyama et al. 2004; Lincoln et al. 2007), glial cells (Stolt et al. 2003), and neural crest cells (Cheung and Briscoe 2003; Lee et al. 2004; Cheung et al. 2005; Sakai et al. 2006). While cardiovascular defects have been reported in *Sox9* mutants (Bi et al. 2001; Akiyama et al. 2004) no epicardial phenotype has been described.

We found that Sox9 is expressed in the developing epicardium during the epicardial EMT stage and restoring its expression can rescue the PDGF receptor driven EMT defect. These data are consistent with the role of Sox9 in inducing avian neural crest cell EMT (Sakai et al. 2006). However, the link between PDGF and Sox9 is still unclear.

I have shown that one potential mechanism by which PDGF regulates epicardial EMT is through controlling *Sox9* transcript levels and protein, which is consistent with a previous report showing upregulation of *Sox9* after PDGF stimulation in primary astrocytes (Gris et al. 2007). Currently, the mechanism of this regulation remains unclear. However, two well-studied pathways downstream of PDGF signaling can lead to stabilization of *Sox9* transcript levels independent of transcription in chondrocytes (MAPK (Murakami et al. 2000) and ROCK (Tew and Hardingham 2006)). In addition, it was recently shown that inhibition of RhoA and p38 MAPK in the epicardium/proepicardial organ of the chick leads to disruption of EMT (Lu et al. 2001; Compton et al. 2006). It would be interesting to determine if either of these pathways are responsible for the increase in Sox9 transcript and protein levels that we observe in response to PDGF stimulation. Another possibility is that PDGF stimulation might lead to direct phosphorylation of Sox9, establishing an autoregulatory loop that could also account for increased transcript levels (Kumar and Lassar 2009) or increased protein

stability. This potential phosphorylation might also induce interactions with other transcriptional components that can induce EMT (Snai2 (Sakai et al. 2006)). The importance of these signaling pathways during epicardial EMT is poorly understood and must be elucidated by use of pathway specific inhibitors to determine how PDGF regulates *Sox9* transcription and protein levels.

The most important question that remains unanswered is whether or not Sox9 is also required for epicardial EMT. Based on my above work, if Sox9 is a major component of PDGF mediated regulation of EMT, then a loss of Sox9 would show a similar EMT defect to PDGF receptor double mutant animals. Through several attempts, I have discovered that Sox9 is a very stable protein and to achieve conditions to test this hypothesis, primary epicardial cultures are insufficient. Sox9 conditional animals are not readily available., Therefore, we cannot test the in vivo role at this time. However, recent work using cancer cell lines, where the EMT process is better defined, has revealed a similar regulation of Sox9 protein levels in response to PDGF stimulation (Figure 4.1). Further studies will be focused on using these techniques to address whether or not Sox9 is required during the EMT process.

Regardless of Sox9's requirement during EMT, it is still sufficient to rescue the PDGF receptor mutant defect in vitro. Therefore, another question that remains is what potential targets are mediating Sox9's function. To answer this question, a screening based approach needs to be conducted. Using the new CHIP-seq technique technology, we can determine Sox9 targets across the genome without bias. However, from previous studies, it is clear that binding does not always indicate it plays a functional role (Cao et al. 2010). Therefore, we need to compare Sox9 targets in basal epithelial cells and cells

undergoing EMT. In addition, we can determine the activity of the targets by conducting CHIP against known active and repressive histone markers. Finally, a concurrent microarray can be done to determine if this ultimately leads to alteration of the target transcript. This experiment will help identify potential targets of Sox9 that can lead to the PDGF receptor mutant rescue, but further experiments are necessary to see if those genes also play a role in the EMT process.

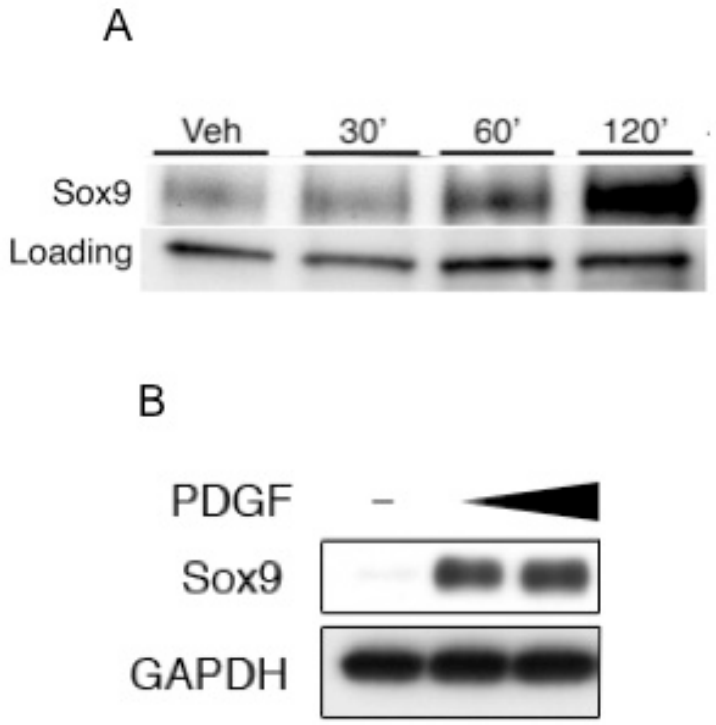

Figure 4.1. PDGF stimulation increases Sox9 protein
(A) Primary epicardial cultures stimulated with 20ng/ml PDGFBB for indicated time points. (B) HT-29 colon cancer cells stimulated with 10ng/ml and 20ng/ml of PDGFBB for 18h.

Physiology

Under non-pathological conditions, the cardiac fibroblast functions to regulate extracellular matrix (ECM) homeostasis and is the major non-myocyte in the heart (Nag 1980). The ECM is essential for structure, connectivity, and mechanical signaling in the heart, and fibroblasts are believed to secrete and organize the majority of the cardiac ECM; the major components are type I and type III collagens(Chapman and Eghbali 1990; Weber et al. 1994)(Baudino et al. 2006). Although important to the normal architecture of the heart's blood vessels and myocardium, these cells are better known for the adverse effects they cause during pathological heart conditions. Congestive heart failure, hypertension, and myocardial infarction are known cardiac insults that can lead to cardiac fibrosis(Brown et al. 2005). This reactive process initially creates stability within the myocardial wall, but eventually leads to reduced contractility, electrical conductivity, and oxygen availability, as well as, myocyte disorganization, and subsequent ventricular dysfunction(Brown et al. 2005). While the contribution of cardiac fibroblasts to heart disease is well established, few studies have aimed to examine the signaling pathways required for development and function of this cell population in vivo. In addition, most cardiac fibroblast functions have only been inferred and have not been directly tested.

I have initially characterized a unique mouse model that can be used to specifically study the impact on cardiac physiology in the absence of the cardiac fibroblast population. Our initial results suggested that in the absense of cardiac fibroblasts (PDGFRα epicardial knockout animals) there was no defect in cardiac hypertrophy or function under basal conditions. This suggests that there might be a second cell population capable of rescuing these defects. However, there was still a

substantial reduction in adventitial collagen, so even if a second population of cardiac fibroblasts were present, they could not rescue the ECM defect.

There are a couple of explanations for the lack of an observable phenotype. First, further study has revealed the cre lines we are using might vary in efficiency between animals leading to varying levels of PDGF receptor deletion. Future studies will need to examine deletion status to determine if this is confounding our results. Second, a much more thorough array of tests is needed to measure function, including higher sensitivity MRI and echocardiography machines. We were also not able to gather a large cohort of animals for those studies and increasing our N value will increase the power of our study. Finally, it is likely that the lack of cardiac fibroblasts might not significantly affect function without cardiac injury or stress.

From initial thoracic aortic banding (TAB) studies, I determined that there was reduced matrix deposition, as expected, in animals lacking EPDC (Figure 4.2). However, the contribution of the cVSMC versus cardiac fibroblasts could not be determined from this study, as both PDGF receptors were deleted. In addition, we did not observe any measurable differences in cardiac function of these animals, despite the presence of a large aneurysm (Figure 4.2). The large aneurysm is consistent with the hypothesis of a lack of fibroblasts leading to a weakened myocardial wall and susceptibility to aneurysm formation. However, this only occurred in 1/6 TAB PDGF receptor mutant animals. Again, further experimentation is needed to increase the power of these initial observations. To remove complications of studying both EPDC populations, they will be conducted in the PDGFRα epicardial mutant mice (lacking only cardiac fibroblasts).

These changes are necessary to accurately and adequately determine the role of cardiac fibroblasts have on cardiac function during cardiac injury.

One interesting aspect we were unable to test was the impact of cardiac fibroblasts on the electrical conductivity of the heart. As previously reported, fibroblasts and myocytes can communicate through gap junctions and this can impact myocyte conductivity (Brown et al. 2005). In addition, collagen acts as an insulator within the heart, and our animals have severely reduced matrix deposition. It will be interesting to determine if this has an impact on the conduction system and detailed ECG analysis will help reveal if there are any defects.

Using these animal models, the contribution of cVSMC and cardiac fibroblasts can be elucidated under basal and pathological conditions. These data will help us to understand the processes that occur during cardiac injury and may lead to better insight into developing therapeutics to reduce the adverse pathological events that occur during cardiac injury.

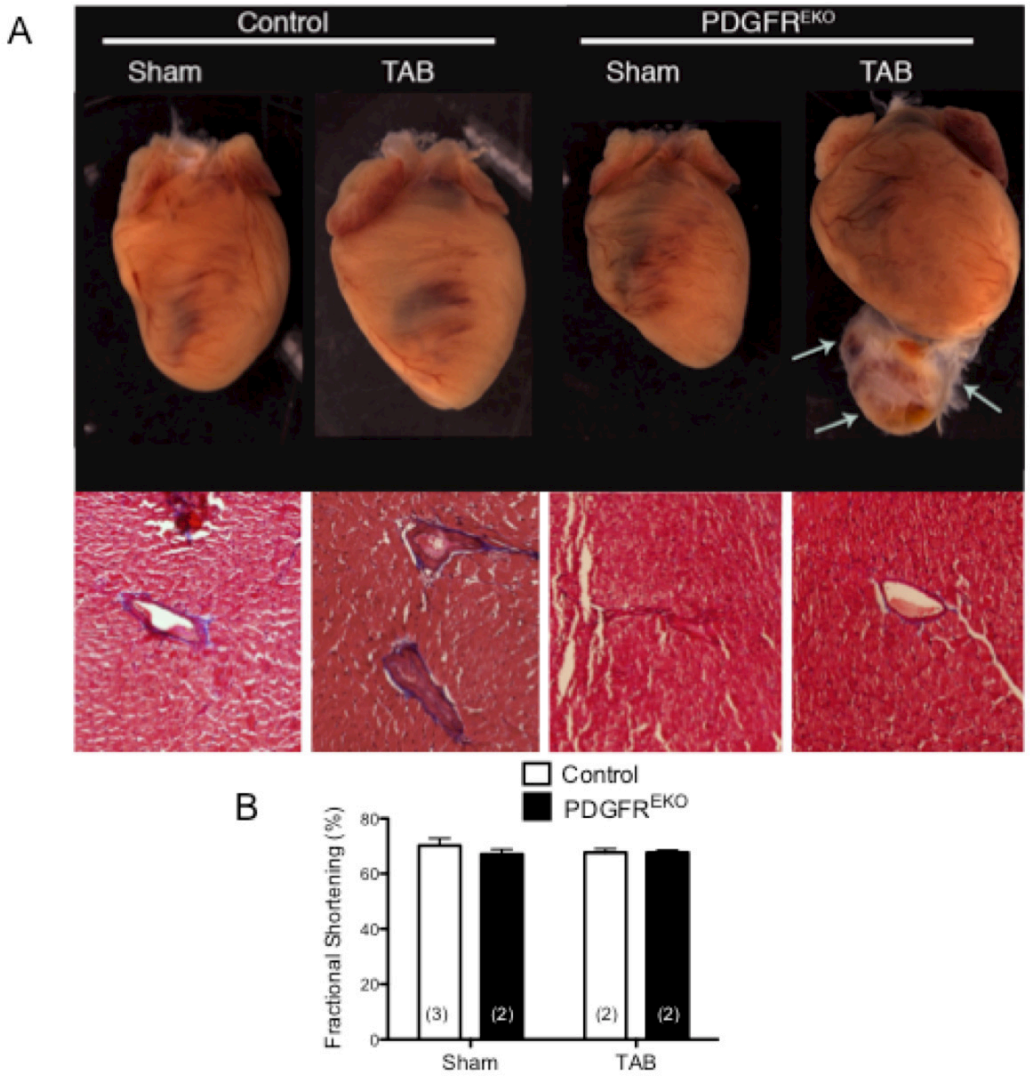

Figure 4.2. Collagen deposition and cardiac function in *PDGFR^{EKO}* animals
(A) Wholemount images of indicated genotypes subjected to thoracic aortic banding (TAB) for 3 weeks. Arrows denote aneurysm. Below, Masson Trichrome staining showing lack of fibrosis in PDGFR mutant animals. (B) Echocardiography data showing no difference in cardiac function.

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