# SRC KINASE REGULATES TGFβ AND HYALURONAN INDUCED EPICARDIAL CELL INVASION, DIFFERENTIATION AND MIGRATION.

By

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# LIST OF ABBREVIATIONS

ALK = Activin-Like Kinase

ASD = Atrioventricular Septal Defect

BMP2 = Bone Morphogenic Protein 2

cdc42 = Cell division control protein 42

ECM = Extracellular Matrix

EMT = Epithelial to Mesenchymal Transition

f-actin = Filamentous Actin

g-actin = Globular Actin

GFP = Green Fluorescent Protein

HA = Hyaluronan

Has2 = Hyaluronan Synthase 2

HEK293 = Human Embryonic Kidney Cells

HMWHA = High-Molecular Weight Hyaluronan

µM = Micro Molar

mL = Milliliter

RT-PCR = Real-Time Polymerase Chain Reaction

Rac1 = Ras-related C3 botulinum toxin substrate 1

RTK = Receptor Tyrosine Kinase

RhoA = Ras homolog gene family, member A

PECAM = Platelet Endothelial Cell Adhesion Molecule

PP2 = Src Kinase Inhibitor

Src= Proto-oncogene tyrosine-protein kinase Src

TGFβ = Transforming Growth Factor Beta

TGFβR3 = Type III TGFβ Receptor

TGFβR3-T841A = Constitutively Active Type III TGFβ Receptor with Threonine 841 Mutated to Alanine

Tβ4 = Thymosin Beta 4

SM22α = Smooth Muscle Actin 22 Alpha

WB = Western Blot

VCAM = Vascular Cell Adhesion Molecule

Y527FSrc = Constitutively Active Src Kinase with tyrosine 527 Mutated to Phenylalanine

# ABSTRACT

The development of the mature cardiovascular system is one of the most captivating stories in embryonic development. The heart is the first organ to form in embryogenesis, and is functional early in development to perfuse the embryo with blood supplying oxygen and the nutrients required for organogenesis. The structural changes in heart development required for formation of the mature four chambered heart are under tight molecular regulation. Severe defects manifest as gross structural malformations of the valves, septa, or vessels that result in physiological consequences that my include hypertension, arrhythmia, or heart failure and may ultimately lead to lethality. According to the American Heart Association, cardiovascular disease is the leading cause of mortality worldwide. A more detailed understanding of the origin of congenital heart defects is necessary for improving prediction, diagnosis, and treatment of cardiovascular disease.

Derived from the epicardium, coronary vessel formation relies on growth factor as well as extracellular matrix (ECM) influences on cells of the epicardium that regulate proliferation, motility, invasion and differentiation. The Transforming Growth Factor β (TGFβ) family of receptors have been well described in regulating cardiovascular development. The Type III TGFβ receptor (TGFβR3) has been shown to be required for development of the coronary vessels. Mouse embryos lacking TGFβR3 exhibit inhibited invasion of epicardially derived cells (EPDCs) into the myocardium. This delay of cell invasion of EPDCs and

formation of coronary vessels is lethal at E 14.5. Relative to *Tgfbr3*$^{+/+}$ cells, epicardial cells lacking TGFβR3 are hypo-proliferative, deficient in cell invasion, and deficient in executing TGFβ ligand and High-Molecular Weight Hyaluronan (HMWHA) stimulated cell invasion. Hyaluronan (HMWHA) is a glycosaminoglycan unmodified sugar extracellular matrix (ECM) molecule synthesized by the Hyaluronan Synthase (Has) family of enzymes. Mouse embryos lacking Hyaluronan Synthase 2 (Has2) are lethal at E 9.5 as a result of severely blocked cardiogenesis due to insufficient endocardial EMT. HA serves structural and bioactive functions in its capacity to stimulate signal transduction pathways required for EMT. Src kinase is a non-receptor tyrosine kinase well characterized to function in growth factor as well as ECM signal transduction, but its role in epicardial cell biology is unclear. **Our hypothesis is that Src kinase is a critical regulator of TGFβ and Hyaluronan induced epicardial cell invasion, differentiation and migration during coronary vessel development.**

Our studies reveal that Src activity is required for TGFβ2-induced synthesis of HA in epicardial cells. We show Src is required for TGFβ2-induced vascular smooth muscle differentiation as well as TGFβ2-induced EMT, cell invasion, and filamentous actin polymerization. Src activity is sufficient to drive epicardial activation of EMT, but not vascular smooth muscle differentiation. These data show that Src is required in the context of TGFβ2-stimulated invasion and differentiation, and sufficient to drive activation of EMT.

Next we demonstrate that TGFβR3 and Src are required for HMWHA-induced cell invasion and filamentous actin polymerization in epicardial cells. HMWHA induces activation of Src kinase in *Tgfbr3*$^{+/+}$ epicardial cells, but not *Tgfbr3*$^{-/-}$ epicardial cells. siRNA knockdown of TGFβR3 in *Tgfbr3*$^{+/+}$ epicardial cells subsequently stimulated with HMWHA phenocopy this deficit in Src activation. *Tgfbr3*$^{-/-}$ epicardial cells fail to activate Rac1 or RhoA GTPases in the presence of HMWHA. Finally, we demonstrate stimulus independent activation of TGFβR3 is sufficient to activate Src. Taken together, these constitute novel findings establishing TGFβR3 as an HMWHA responsive receptor that is upstream of Src signal transduction.

Migration of the epicardium to cover the looped and functioning heart tube is an early step required for development of the coronary vessels. We demonstrate that *Tgfbr3*$^{-/-}$ epicardial cells are delayed in cell migration relative to *Tgfbr3*$^{+/+}$ cells in a wound healing model of cell migration. *Tgfbr3*$^{-/-}$ cells lack expression of BMP2 mRNA, we found that exogenous BMP2 is sufficient to drive *Tgfbr3*$^{-/-}$ (but not *Tgfbr3*$^{+/+}$) cell migration to levels comparable to unstimulated *Tgfbr3*$^{+/+}$ epicardial cells, without enhancing cell proliferation. We demonstrate that Src is required for this BMP2 induced cell migration and filamentous actin polymerization in *Tgfbr3*$^{-/-}$ cells.

These studies demonstrate mechanisms required for TGFβ ligand as well as HMWHA stimulated epicardial cell behavior changes have a common mediator in Src kinase, and provide novel insights into early events in the development of the cardiovascular system. The adult epicardium has been

demonstrated to participate in repair of ischemic myocardium in mouse models of myocardial infarction. Expression of molecules required for coronary vessel development are re-expressed in this regeneration (as discussed in chapter 5). Elucidating these pathways will constitute important future targets in aiding in adult cardiovascular regeneration and cardioprotection in adult heart disease.

# CHAPTER 1

# INTRODUCTION

## 1.1 Cardiovascular Disease

Cardiovascular disease is the leading cause of lethality in the United States and worldwide (1). Heart disease results in 600,000 deaths annually nationwide, and account for 1 out of every 4 deaths (2). Cardiovascular diseases are classified as any pathology affecting the structure and function of the heart, arteries, and veins of the vascular system. Common syndromes affecting the cardiovascular system include hypertension, peripheral arterial disease, and coronary heart disease. Coronary heart disease (CHD) is the most lethal of all cardiovascular pathologies resulting in 380,000 annual deaths (2). CHD can ultimately result in ischemic injury to the heart muscle consequence of a myocardial infarction (MI) that can end in lethality. Recent studies have demonstrated that the adult zebrafish heart has a regenerative capacity and can reactivate molecular pathways required for coronary development in response to myocardial injury (3). There is a limited regenerative function of the mammalian heart to undergo neovascularization and cardiomyocyte replacement in MI (4). Understanding the molecular basis of embryonic heart development will aide in identifying target pathways that may be therapeutically enhanced in cardiac repair.

Malformations of the heart during embryonic development are classified as congenital heart defects. Of all lethal birth defects, 26.6% were the result of

defects of the heart. Non-lethal congenital heart defects are present in 1 out of 150 births in the US (5), and can lead to physiological consequences in adulthood. There is a large body of work demonstrating a developmental basis for adult cardiovascular pathologies as a result of structural malformations of the heart (6). Atrioventricular septal defect (ASD) is characterized as a deficiency of the septa that separates the left and right ventricles. This results in mixing of oxygenated and deoxygenated blood that can lead to hypertension, arrhythmia, and congestive heart failure (7). Trisomy 21 has a high incidence of ASD, and novel candidate genes associated with ASD are emerging (8). Common valvular defects include hypoplasia, atresia, and stenosis resulting from valvular enlargement. Noonan's syndrome characterized by a gain of function mutation in *Ptpn11* (SHP2 phosphatase) resulting in enhanced signaling of receptor tyrosine kinases (9). This pulmonary valve stenosis observed in Noonan's syndrome results in slowed blood flow to the lungs. Attenuating this pathway in a mouse model of Noonan's syndrome is sufficient to rescue the cardiac phenotype (170).

Understanding the molecular basis of heart development and relevance to congenital heart defects and adult cardiovascular pathologies is necessary to improve diagnosis and treatment of cardiovascular disease.

### 1.2 Early Events in Heart Development

The mammalian heart is the first organ to form and is the earliest to function. A population of cells of anterior lateral plate mesoderm migrates and commits to a cardiogenic lineage under molecular influences from the endoderm. This transformation leads to the formation of the cardiac crescent that ultimately

results in formation of the functional heart tube (10). The heart tube consists of two distinct cell layers: the endocardium comprising the endothelial lining of the inside of the heart, and the myocardium that functions in pumping blood through the developing embryo (11). The heart tube undergoes a rearrangement known as looping to give rise to four distinct chambers of the heart, the right and left ventricles separated by the septa, and the right and left atria that are separated from the ventricles by the mature valve leaflets.

The primitive valves and septa arise from a structure known as the atrioventricular and outflow tract cushion during heart looping. Endocardial cells synthesize extracellular matrix in the atrioventricular canal forming the atrioventricular cushion (fig. 1.1). Endocardial cells undergo epithelial to mesenchymal transition (EMT) directed by secreted growth factors from the adjacent myocardium (12). Transformed endocardial cells invade into the atrioventricular cushion, and once resident in the primitive valve, direct valvular remodeling to give rise to the mature valve structures which function throughout the life of the organism (13). Perturbations in EMT or cell invasion may lead to aberrant valve morphologies that often result in physiological consequences.

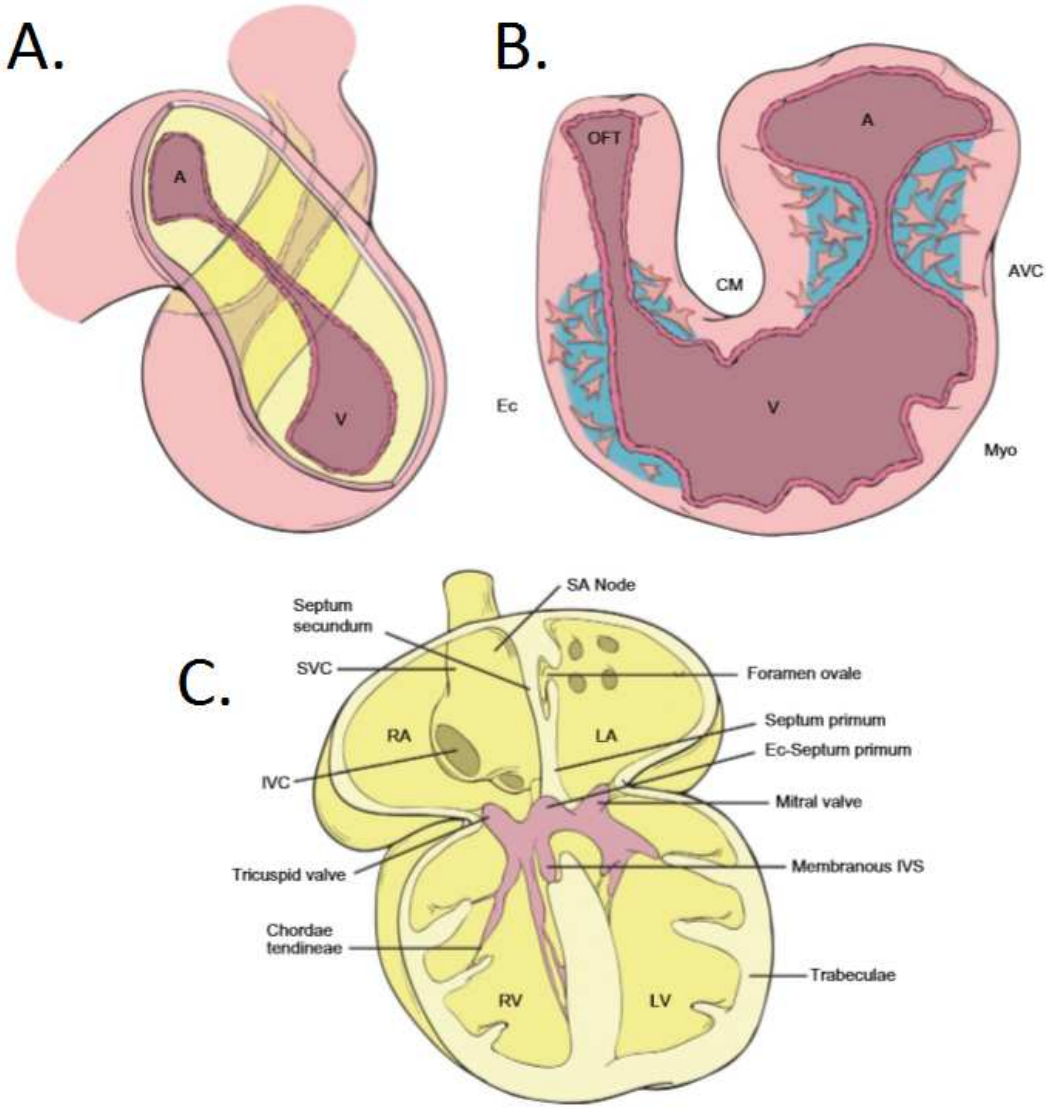


**Figure 1.1 – EMT in valve development.**

(A) The looped heart consists of two layers: myocardial cells (outer pink) endocardial cells (inner red) synthesizing ECM (yellow). (B) Endocardial cells undergo EMT and invasion into the cardiac cushion of the atrioventricular canal (AVC) forming primitive valve leaflets. (C) Mature structures derived from AVC mesenchyme (colored in pink): mitral and tricuspid valves, interventricular septum and septum intermedium. Taken from Harvey and Rosenthal (169).

## 1.3 The Epithelial to Mesenchymal Transition (EMT)

EMT is an important precursor to cell differentiation which results in a diverse array of cell types and expansion of tissues in organogenesis. Epithelial cells are characterized by a definite cell polarity and maintaining tight junctions with other epithelial cells (14). Under growth factor or matrix direction, a population of epithelial cells will lose cell polarity and disassemble tight junctions. This disassembly is known as activation, identified by loss of epithelial cell character. Activated cells may be identified by immunofluorescence visualization of Zona Occludens 1 (ZO-1) or Beta catenin (15), whose expression is localized to tight junctions in epithelial cells, and reorganized in transformed cells. Activated cells will then adopt an elongated morphology, and differentiate into a mesenchymal (fibroblast-like) phenotype. Mesenchymal cells may be identified by expression of ZEB1, Twist, or Snail transcription factors (16), and the intermediate filament vimentin (17). In our experiments, epicardial cells that are elongated and expressing vimentin are considered cells that have undergone EMT. Mesenchymal cells may then undergo cell invasion into adjacent tissues or cell-free matrix. Mesenchymal cells resident in the matrix below the plane of the epithelial monolayer from which they originated are considered to have completed cell invasion in our studies. In cardiogenesis, developmental EMT is documented to occur and be required for development of the valves, septa, and coronary vessels (18).

## 1.4 The Epicardium

The epicardium originates from a group of mesothelial cells known as the proepicardium. Under guidance of pro-migratory secreted growth factors from the myocardium, the epicardium migrates towards and attaches to the myocardium (fig. 1.2A) (19). The epicardium then undergoes proliferation and expansion to cover the heart in an epithelial sheet (20). Secreted growth factors from the myocardium induce the synthesis of extracellular matrix to be deposited into the sub epicardial space. A small subset of epicardial cells undergo invasion through the subepicardial space with some cells continuing into the myocardium (21). Once resident in the myocardium, epicardially-derived cells (EPDCs) undergo differentiation into several cell types including vascular smooth muscle and cardiac fibroblasts. EPDCs then form the capillary plexus to organize structures that will give rise to the mature coronary arteries (22). EPDCs give rise to vascular smooth muscle cells that contribute to the coronary vessels (fig 1.2B). EPDCs also differentiate into cardiac fibroblasts that remain resident in the myocardium that contribute to cardiovascular repair (23).

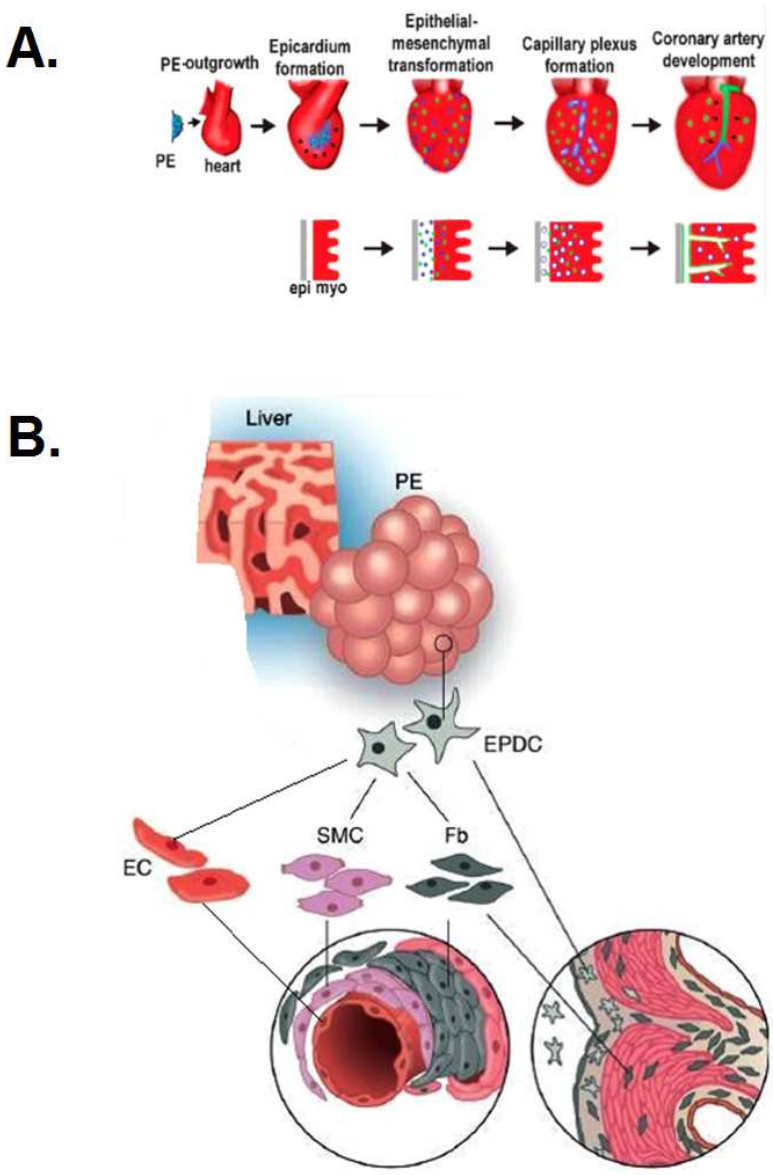


**Figure 1.2- Development and Fate of the Epicardium.**

(A) The epicardium must undergo migration, proliferation, invasion and differentiation to give rise to the coronary vasculature. Adapted from Reese et al (22). (B) Epicardially derived cells differentiate into vascular endothelial cells, vascular smooth muscle cells, and cardiac fibroblasts resident in the myocardium. Adapted from Lie-Venema et al (19).

In avian models of cardiogenesis, the proepicardium contributes to the coronary endothelium (24). Although EPDCs contribution to vascular endothelial cells of the coronary vessels has been demonstrated in mouse cardiovascular development (25), these studies remain controversial as subsequent work has not demonstrated a significant contribution of EPDCs to coronary vascular endothelial cells (26, 27). Work by Red-Horse et al demonstrates the major source of murine coronary endothelial cells arises from the sinus venosus, rather than EPDCs, which gives rise to the coronary plexus (28). There is evidence that epicardially derived fibroblasts and smooth muscle cells contribute to structures of the valves and septa (19), demonstrating a diverse role for the epicardium in cardiovascular development. Although this contribution is observed, whether or not EPDCs are dispensable in valve and septal development remains under investigation.

This complex transformation of the epicardium is required for development of the coronary vessels which will function to perfuse the heart with oxygenated blood and nutrients throughout development and the life of the organism. Several families of growth factor ligand and receptor families have been described in cardiogenesis, the one of primary interest in this study is the Transforming Growth Factor β (TGFβ) family of ligands and receptors.

### 1.5 TGFβ Ligands and Receptors

The TGFβ family of ligands and receptors induce a variety of context-dependent processes in development and adult disease including proliferation, apoptosis, differentiation and cell migration (29). TGFβ1, TGFβ2 and TGFβ3

ligands share high sequence homology. Upon proteolytic cleavage and secretion of active TGFβ ligands, they may bind to the Type I and II (TGFβRI, II) receptors or Type III (TGFβR3) receptor. Transphosphorylation of TGFβRII and I receptors occurs upon ligand binding, subsequently activating multiple cell-signaling cascades that are separated into two types: Canonical (Smad-dependent) and Non-canonical (Smad-independent) pathways.

Upon stimulation with TGFβ ligand, Type II and Type I TGFβ receptors associate with and phosphorylate Smad2/3 proteins, phosphorylated Smads will complex with Smad4, the common mediator Smad (fig. 1.3) (30). This complex will translocate to the nucleus to interact with transcriptional co-activators to execute gene transcription related to proliferation, differentiation and migration. The canonical pathway has been studied in TGFβ dependent processes in development, as well as cancer progression. A growing body of work is revealing the importance of non-canonical aspects of TGFβ signaling for cell differentiation and migration (31).

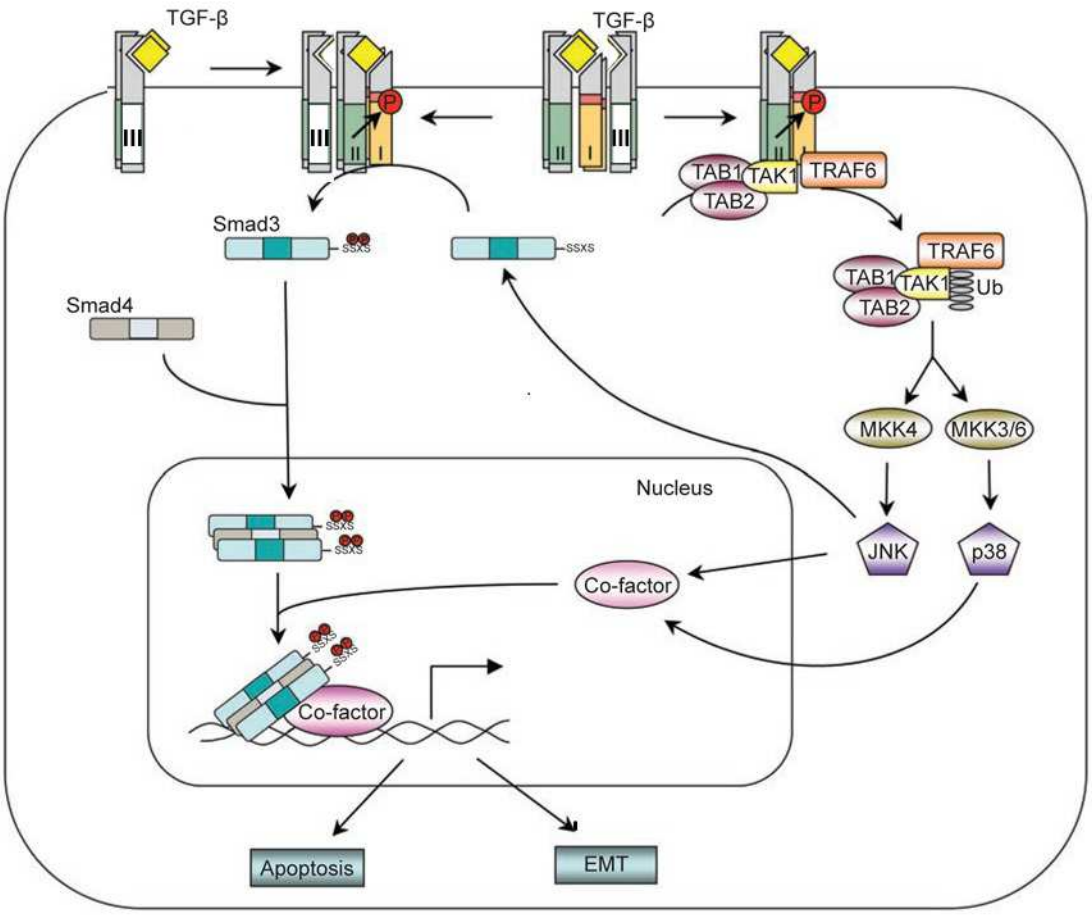


**Figure 1.3- Canonical and Non-canonical TGFβ signaling.**

TGFβ ligands execute canonical (Smad-dependent) and non-canonical (Smad-independent) signal transduction pathways through Type III, II, and I TGFβ receptors. Adapted from Zhang et al (31).

Activation of non-canonical TGFβ signaling pathways are independent of the Smad family of proteins. TGFβs induce the ras/raf/MEK/ERK pathway which is required for TGFβ-induced EMT (31). TGFβs can induce reorganization of the actin cytoskeleton through RhoA (32) and cdc42 (33) GTPases, contributing to TGFβ-dependent cell motility. TGFβs are also potent activators of the Mitogen Activated Protein Kinase (MAPK) pathway through TAK1 and subsequent stimulation of MKK4, which activates JNK that can act synergistically with the canonical pathway, and MKK3/6 upstream of p38 MAPK activation shown to be important in invasive cell motility in TGFβ dependent cancers (31, 34). Activation of Src kinase by TGFβ ligands and receptors is less well understood, though a critical phosphorylation of Y284 of TGFβRI is required for Src and subsequent p38 MAPK activation by TGFβ1 in a human breast cancer cell model (35). Furthermore, TGFβ1 activation of Src requires TGFβRI (ALK5) kinase activity in porcine ventricular myofibroblasts (36).

Roles for TGFβ ligands and receptors have been defined for EMT in heart development. Conventional deletion of TGFβ2 in mice results in valve and septal malformations consistent with altered EMT (37). TGFβ1 and TGFβ3 knockout mice lack a cardiac phenotype (38, 39), highlighting a non-redundant role for TGFβ2 in endocardial EMT. Previous work in our laboratory demonstrates TGFβ2-stimulated epicardial cell invasion requires induction of Has2 expression and HA synthesis (40). Smad4 is required for TGFβ-induced epicardial cell invasion (41), but the requirement of non-canonical effectors in this process is not yet clear.

Study of TGFβ receptors in cardiogenesis is challenging as several knockout phenotypes are embryonic lethal prior to complete cardiovascular development. Investigators have pursued tissue specific knockouts in order to define the role of specific receptors in heart development. ALK2 deletion in neural crest derived cells results in outflow tract abnormalities and dysregulated formation of the aortic arch (42). Endothelial specific knockout of ALK2 results in valve and septal defects as a consequence of insufficient endocardial EMT (43). ALK3 deletion in the myocardium (44) and endothelium (45) result in defective atrioventricular cushion EMT, the endothelial knockout phenotype being most severe. ALK5 is not required in the myocardium for proper cardiogenesis, but ablation of ALK5 in the endothelium results in loss of endocardial EMT, and epicardial specific knockout results in decreased formation of coronary vessels and insensitivity to TGFβ-induced EMT *in vitro* (46).

Bone morphogenic proteins (BMPs) are members of the TGFβ superfamily of ligands that are most well characterized as inducers of bone formation and osteogenesis (47). BMP ligands first bind Type I then Type II receptors stimulating receptor transphosphorylation, subsequent phosphorylation and activation of Smad 1/5/8 canonical signaling effectors (47). Although genomic deletion of BMP2 is embryonic lethal at E7.5-8.5, conditional deletion of BMP2 in the myocardium blocks AVC mesenchyme formation, and results in decreased expression of TGFβ2 and Has2 (48-50). The Par6/RhoA pathway is required for BMP2-stimulated endocardial cell invasion (51, 52). BMP2 is been shown to be a directional signal in epicardial attachment to the myocardium in

chick (53). BMP2 requires Src for expression of target gene *Idl1* in cancer (54) and regulation of cell invasion, and cdc42 augmentation of actin cytoskeleton (55). These findings highlight the emerging importance of non-canonical signaling in BMP2-stimulated cell transformation, invasion, and migration. The role of Src in this process is not well defined in the context of BMP2-dependent EMT and cardiovascular development.

TGFβRII is required for chick endocardial EMT and cell invasion (56), endothelial-specific knockout of TGFβRII in mouse shows a deficit in endocardial EMT in collagen gel based assays (57). TGFβRII is required and sufficient for endocardial EMT in both chick and mouse models of valve development (58, 59). In humans, single point mutations in both TGFβRI and TGFβRII have been associated with including Loeys-Dietz syndrome and Marfan syndrome II (60, 61), this highlights a clinical relevance of TGFβ signaling in adult cardiovascular pathologies. The requirement for Type I and II TGFβ receptors has been studied in endocardial EMT, the requirement of Type III TGFβ receptor in cardiovascular development had not been elucidated until relatively recently.

## 1.6 Type III TGFβ Receptor

TGFβR3 is most well characterized in functioning in ligand presentation to TGFβRI and TGFβRII complexes to stimulate canonical TGFβ signaling cascade (41, 62). TGFβR3 is a transmembrane receptor that consists of a ligand binding domain, and a cytoplasmic domain required for directing epicardial cell invasion through GIPC (63), indicative of a non-canonical intracellular signaling role of TGFβR3. TGFβR3 is required and sufficient for avian endocardial EMT and cell invasion (64). It was later demonstrated that TGFβR3 is required for BMP2 and TGFβ2-stimulated chick endocardial EMT and invasion requires canonical effectors Smad1 and Smad4 (65), and activation of the Par6/RhoA non-canonical pathway (51).

In order to assess the role of TGFβR3 in embryonic development, TGFβR3 was deleted in mouse. The *Tgfbr3*$^{-/-}$ phenotype is embryonic lethal at E14.5 due to insufficient formation of the coronary vessels (66). Visualization of the coronary vessels by PECAM staining reveals normal formation of the coronary vasculature in E13.5 *Tgfbr3*$^{+/+}$ hearts (fig 1.4 A). In contrast, complete formation of the coronary vasculature is absent in hearts of *Tgfbr3*$^{-/-}$ E 13.5 embryos (fig 1.4 B). Wilm's-Tumor Suppressor 1 (WT1) is a marker for EPDCs, and staining for WT1 in *Tgfbr3*$^{+/+}$ hearts reveal EPDCs resident in the myocardium, indicative of cell invasion of EPDCs (fig 1.4C, E). In *Tgfbr3*$^{-/-}$ embryos, EPDCs are shown to retain their ability to attach and cover the

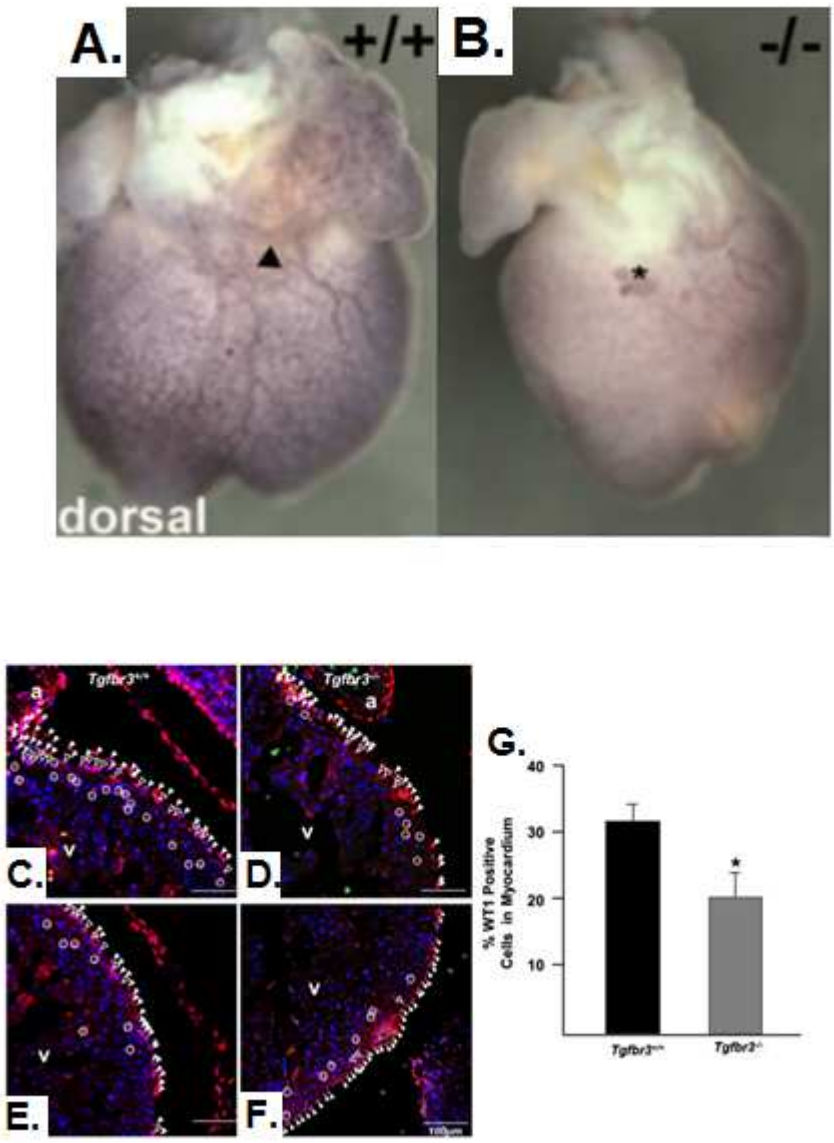


**Figure 1.4- TGFβR3 is required for coronary vessel development.**

(A-B) PECAM (CD31) staining of E14.5 *Tgfbr3*$^{+/+}$ (A) and *Tgfbr3*$^{-/-}$ (B) hearts for visualization of the coronary vasculature. Adapted from Compton et al (66). (C-F) WT1 staining of E13.5 *Tgfbr3*$^{+/+}$ (C, E) and *Tgfbr3*$^{-/-}$ (D, F) heart for visualization of EPDCs resident in myocardium. (G) Quantitation of WT1 positive EPDCs resident in the myocardium of *Tgfbr3*$^{+/+}$ (black bar) and *Tgfbr3*$^{-/-}$ embryos (gray bar). Adapted from Sanchez et al (63).

myocardium, but are delayed in cell invasion into the myocardium (63) (fig 1.4D, F). *In vitro, Tgfbr3*$^{-/-}$ epicardial cells do not undergo cell invasion in response to TGFβ1, TGFβ2 or BMP2, and exhibit delayed two dimensional cell motility in a wound healing model of cell migration (63, 41). Although a deficit in cell invasion is detected, *Tgfbr3*$^{-/-}$ cells undergo EMT and differentiation into vascular smooth muscle cells as observed in *Tgfbr3*$^{+/+}$ cells. BMP2 requires TGFβR3 and canonical effectors Smad1 and Smad4 for epicardial cell invasion, though the importance of non-canonical pathways has been less studied (41). BMP2 does not induce epicardial smooth muscle differentiation, is inhibitory to TGFβ2 induction of smooth muscle differentiation, and does not require TGFβR3 to induce epicardial EMT. *Tgfbr3*$^{-/-}$ epicardial cells fail to undergo cell invasion in response to high-molecular weight hyaluronan (HMWHA). This is an unexpected finding, in that TGFβR3 is thought to be required for TGFβ ligand binding only, and has not been previously reported to be important for cell responsiveness to extracellular matrix.

Additional roles for TGFβR3 are beginning to be elucidated. TGFβR3 binds FGF2 (67), functioning as an FGF co-receptor and enhancing FGFR1 mediated cell differentiation (68). *Tgfbr3*$^{-/-}$ epicardial cells fail to undergo cell invasion in the presence of FGF2 relative to *Tgfbr3*$^{+/+}$ cells (63). TGFβR3 regulates cancer cell migration, through augmenting filamentous actin polymerization via TGFβ1 dependent activation of Rac1 and cdc42 GTPases (69). Substitution of Threonine 841 for Alanine in TGFβR3 prevents β-Arrestin binding to TGFβR3, resulting in inhibited receptor internalization and relief of

NFκB activity suppression; though TGFβR3-T841A has no effect on ligand independent activation of the canonical effector Smad2 (70, 71). This activating substitution in TGFβR3 confers ligand-independent epicardial cell invasion (Barnett Unpublished). These observations suggest that non-canonical TGFβR signal transduction may contribute to induction of ligand stimulated epicardial EMT and cell motility which we address in Chapters 2 and 4.

### 1.7 Hyaluronan

Hyaluronan (HA) is a long-chain glycosaminoglycan polymer extracellular matrix molecule synthesized by the hyaluronan synthase family of enzymes (Has1, Has2, Has3) (72). HA serves structural as well as biochemical signaling functions in the developing embryo. HA is composed of alternating N-D-acetyl glucosamine and D-glucuronic acid and retains a negatively charged state (73). Degradation of HA whole embryo *in situ* cultures results in ablation of the endocardial cushion mesenchyme (74). The Has2 enzyme is considered to be the major source of HA biosynthesis during development (75). In order to assess the role of Has2 in embryonic development, Has2 was deleted in mouse. The mouse *Has2*$^{-/-}$ phenotype is embryonic lethal at 9.5 days of gestation due to blocked cardiac development as a consequence of deficient EMT (76). Whole-mount PECAM staining of *Has2*$^{-/-}$embryos (fig. 1.5A, B) reveal insufficient heart looping and inhibited vasculogenesis relative to *wild-type*. HABP staining of *wild-type* and *Has2*$^{-/-}$ atrioventricular cushions show *Has2*$^{-/-}$ lack hyaluronan and exhibit severely inhibited cardiac cushion formation relative to *wild-type* (fig. 1.5C, D). Atrioventricular cushion EMT assays show that *Has2*$^{-/-}$

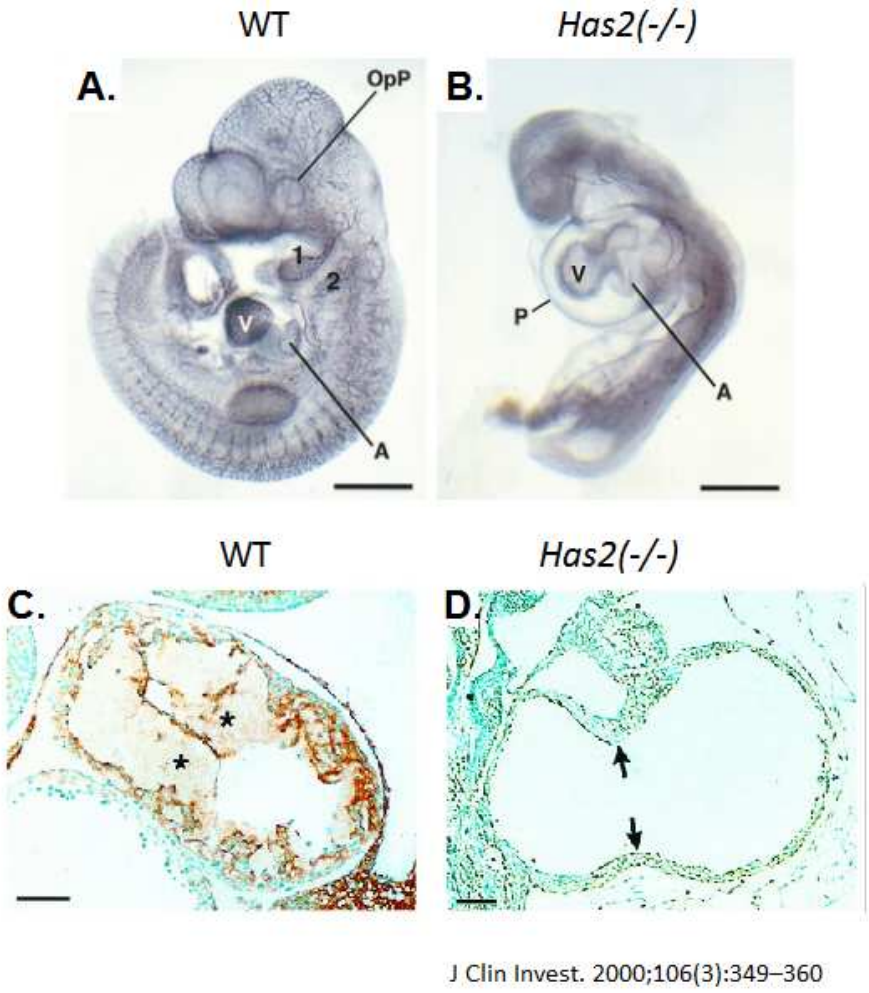


**Figure 1.5 – Has2 and Hyaluronan are required for cardiovascular development.**

(A-B) Whole mount PECAM (CD31) staining of E9.5 *Wild-type* (A) and *Has2*$^{-/-}$ (B) embryos to visualize vasculature. A=Atria, V=Ventricle, P=Pericardium, OpP=Otic placode, 1, 2= First and second pharyngeal pouches. (C-D) HABP staining (brown) of E9.5 *Wild-type* (C) and *Has2*$^{-/-}$(D) to visualize Hyaluronan deposition in atrioventricular cushions. Adapted from Camenisch et al (77).

explants fail to undergo endocardial EMT, addition of exogenous HA was sufficient to rescue this phenotype. Thus, Has2 synthesis of HA is required for endocardial EMT and cardiac development.

Glycosaminoglycans are present during epicardial attachment to the myocardium and in the subepicardial space (78). As the *Has2*$^{-/-}$ phenotype is embryonic lethal before the formation of the epicardium, cellular techniques are required to assess the role of HA in the development of the coronary vasculature. Previous work has shown that HA is required for TGFβ2-induced epicardial EMT and cell invasion (40). HMWHA, but not low-molecular weight hyaluronan, is sufficient to drive cell invasion, and only partially requires the receptor cluster determinant 44 (CD44) (79). This observation suggests that there are other HA-responsive receptors independent of CD44 required for epicardial cell invasion. Taken together, these data suggest HA is obligate for epicardial EMT, but that further investigation is required to uncover signaling pathways required for HA stimulated epicardial cell invasion.

HA engages CD44 to stimulate intracellular signaling required for induction of EMT and cell invasion (80, 81). HA signaling through CD44 can stimulate Ras/Raf/MEK/ERK, Rho GTPases, and c-Src pathways to modulate HA-dependent cell motility and augmentation of the actin cytoskeleton (fig. 1.6). MEKK1 and ERK1/2 are required for HMWHA-induced epicardial EMT and cell invasion in a CD44 dependent fashion (79). HMWHA-stimulated NFκB activity is required for epicardial cell invasion and partially requires CD44 (80). HMWHA-mediated intracellular signal transduction executed through CD44 can enhance

canonical TGFβ Type I receptor signaling (82). Previous work in our laboratory describes that TGFβR3 is required for HMWHA-stimulated epicardial cell invasion (63), though a molecular explanation for this requirement has not yet been elucidated. HMWHA is a driver of Src-dependent cell motility via activation of Rho GTPase family members and filamentous actin polymerization in several tumor-derived cell lines (83, 84). A role for Src kinase in HMWHA-stimulated epicardial cell invasion and differentiation has not yet been investigated.

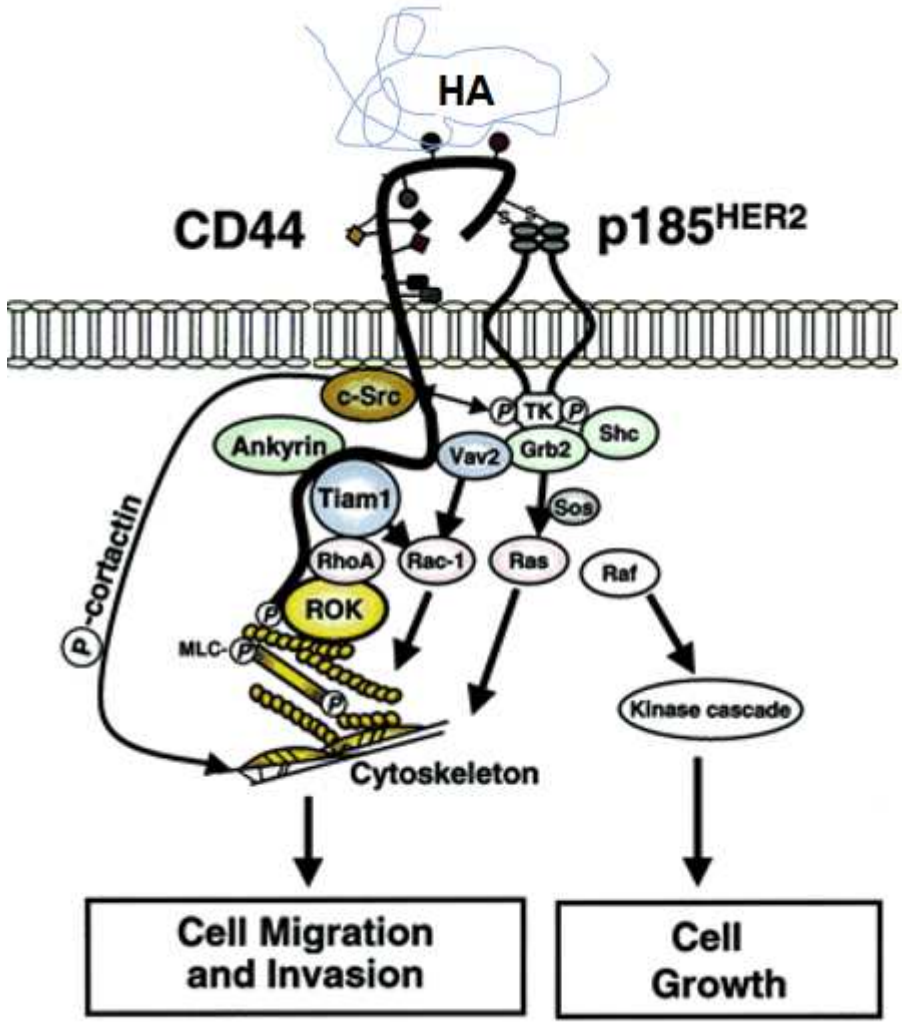


**Figure 1.6 – HA Receptors and Signal Transduction.**

Hyaluronan stimulates intracellular signaling cascades required for migration, invasion, and proliferation through its receptor CD44 and associated receptors including Her2 receptor tyrosine kinase. CD44 is catalytically inactive, but acts as a scaffolding molecule for signaling effectors required for migration as well as interacting with elements of the actin cytoskeleton. Adapted from Turley, et al (85).

### 1.8 Src Kinase

The Avian Rous Sarcoma Virus (RSV) was the first virus described to cause cancer (86). RSV causes fibrosarcoma, characterized by malignant tumors arising from mesenchymal fibroblasts. The primary driver of RSV induced cancer was discovered to be a tyrosine kinase, subsequently named v-Src (Viral-Sarcoma). v-Src is an oncogene, as it confers uncontrolled cell proliferation and an invasive phenotype in cancer progression (87). c-Src (Cellular Src) is the human homologue of v-Src, and is considered a proto-oncogene as overexpression or mutation of c-Src contributes to carcinogenesis in humans (88). Once v-Src and c-Src had been sequenced, putative regulatory tyrosines were identified. Phosphorylated Y527 of Src negatively regulates Src kinase activity (89), loss of this tyrosine leads to constituitive activation of the kinase. v-Src lacks a portion of the C-terminal domain containing 527 tyrosine (90), conferring uninhibited kinase activity.

The Src family of kinases (SFKs) are ubiquitously expressed non-receptor tyrosine kinases that modulate a variety of context-dependent functions of cell behavior. Src consists of SH2 (phospho-tyrosine recognition) and SH3 (proline-rich domain recognition) domains required for subsrate interactions, and a kinase domain required for substrate phospohorylation (91). There are two tyrosine residues important for regulating Src kinase activity: Y527 and Y416. Phosphorylation of Y527 is inhibitory to Src activity (absent in v-Src) since the SH2 domain binds pY527 maintaining the enzyme in an inactive confirmation

(89). Upon dephosphorylation of pY527 by protein phosphatases, autoinhibition is releived, followed by Src SH2 recognition and binding to target substrates. Phosphorylation of Y416 activates Src by displacing Y416 from the kinase active site binding pocket to allow Src to phosphorylate substrates (92).

c-Src activity is localized to the plasma membrane via 14-C myrostylation (93). This localization of Src provides for close proximity to cell-surface transmembrane receptors to support signal transduction. Src activity can be stimulated by several growth factor receptors and receptors that interact with elements of the extracellular matrix (fig. 1.7). Src is activated by, and subsequently enhances the signaling of receptor tyrosine kinases (RTKs) (fig. 1.7). Src associates with activated RTKs such as ErbB and FGFRs via SH2 binding to phosphorylated tyrosines on intracellular domains to enhance mitogenic and migratory signaling (94, 95). Integrins are transmembrane receptors that bind an array of substrates including VCAM, Fibronectin, and Collagen (96). Integrin-dependent cell motility requires Src kinase-mediated activating crosstalk with Rho GTPases (97).

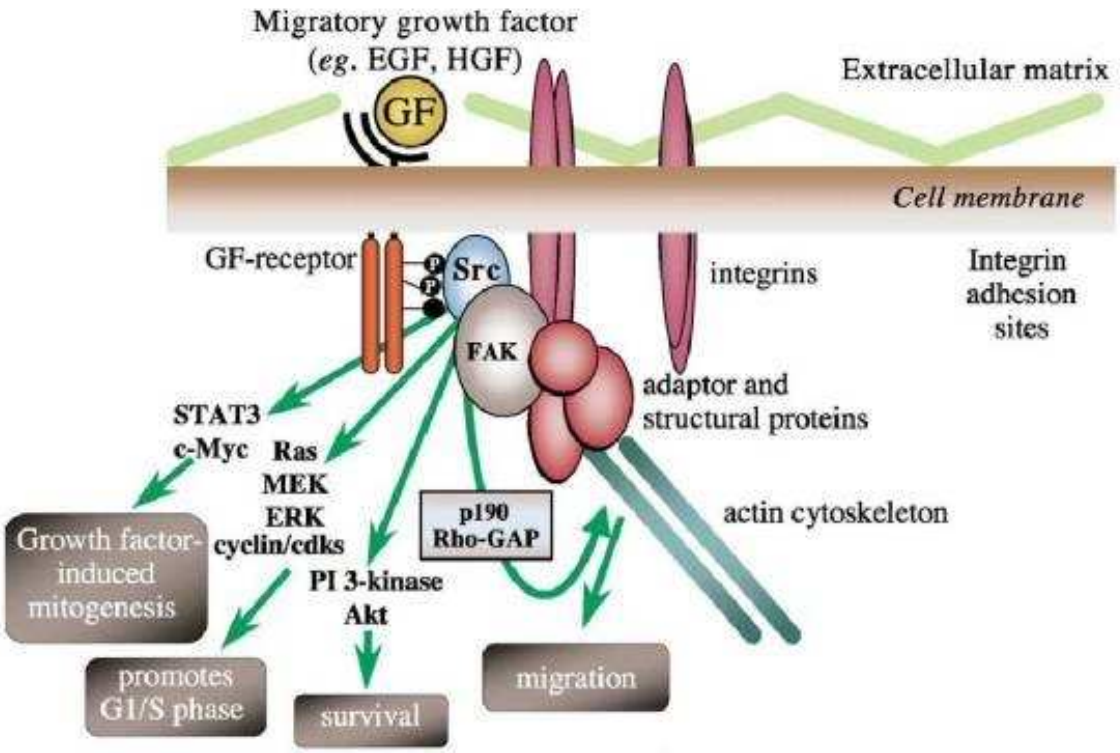


**Figure 1.7- Src Signaling Pathways.**

Src kinase can be activated by of growth factor ligand and receptor families as well as components of the extracellular matrix. Src can promote proliferation, survival, cell migration, and modulation of the actin cytoskeleton through a variety of cell signaling cascades. Taken from Frame et al (88).

*c-Src* $^{-/-}$ embryos do not display a vascular or cardiac phenotype (98). It is postulated that expression of other SFKs (Fyn, Yes) in the heart are sufficent to compensate for loss of c-Src. cSrc$^{-/-}$ fibroblasts demonstrate deficent activation of Focal Adhesion Kinase (FAK) and fail to dissasmble focal adhesions required for cell motility (99). Another major substrate of Src is p190 Rho GAP activation, subsequently leading to GTP-binding and activation of RhoGTPase family members, initiation of filamentous actin polymerization, and cell motility (100).

Several receptors upstream of Src activation are required for EMT during heart development. The ErbB family of RTKs have been well described in cardiogenesis (101). Expression of ErbB3 is restricted to the atrioventricular canal during cardiac cushion formation, and *erbb3*$^{-/-}$ phenotype is embryonic lethal at E13.5 due to defects in epicardial EMT (102). *Egfr*$^{-/-}$ (103) and *erbb2*$^{-/-}$ (104) embryos exhibit inhibited formation of endocaridal cushion mesenchyme.

TGFβ ligands induce Src activation, but the specific mechanism for this activation remains unclear. Tyrosine 284 phosphorylation of the Type II TGFβ receptor by Src is required for activation of p38 MAPK in a human breast cancer cell model (35). Although the mechanism for Src activation by TGFβs is largely unclear, it has been reported that TGFβ1-induced Src activation requires TGFβRI (ALK5) kinase activity (36). HMWHA can engage cell surface receptors CD44 and RHAMM (85) to stimulate intracellular signaling to drive EMT and cell invasion (80, 81). HA-mediated intracellular signal transduction executed through CD44 can enhance canonical TGFβ Type I receptor signaling (82). HA is a driver

of Src-dependent cell motility via activation of Rho GTPase family members and filamentous actin polymerization in several tumor-derived cell lines (83, 84).

### 1.9 Actin and RhoGTPases

The ability of a cell to regulate the actin cytoskeleton is required for cell division, survival, adhesion, contractility and motility (105). Actin is a cytosolic 42 KDa protein found in all eukaryotic cells. α-actin controls contractility in the myocardium and vascular smooth muscle, β and γ actin are the principle molecules modulating cell invasion and motility (106). Actin may exist as monomer, referred to as globular actin (g-actin), or in linear polymers known as filamentous actin (f-actin). Cells alter the structure of the actin cytoskeleton rapidly in response to extracellular stimuli including chemokines, extracellular matrix molecules, and secreted growth factors.

The Rho family of GTPases modulate f-actin polymerization to form distinct structures required for invasive cell motility. Upon activation by Guanine Nucleotide Exchange Factors (GEFs), GTP binding to Rho GTPase family proteins activates downstream signaling cascades to modulate filamentous actin polymerization (107). Binding of GTP is a rapid molecular switch, GTPases hydrolyze GTP to GDP to down-regulate signaling via intrinsic GTPase activity. The Rho family of GTPases consists of three proteins that mediate the formation of distinct actin structures: Rac1, Rho, and cdc42. Rac1 induces lamellipodia formation at the leading edge of motile cells, cdc42 forms filopodial structures extending beyond leading edge, and Rho is required for stress fiber formation

and turnover of focal adhesions that mediate cell-substrate contacts (fig. 1.8) (108).

GTP bound Rac1 activates PAK p21 ((Cdc42/Rac)-activated kinase 1) and subsequently filamin that directly binds and crosslinks actin filaments (109). Rac1 also activates Wiskott-Aldrich Protein (WASp) that interacts with and activates Actin Related 2/3 complex of proteins (ARP2/3) complex that directly nucleates actin branching at leading edge of lamellae (110). Activation of Rac1 inhibits Myosin Light Chain Kinase (MLCK) activity, this leads to dissolution of stress fibers and turnover of focal adhesions permissive of cell motility. Cdc42 similarly activates WASp and APR2/3 complex formation and nucleation of actin filaments to form filopodial structures (108). Rho activation subsequently stimulates Rho kinase (ROCK) activity and subsequent activation of Myosin Light Chain Phosphatase (MLC (P)) phosphatase that results in dissolution of existing, and formation of new focal adhesions in a motile cell (111).

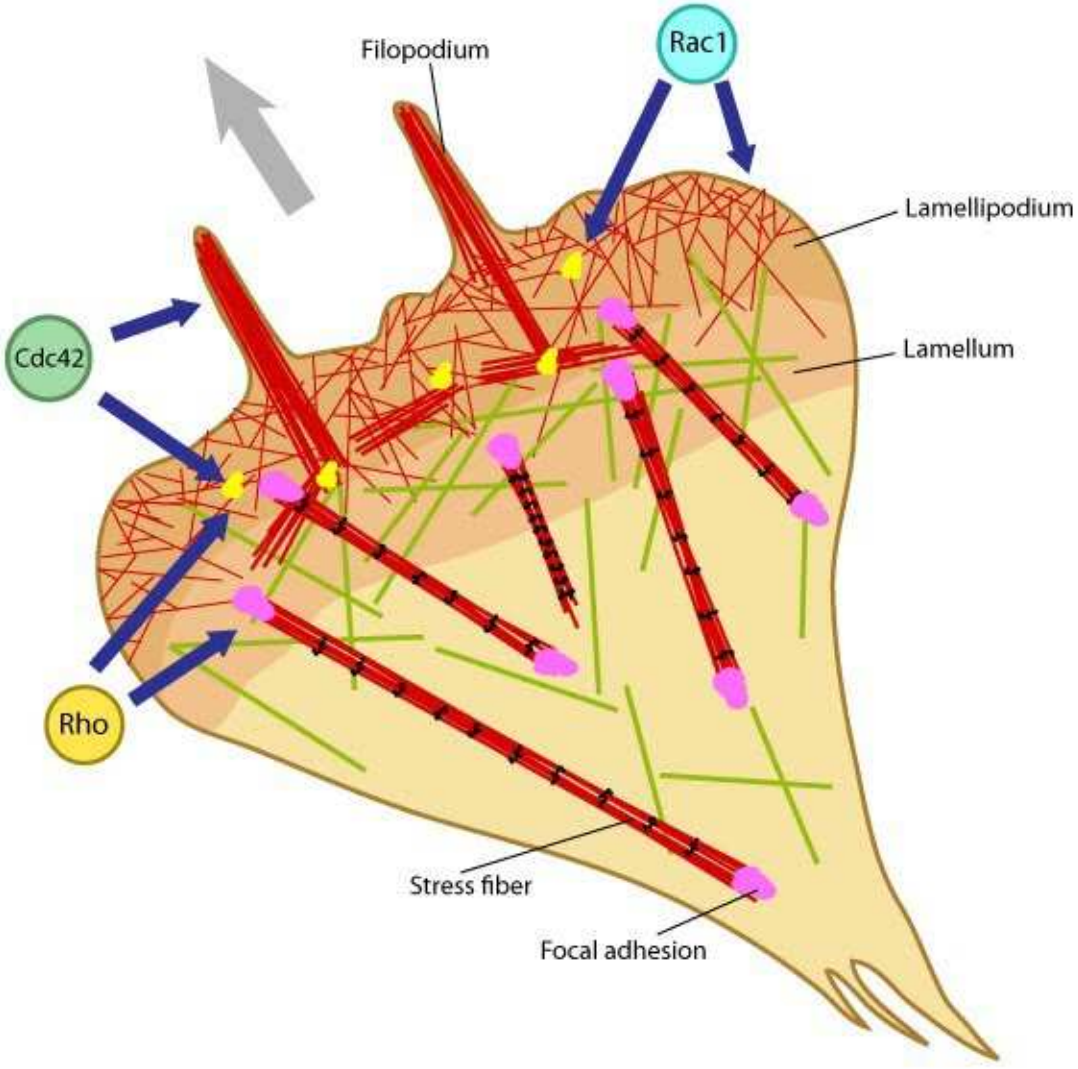


**Figure 1.8 – Rho Family GTPases Modulate Actin Dynamics**

The Rho family of GTPases are directly upstream of regulating cell motility by modulating distinct structures of the actin cytoskeleton. Cdc42 is required for filopodial extensions, Rac1 forms lamellipodia at the leading edge of cells, and RhoA regulates the maintenance and turnover of focal adhesions (108).

TGFβR3 regulates cancer cell migration, by augmenting filamentous actin polymerization via TGFβ1 dependent activation of Rac1 and cdc42 GTPases (69). TGFβ access to the Par6 RhoA pathway is required for endocardial and epicardial cell invasion (52, 65), though Rac1 and cdc42 are not sufficient to drive invasion in either epicardial or endocardial cells. TGFβ ligand activation of RhoA, Rac1, and cdc42 activity have been demonstrated to be independent of the canonical TGFβ pathway (32, 112).

Work reviewed in this chapter demonstrates that there is a role for non-canonical aspects of TGFβ signaling in endocardial and epicardial cell transformation in development, as well as cancer progression in adult disease. The role of Src kinase in TGFβ ligand and HMWHA dependent epicardial cell EMT, invasion, and migration has not yet been addressed. TGFβR3 has several signaling functions independent of the canonical pathway, and more investigation is necessary to elucidate other key effectors of this unique receptor. It is the goal of this work to evaluate the importance of Src in epicardial EMT, cell invasion, and migration in the context of these regulators of cardiovascular development.

# CHAPTER 2

# SRC IS REQUIRED FOR TGFβ2-INDUCED EPICARDIAL CELL INVASION AND VASCULAR SMOOTH MUSCLE DIFFERENTIATION

## 2.1 Introduction

The coronary vasculature is required for proper development and function of the heart. In embryonic development, the coronary vessels arise from a tissue known as the proepicardium (113). Cells from the proepicardium are transferred to surface of the heart to form the epicardium that contribute to coronary vessel development. The epicardium undergoes migration, differentiation and cell invasion into the primitive myocardium. The epicardium forms an epithelial sheet that covers the myocardium, and secreted growth factors from the myocardium stimulate synthesis of extracellular matrix molecules including hyaluronan (HA) in the subepicardial space (78, 114). These matrix and growth factors induce Epithelial to Mesenchymal Transition (EMT), in which transformed epicardial cells migrate through the subepicardial space, invade the myocardium, and differentiate into cardiac fibroblasts resident in the myocardium that contribute to repair, and vascular smooth muscle cells that constitute the mature coronary vessels (115). As EMT and cell invasion is required for heart development, perturbations in this process can lead to congenital defects or adult coronary artery disease, the leading cause of lethality in the United States (2).

The requirement for transforming growth factor β (TGFβ) family of ligands and receptors for epicardial (EMT) and invasion has been reported (116, 40). TGFβ2, but not TGFβ1 or TGFβ3, has an exclusive function in heart development and is required for valve maturation (117-119). The Type III TGFβ receptor (TGFβR3) binds TGFβ2 at picomolar concentrations (120), making TGFβ2 the highest affinity ligand binding partner of TGFβR3. Conventional gene deletion of *Tgfbr3* results in aborted coronary vessel morphogenesis likely due to a deficit of EPDCs resident in the myocardium (66). We have shown that TGFβ2 induces epicardial cell invasion and requires HA (40). The canonical TGFβ signaling pathway is required for TGFβ ligand stimulated epicardial invasion (41), though non-canonical signaling pathways are less well described.

Src is a ubiquitously expressed non-receptor tyrosine kinase that has been identified as a driver of cell motility in many cell systems (91). Src is activated by a variety of stimuli including receptor tyrosine kinases, Integrins and TGFβs (121). These effectors function in the onset and progression of cancer, but the role of Src in TGFβ directed cell transformations and motility in the context of cardiovascular development has not been addressed. Src is an attractive preclinical target for suppression of tumor cell motility and differentiation in TGFβ-dependent cancers. It has been recently reported that phosphorylation of tyrosine 284 in the Type II TGFβ receptor by Src is required for activation of p38 MAPK (35). Although the mechanism for Src activation by TGFβs is largely unclear, it is known that ALK5 (Type I TGFβ receptor) kinase activity is required for Src activation (36).

In this study, we assessed the role of Src in TGFβ2-stimulated epicardial EMT, cell invasion and differentiation. Finally, we determined if Src activity was sufficient to drive activation of epicardial EMT and smooth muscle differentiation.

## 2.2 Materials and Methods

### Cell line and culture conditions

Immortalized murine Epicardial cells were originally provided by Dr. Joey Barnett (Vanderbilt Medical University). Cell culture conditions were used as previously described (40). Briefly, these cells are conditionally immortal at 33℃ by the interferon stimulated expression of temperature sensitive Large T Antigen (TAg) (122). Cells are cultured at 37℃ in the absence of interferon to revert cells into a primary state for all experiments.

### Reagents

Human recombinant TGFβ2 was purchased from R&D systems (R&D #302-B2). Antibodies for western blotting were purchased from the following manufacturers: pY416 Src (Cell Signaling 2101), cSrc (Santa Cruz, sc-19), pSmad2/3 (Santa Cruz sc-1176), Smad2/3 (Santa Cruz sc-6032). Vimentin (Santa Cruz sc-7557) was used for immunostaining. Src inhibitor PP2 ($IC_{50}$= 100 nM) was obtained from Calbiochem (CalBiochem #529573). Y527F-Src mutant was kindly provided by Dr. David Merryman (Vanderbilt Medical University).

**RT-PCR**

RNA was isolated and purified using Trizol RNA isolation reagent (Invitrogen) according to manufacturer's instructions. cDNA was generated using first strand cDNA synthesis kit (Roche) from 1µg RNA isolated from each experimental condition. Real-Time PCR was performed as previously described using TaqMan Master primer-probe system (Roche). 40S Ribosomal Protein 7 (RPS7) was used as a housekeeping gene. The following oligonucleotides for murine primer sets were used for detection of indicated genes:

| | |
|---|---|
| *RPS7 (f)* | 5'-AGCACGTGGTCTTCATTGCT-3' |
| *RPS7 (r)* | 5'-CTGTCAGGGTACGGCTTCTG-3' |
| *CD44 (f)* | 5'-TCCTTCTTTATCCGGAGCAC-3' |
| *CD44 (r)* | 5'-AGCTGCTGCTTCTGCTGTACT-3' |
| *Has2 (f)* | 5'-GGCGGAGGACGAGTCTATG-3' |
| *Has2 (r)* | 5'-ACACATAGAAACCTCTCACAATGC-3' |
| *Tgfbr3 (f)* | 5'-TCCAAACATGAAGGAGTCCA-3' |
| *Tgfbr3 (r)* | 5'-GTCCAGGCCGTGGAAAAT-3' |

**Hyaluronan Assay**

Epicardial cells were seeded onto a 96-well tissue culture plate in DMEM 10% FBS and allowed to adhere overnight, subsequently serum starved overnight (DMEM 0% FBS) prior to described experiments. Media was collected

after 48 hours TGFβ2 stimulation in the presence or absence of PP2 for use in the Hyaluronan Capture Assay (DuoSet ELISA Development kit, R&D Systems DY3614) to allow for quantitation of HA synthesis by epicardial cells. Experiments were performed in triplicate three independent times, data is shown relative to untreated control.

**SM22α Reporter assay**

Epicardial cells isolated from SM22α-*LacZ* transgenic mice were generated and isolated as previously described (41) and were used to determine SM22α promoter activity and epicardial cell differentiation into a smooth muscle cell lineage *in vitro*. SM22α-*LacZ* epicardial cells were stimulated with 2 ng/mL TGFβ2 in the presence or absence of PP2 for 24 hours and allowed to transform.

β-galactosidase staining and luminesce detection of β-galactosidase assays were performed in parallel. Visualization of β-galactosidase expression in SM22α-*LacZ* epicardial cells was performed by using β-galactosidase Reporter Gene Staining Kit (Sigma Aldrich, GALS-1KT) according to manufacturer's instructions. Chemiluminescence detection of β-galactosidase activity in cell lysates was performed using the Galacto Light-Plus System (Applied Biosystems) and BioTek2 micro plate luminometer.

**Collagen Gel Invasion Assay and Immunofluorescence**

To recapitulate *in vivo* epicardial cell EMT and cell invasion, epicardial cells were cultured on Rat Tail Type I collagen gels at 1 mg/ml collagen (BD Biosciences, Franklin Lakes, New Jersey). Epicardial cells were seeded onto

gels 24 hours prior to treatment. Cells were incubated with PP2 for 1 hour prior to 2 ng/ml TGFβ2 stimulation for 48 hours. Cells were fixed in 4% paraformaldehyde prior to immunofluorescence detection of vimentin, a marker for cardiac mesenchyme. Collagen gels were mounted on glass slides and vimentin positive transformed epicardial cells were observed using fluorescence microscopy using a Leica DMLB microscope and documented using a Retiga 200R camera and ImagePro Plus 5.1 software. Cell counting of transformed, invaded epicardial cells using phase-contrast microscopy by observers blinded to conditions was performed.

## 2.3 Results

### 2.3.1 Src is required for TGFβ2-induced expression of pro-EMT genes.

TGFβ2-stimulated HA synthesis is required for epicardial cell invasion (40), we detect that Src kinase activity is required for this process. TGFβ2 induces Src tyrosine 416 phosphorylation (activating Src kinase activity) and phosphorylation of Smad2/3 (canonical TGFβ signaling effector) (fig. 2.1A). Epicardial cells were stimulated with 2 ng/mL TGFβ2 for 48 hours in the presence or absence of PP2, a potent Src inhibitor. RNA was isolated and analyzed by RT-PCR for genes implicated in the regulation of epicardial EMT. TGFβ2 stimulation induces Has2 mRNA expression, consistent with our previous report (40). In the presence of PP2, this induction is severely blocked (fig. 2.1B). Inhibition of Src in the presence of TGFβ2 results in a decrease of basal and TGFβ2-stimulated production of HA by 71.4% (fig. 2.1C). Blocking Src in the presence of TGFβ2 also leads to the attenuation of CD44 expression (fig. 2.1D.). Expression of TGFβR3 has been shown to play a role in the maintenance of an epithelial phenotype, and loss of TGFβR3 is associated with a motile phenotype in cancer (69). Src inhibition during TGFβ2 stimulation attenuates down-regulation of TGFβR3 (fig. 2.1E). Taken together, these data suggest a role for Src in TGFβ2-induced epicardial EMT and cell invasion.

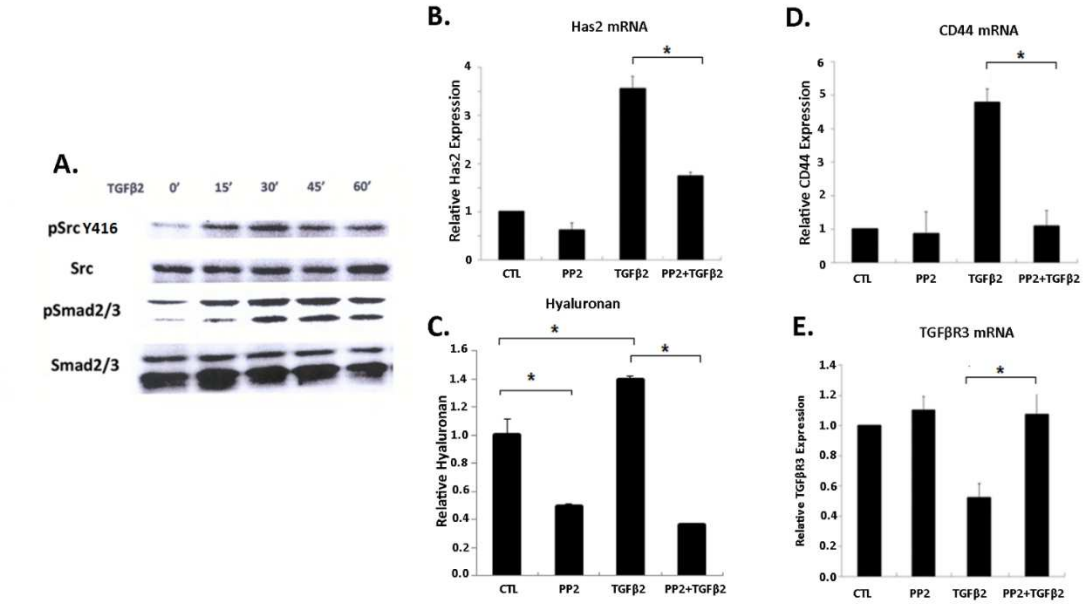


**Figure 2.1 – Src required for TGFβ2 induction of pro-EMT gene expression.**

(A) Western blot detection of pY416 Src and pSmad2/3 in epicardial cells stimulated with 2 ng/mL TGFβ2. (B) Detection of Has2 mRNA in epicardial cells stimulated with 2 ng/mL TGFβ2 in the presence or absence of 1 μM PP2 for 48 hours. (C) Detection of HA from epicardial cells stimulated with 2 ng/mL TGFβ2 in the presence or absence of 1 μM PP2 for 48 hours. (D, E) Detection of CD44 and TGFβR3 mRNA in epicardial cells stimulated with 2 ng/mL TGFβ2 in the presence or absence of 1 μM PP2 for 48 hours. (*p=<0.05).

### 2.3.2 Src is required for TGFβ2-induced EMT and cell invasion

We have previously determined TGFβ2 requires Src activity to induce expression of pro-EMT genes, the functional consequences of this observation was determined by evaluating the role of Src in TGFβ2-induced epicardial cell EMT and cell invasion. The Type I collagen gel invasion assay is a widely accepted system for assessing cell invasion in primary embryonic tissues (13). Epicardial cells were cultured on hydrated Type I collagen gels and stimulated with 2 ng/mL TGFβ2 in the presence or absence of PP2 for 48 hours, and assayed by immunofluorescence detection of the intermediate filament protein vimentin serves to identify mesenchymal cells. Epicardial cells stimulated with TGFβ2 induce the expression of vimentin, undergo EMT as defined by an elongated morphology and expression of vimentin (fig. 2.2C, arrows), and invade into the collagen gel (fig. 2.2E). In the presence of PP2, there is diminished detection of vimentin than those stimulated with TGFβ2, and subsequently fail to activate EMT or undergo cell invasion (fig. 2.2D, arrows). Invaded cells under the same conditions were observed by phase contrast microscopy, and counted. TGFβ2 stimulation resulted in a 5-fold increase in in the number of cells entering the matrix relative to vehicle incubated controls (fig. 2.2E). In the presence of PP2 and TGFβ2, cells fail to undergo cell invasion as a result of blocked activation of EMT. These data demonstrate that Src is required for activation of TGFβ2-stimulated epicardial EMT and cell invasion.

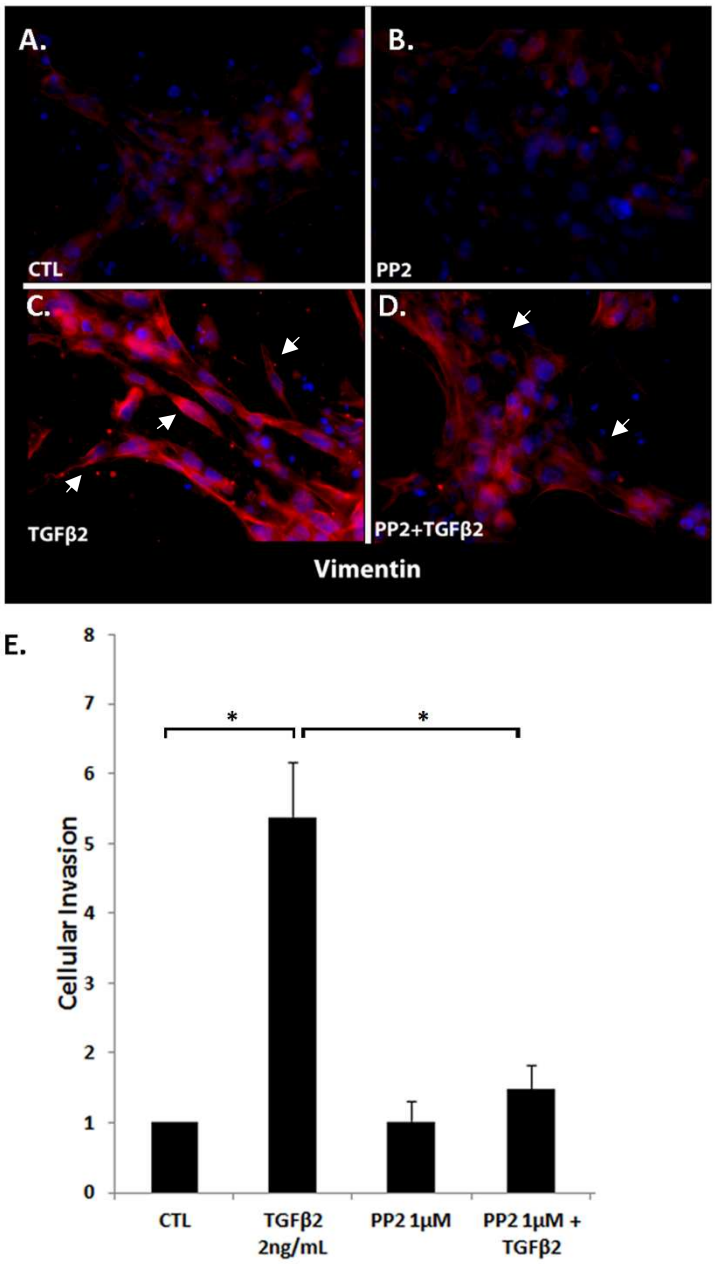


**Figure 2.2- Src is required for TGFβ2-stimulated epicardial EMT and cell invasion.**

(A-D) Immunofluorescence detection of vimentin in epicardial cells stimulated with 2 ng/mL TGFβ2 in the presence or absence of 1 μM PP2 on collagen gels. (E) Counting of invaded epicardial cells under identical conditions relative to unstimulated control (*=$p<0.005$).

### 2.3.3 Src is required for TGFβ2-induced smooth muscle differentiation.

Epicardial EMT and cell invasion is an early step in the formation of the coronary vessels. Once invaded into the myocardium, epicardial cells undergo differentiation into vascular smooth muscle cells that will contribute to the coronary vasculature. As TGFβ2 has previously been shown to induce smooth muscle differentiation (116), we evaluated the role of Src in this process. Immortalized SM22α-*LacZ* epicardial cells were stimulated with TGFβ2 in the presence or absence of PP2 and allowed to undergo differentiation. β-galactosidase expression is indicative of a transcriptionally active SM22α promoter. As expected, TGFβ2 induces expression of β-galactosidase as visualized by X-GAL staining (fig. 2.3C, arrows). In the presence of PP2, there is a significant blockade of TGFβ2-stimulated smooth muscle differentiation as observed by a marked reduction in blue colorimetric detection of XGAL (fig. 2.3D, arrows). To confirm this observation, whole cell lysates were prepared under identical conditions and subject to luminescence-based detection of β-galactosidase activity. TGFβ2-stimulated epicardial cells induce β-galactosidase activity 3.5 fold over unstimulated controls (fig. 2.3E). In the presence of PP2, this induction is significantly blocked relative to cells stimulated with TGFβ2. Thus, vascular smooth muscle differentiation as induced by TGFβ2 is dependent on Src.

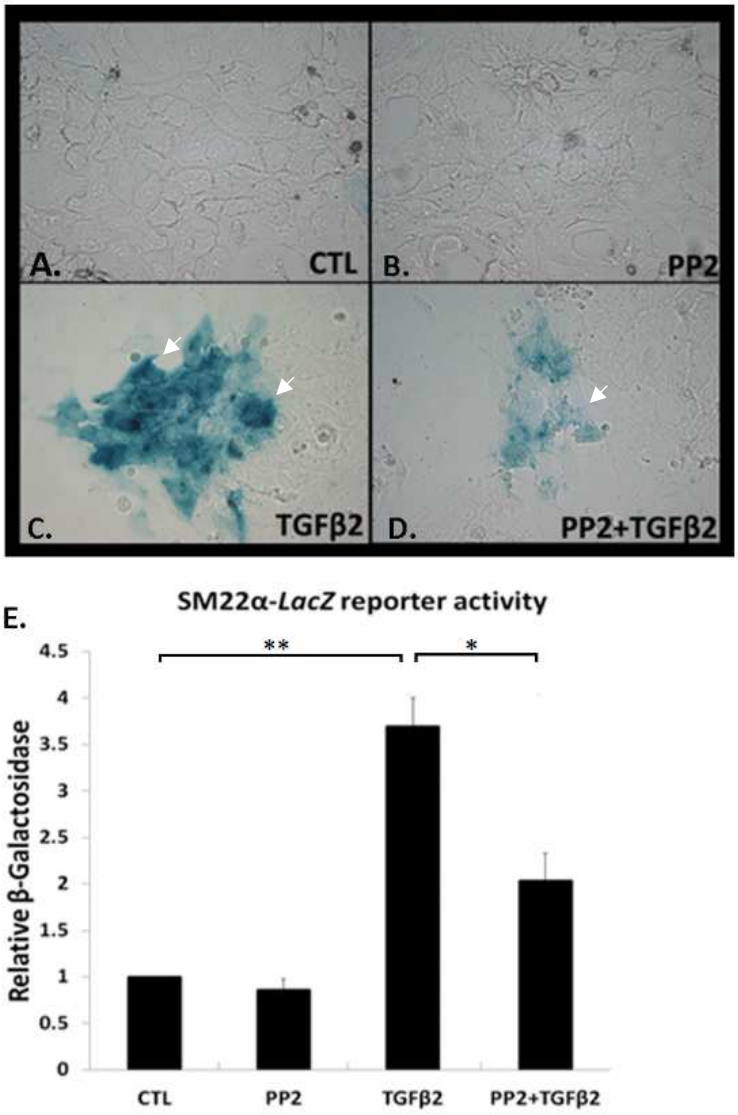


**Figure 2.3- Src required for TGFβ2-induced smooth muscle differentiation.**

(A-D) X-GAL staining for β-galactosidase expression in SM22α-*LacZ* epicardial cells stimulated with 2 ng/mL TGFβ2 for 48 hours in the presence or absence of 1 μM PP2. (E) Detection of β-galactosidase in whole cell lysates from SM22α-*LacZ* epicardial cells stimulated with 2 ng/mL TGFβ2 for 48 hours in the presence or absence of 1 μM PP2. (*=$p<0.05$. **=$p<0.005$).

### 2.3.4 Src is required for TGFβ2-induced filamentous actin polymerization

Mobilization of the actin cytoskeleton is universally required for cell invasion and motility (105). Src is known to modulate cell motility through stimulating filamentous actin (f-actin) polymerization (100). Since TGFβ2-stimulated cell invasion is blocked in the presence of PP2, we determined the role of Src in TGFβ2-stimulated f-actin polymerization. Epicardial cells were pre-incubated with PP2 for 1 hour, and subsequently stimulated with TGFβ2 for 1 hour. Phalloidin staining for the visualization of actin filaments was performed. Vehicle incubated epicardial cells have a low-basal level of f-actin polymerization (fig. 2.4A). TGFβ2 stimulates f-actin polymerization and formation of dense actin filaments (2.4B, arrows). Cells incubated with PP2 appear to reorganize actin filaments into cortical actin at the plasma membrane (fig. 2.4C, arrows), which functions in support of the cell membrane rather than active cell motility (123). In the presence of PP2, TGFβ2-stimulated formation of dense actin filaments is completely inhibited (fig. 2.4D, arrows). Thus, filamentous actin polymerization as induced by TGFβ2 is dependent on Src.

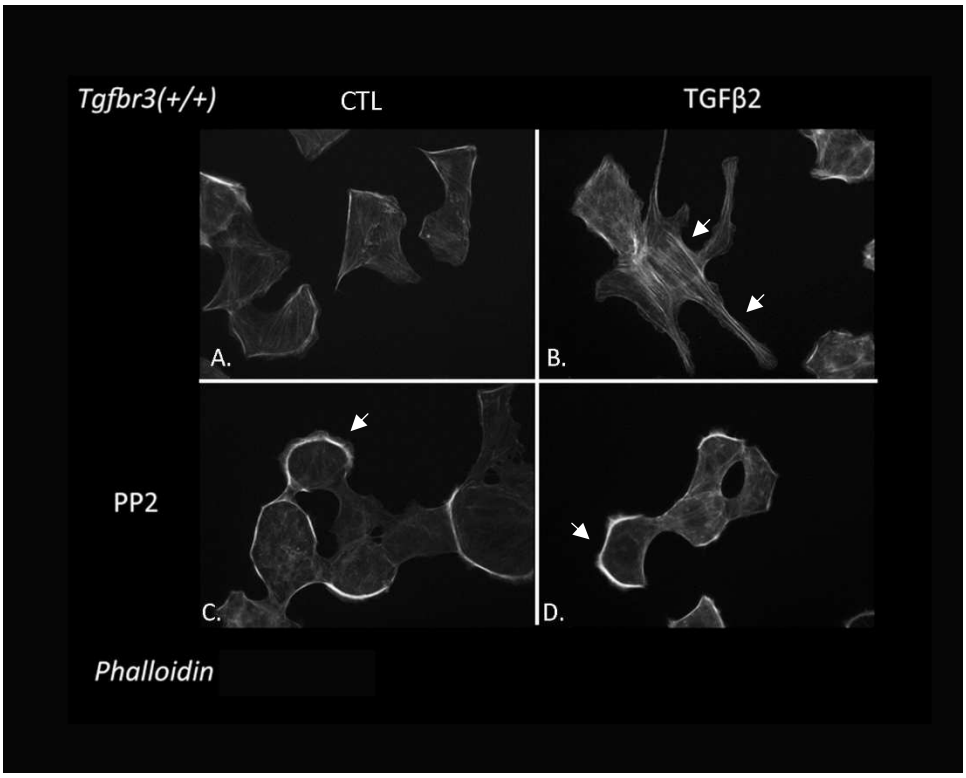


**Figure 2.4 – Src required for TGFβ2-stimulated filamentous actin polymerization**.

(A-D) Phalloidin staining for visualization of filamentous actin polymerization in epicardial cells stimulated with 2 ng/mL TGFβ2 for 1 hour in the presence (D) or absence (B) of 1μM PP2.

### 2.3.5 Src kinase drives epicardial EMT, but not smooth muscle differentiation.

Since Src is required for TGFβ2-induced invasion and smooth muscle differentiation, we next asked if Src activity is sufficient to drive EMT and smooth muscle differentiation. It has previously been reported that Src activity is sufficient to drive smooth muscle differentiation in porcine valve myofibroblasts (36). Phosphorylation of tyrosine 527 of Src kinase is auto inhibitory to enzymatic activity and leads to a closed and inactive confirmation of Src (92). Mutation of Y527 to phenylalanine prevents this inhibition, resulting in a constitutively active Src (Y527F-Src). Epicardial cells were transfected with EGFPN1 vector or EGFPN1-Y527F-Src construct encoding the constitutively active mutant of Src. Cells expressing EGFP vector or *wild-type*-Src-EGFP (data not shown) retain an epithelial cell morphology (fig. 2.5C-D, arrows). Cells expressing Y527F-Src adopt of an elongated morphology consistent with activation of EMT (fig. 2.5E-F, arrows). RT-PCR analysis reveals that Y527F-Src induces the expression of Has2 and CD44 independent of stimulus (fig. 2.5G), two molecules we have previously shown to be required for epicardial EMT and cell invasion. Src activity was not sufficient to drive smooth muscle differentiation as evidenced by the expression of SM22α. Thus, Src activity is sufficient to drive activation of EMT, but not smooth muscle differentiation in epicardial cells.

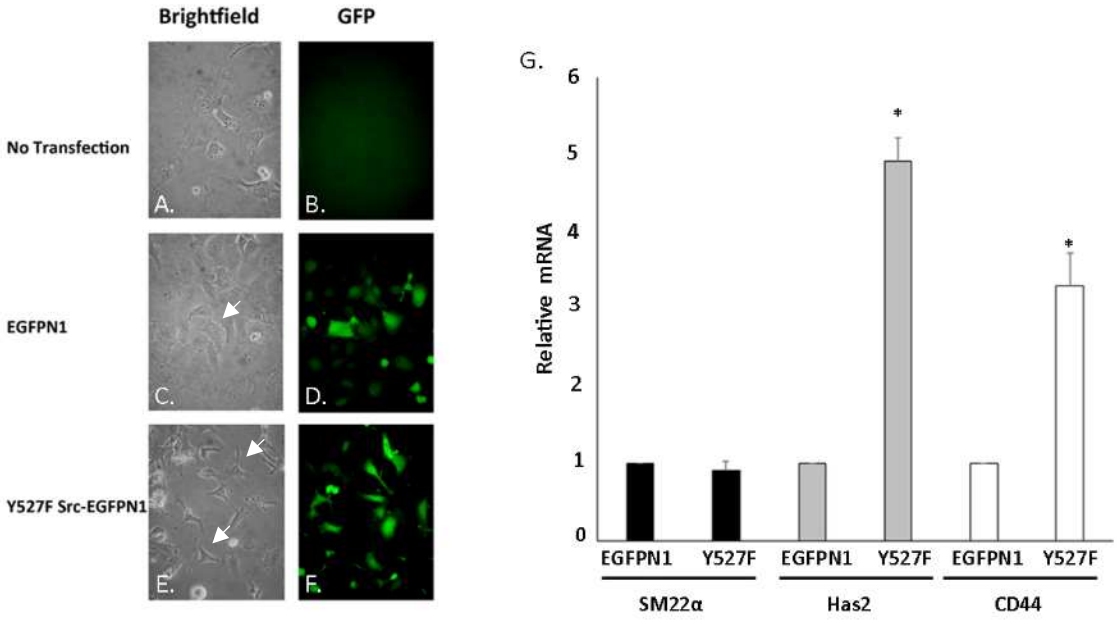


**Figure 2.5 Constitutively active Src drives activation of EMT, but not smooth muscle differentiation.**

(A-F) Epicardial cells were transfected with EGFPN1 (empty-GFP vector) or EGFPN1-Y527F-Src containing a constitutively active mutant of Src kinase. Phase contrast microscopy to visualize cell morphology (A, C, E), and GFP expression indicates expression of each vector (B, D, F). (G) RT-PCR analysis of mRNA from epicardial cells expressing vector or Y527F-Src. (*=p<0.005).

## 2.4 Discussion

The TGFβ family of ligands and receptors are directly implicated in epicardial EMT. TGFβ2 serves a non-redundant function in endothelial cell EMT that is required for valve formation in the heart. The requirement of canonical TGFβ signaling in these processes have been described (41), the importance of non-canonical signaling pathways of TGFβ2-induced epicardial EMT remain unclear. In this study, we assessed the role of Src in TGFβ2-mediated epicardial cell matrix synthesis, smooth muscle differentiation, and cell invasion.

We demonstrate that Src activity is required for TGFβ2-induced Has2 expression and HA synthesis, as well as expression of the HA receptor CD44 (fig. 2.1). As HA has been previously shown to be required for epicardial EMT (40), this is an important finding in that Src is required for not only synthesis of HA matrix, but CD44 expression required for epicardial autocrine responsiveness to HA. We show that PP2 inhibited unstimulated epicardial HA synthesis significantly without significantly down-regulating Has2 mRNA. This result is indicative of a possible role for Src in modulating Has2 enzyme activity by unknown post-translational mechanisms. Has2 activity can be stimulated by Mono Ubiquitination of Lysine 190 of Has2 protein (124) and negatively regulated by phosphorylation of Threonine 110 by AMPK (125). Previous work in the laboratory has shown that Src is required for EGF induced tyrosine phosphorylation of Has2 (Craig Unpublished), though specific residues that are phosphorylated and their effect on Has2 enzymatic activity has yet to be elucidated.

TGFβ2 induces down-regulation of TGFβR3 in epicardial cells as previously shown in cancer (126), this is expected as loss of expression of TGFβR3 is associated with a metastatic and motile phenotype in a mouse model of tumorigenesis (127). Our experiments blocking Src activity in the presence of TGFβ2 suggests a role for Src in transcriptionally regulating TGFβ receptor expression.

We assessed the role of Src in TGFβ2-induced cell invasion. Src activity is required for TGFβ2-induced activation of EMT as well as cell invasion (fig 2.3). Epicardial cells in the presence of PP2 and TGFβ2 do induce expression of the mesenchymal marker vimentin, but still fail to undergo elongation consistent with EMT and cell invasion. This suggests that Src is required for early activation of TGFβ2-induced EMT that precedes cell invasion. This finding is supported by our data demonstrating Src is required for TGFβ2-induced f-actin polymerization required for cell invasion (fig. 2.4). TGFβ2 induces smooth muscle differentiation in epicardial cells (116), the requirement for Src in this transformation had not yet been addressed. TGFβ1 induced smooth muscle differentiation in 10T1/2 mouse embryonic fibroblasts requires Src (128). We demonstrate that Src activity is required for TGFβ2-induced smooth muscle differentiation in epicardial cells (fig. 2.2), constituting a novel finding for Src in the epicardium and suggesting that this pathway is conserved in embryonic tissues.

In order to define the role for Src in epicardial cell differentiation, we asked if Src activity was sufficient to drive EMT and smooth muscle differentiation. Epicardial cells expressing a constitutively active mutant of Src stimulates loss of

epithelial character and elongation consistent with the activation of EMT, and induce expression of Has2 and CD44, but not SM22α (fig. 2.5). This finding is contrary to work that showed Src activity is sufficient to drive smooth muscle differentiation in porcine valve myofibroblasts (36). This is indicative of differential roles for Src in embryonic primary cells in cardiac development compared to terminally differentiated cells in cardiovascular disease. Thus, Src is required for TGFβ2-induced activation of EMT and cell invasion (fig. 2.2), and Src is sufficient to drive activation of epicardial EMT (fig. 2.5). Src activity is required for TGFβ2-induced smooth muscle differentiation (fig. 2.3), but not sufficient to drive smooth muscle differentiation (fig. 2.5). This indicates the requirement of requirement of other TGFβ2 pathways independent of Src activity to induce smooth muscle differentiation in coronary progenitor cells.

# CHAPTER 3

## HYALURONAN REQUIRES THE TYPE III TGFβ RECEPTOR AND SRC KINASE TO DIRECT EPICARDIAL CELL INVASION

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### 3.1 Introduction

The coronary vasculature is required for proper development and function of the heart. During embryonic development the formation of the coronary vessels is dependent upon the transfer of cells from the proepicardium to the surface of the heart to form the epicardium (113). The epicardium is an epithelial sheet that covers the myocardium, and secreted growth factors from the myocardium stimulate synthesis of extracellular matrix molecules including hyaluronan (HA) in the subepicardial space (78, 114). These matrix and growth factors induce Epithelial to Mesenchymal Transition (EMT), in which transformed epicardial cells migrate through the subepicardial space, invade the myocardium and differentiate into vascular smooth muscle cells and cardiac fibroblasts (115). The EMT process is defined by loss of polarity and cell-cell contacts in epithelial cells, and adopting an elongated fibroblast-like morphology. These transformed cells are then competent to undergo cell invasion. This EMT process is a requirement for development several organs including the embryonic heart (129). As EMT is required for heart development, perturbations in this process can lead

to congenital defects leading to adult cardiovascular disease, which is the leading cause of death in the United States (2).

HA is a long-chain glycosaminoglycan extracellular matrix molecule synthesized by the Hyaluronan synthase family of enzymes (Has1, Has2, Has3) (72) and serves structural functions as well as stimulating biochemical signaling cascades in the developing heart. The *Has2*$^{-/-}$ knockout phenotype is embryonic lethal at 9.5 days of gestation due to blocked cardiac development as a consequence of deficient endocardial EMT (76). These knockout embryos lack HA and fail to complete endocardial cushion EMT and maturation with lethality occurring before epicardial development. Since lethality precedes formation of the epicardium in the *Has2*$^{-/-}$ embryo, we used *in vitro* techniques to determine the role of HA in epicardial cell invasion. Well-characterized mouse epicardial cell lines (116) were used to decipher the mechanisms of HA-triggered epicardial EMT and invasive cell motility. Prior work in our laboratory has shown that high-molecular weight hyaluronan (HMWHA) can induce epicardial cell invasion and EMT, and is required for TGFβ2-induced epicardial cell invasion and EMT (40, 79).

HA can engage cell surface receptors CD44 and RHAMM (85) to stimulate intracellular signaling that can modulate epithelial character and cell invasion (80, 81). HA-mediated intracellular signal transduction executed through CD44 can enhance canonical TGFβ Type I receptor signaling (82). HA is a driver of Src-dependent cell motility via activation of Rho GTPase family members and filamentous actin polymerization in several tumor-derived cell lines (83, 84). The

Rho family of GTPases modulate f-actin polymerization to form distinct structures required for invasive cell motility: Rac1 induces lamellipodia formation at the leading edge of motile cells, cdc42 forms filopodial structures extending beyond leading edge, and RhoA required for turnover of focal adhesions (108).

The Type III TGFβ receptor (TGFβR3) lacks catalytic activity and functions in TGFβ ligand presentation to Type I and II TGFβ receptors to stimulate receptor activation (41, 62). It has previously been demonstrated that TGFβR3 is required for endocardial (130) as well as epicardial cell invasion (63). *Tgfbr3*$^{-/-}$ mice die at E14.5 as a result of failed coronary vessel development associated with decreased epicardial cell invasion into the myocardium (66). We have previously shown have shown that *Tgfbr3*$^{-/-}$ epicardial cells do not invade in response to HMWHA (63), the molecular mechanism underlying this phenotype is unknown. TGFβR3 is also known to regulate cancer cell migration, by augmenting filamentous actin polymerization via TGFβ1 dependent activation of Rac1 and cdc42 GTPases (69). Src is a ubiquitously expressed non-receptor tyrosine kinase that has been identified as a driver of cell invasion in many cell systems (91). Src activation has not previously been reported to be involved in TGFβR3 signal transduction, but has been suggested to be activated via Type I TGFβ receptor-dependent pathway in a TGFβ1-dependent manner (36). How these effectors function in epicardial cells and whether they are responsive during HA directed cell invasion have not yet been reported.

This study reveals that HA mediated activation of cell invasion and filamentous actin polymerization is dependent on TGFβR3. Furthermore, Src

kinase is required for HA mediated epicardial cell invasion, and filamentous actin polymerization. In the absence of TGFβR3, HA-stimulated activation of Src kinase, Rac1 and RhoA GTPases are deficient concomitant with defective cell invasion. These data establish a novel role for TGFβR3 and Src as central signaling nodes for HA directed epicardial cell invasion.

## 3.2 Materials and Methods

### Cell lines and Reagents

Conditionally immortal murine epicardial cells were originally provided by Dr. Joey Barnett (Vanderbilt Medical University) as described (116). Cell culture conditions were used as previously described (40). Epicardial cells used in this study were isolated from wild-type (*Tgfbr3*$^{+/+}$) mouse embryos and embryos lacking TGFβR3 (*Tgfbr3*$^{-/-}$). Src kinase inhibitor PP2 was purchased from EMD Millipore (#529573). High-molecular weight hyaluronan (HMWHA) was purchased from R&D Systems (#GLR002).

### Western Blotting

Whole cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a fluoride membrane (PVDF). After blocking in 5% Bovine Serum Albumin (BSA), membranes were probed with different primary and secondary antibodies in 3% BSA. Antibodies for detection of pY416Src (6943S), Src (2109S), and TGFβR3 (2519S) were purchased from Cell signaling technologies (Danvers, MA). Antibodies for the detection of β-Tubulin (sc-9104), Rac1 (sc-217), and cdc42

(sc-087) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). RhoA (ARH03) and Actin (AAN01) antibodies were purchased from Cytoskeleton (Aurora, CO). Densitometry was analyzed by ImageJ, all stimulated conditions were set relative to unstimulated conditions for each cell line and siRNA treatment as internal controls.

**Cell invasion assays**

To assess cell invasion, epicardial cells were cultured in DMEM containing 10% FBS on 1 mg/mL Rat Tail Type I Collagen gels in 4-well tissue culture dishes (BD Biosciences, Franklin Lakes, New Jersey). Cells were seeded at 40,000 cells/gel for each experimental condition, and allowed to attach overnight. Cells were stimulated with 300 µg/mL HMWHA for 30 minutes, and allowed to undergo cell invasion for 24 hours. Cells were fixed with 4% Paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.5% TritonX-100, and subject to AlexaFlour594-conjugated phalloidin staining to visualize filamentous actin and cell morphology (Life Technologies A12381). Invaded, transformed epicardial cells were manually counted by observers blinded to the conditions in three independent experiments.

**Phalloidin staining**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were seeded on glass coverslips at an equal and sub-confluent density and allowed to adhere overnight. Cells were subject to overnight serum starvation (DMEM, 0%FBS), and stimulated with 300 µg/mL HMWHA for 60 minutes. Immunofluorescence visualization of filamentous

actin was accomplished using AlexaFlour594 phalloidin (Life Technologies A12381) and a Leica DMLB microscope. Images were documented using a Retiga 200R camera and ImagePro Plus 5.1 software.

**Filamentous Actin Assay**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were seeded at 800,000 cells per 10 cm tissue culture dish (70% confluence) and allowed to adhere overnight. Cells were grown in serum free conditions for 18 hours prior to stimulation with 300 µg/mL HMWHA for 60 minutes. The extent of filamentous actin (F-actin) polymerization was assess by the F-Actin/G-Actin In Vivo Bioassay Biochem Kit (to be referred to as the f/g actin assay) according to the manufacturer's instructions (Cytoskeleton BK037). Briefly, whole cell lysates were ultracentrifuged (100,000xg) to fractionate globular (g) and filamentous (f) actin fractions. These fractions were then resolved by SDS-PAGE and Western blot detection of actin to allow for comparison of filamentous actin in each treatment. Densitometry of g and f actin bands was performed using Image J, and the f/g actin ratio was obtained dividing f band density by g band density. f/g ratios under stimulated conditions were plotted relative to unstimulated f/g ratios from *Tgfbr3*$^{+/+}$ conditions.

**Detection of GTP-bound RhoA, Rac1, and cdc42.**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were seeded at 800,000 cells per 10 cm tissue culture dish (70% confluence). Following 18 hour serum starvation, cells were stimulated with 300 µg/mL HMWHA for indicated time points. 1 mg of

whole cell lysate was incubated with PAK-PDB beads (Cytoskeleton #PAK02A) for 1 hour to bind activated (GTP-bound) Rac1 and cdc42 for each experimental condition. 1mg of whole cell lysate was incubated with RBD beads (Cytoskeleton #BK036) for 1 hour to bind GTP-bound RhoA for each experimental condition. Beads with precipitated GTP-bound RhoA, Rac1, and cdc42 and 25 µg of total lysate (for loading control) under each experimental condition were resolved by SDS-PAGE and Western blot detection of Rac1, cdc42, and RhoA.

**Introduction of siRNA**

*Tgfbr3*$^{+/+}$ epicardial cells were plated at 50,000 cells per well of a 6-well tissue culture plate (50% confluence) in DMEM containing 10% FBS without antibiotics and were cultured for 48 hours. Cells were then transfected with 2 µg Control siRNA or 2 µg siRNA targeting mouse TGFβR3 (SantaCruz Biotechnologies, sc-40225), and 8 µL of Xtreme Gene siRNA transfection reagent (Roche). The media was changed to remove the reagent 16 hours after transfection. 48 hours post transfection, cells were stimulated with HMWHA and whole cell lysates were analyzed by Western blot. Validation of TGFβR3 knockdown was accomplished via Western blot analysis.

**Expression of DNA Vectors**

HEK293 cells were plated at 50,000 cells per well of a 6-well plate (50% confluence) in DMEM containing 10% FBS without antibiotics and cultured for 48 hours. Cells were then transfected with 2 µg pAdTrack empty vector or pAdTrack vectors containing TGFβR3-T841A (constitutively active TGFβR3) using 3 µL of

X-treme gene HP DNA transfection reagent (Roche). The media was changed to DMEM containing 10% FBS without antibiotics 24 hours after transfection. 48 hours after transfection, whole cell lysates were resolved by SDS-PAGE, and Western blot analysis to detect the indicated molecules.

**Statistical Analysis**

All graphs represent mean values, with error bars showing the standard deviation of the mean. Experiments were performed in duplicate and repeated a minimum of three times. Statistical significance was assessed using a 2-way student’s t-test, with p values below 0.05 considered significant.

## 3.3 Results

### 3.3.1 TGFβR3 and Src are required for HMWHA-induced cell invasion.

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells cultured on collagen gels were stimulated with HMWHA for 30 minutes and the extent of cell invasion was assessed after 24 hours (fig. 3.1). *Tgfbr3*$^{+/+}$ cells stimulated with HMWHA adopt an elongated mesenchymal phenotype and undergo cell invasion into the collagen gel (fig. 3.1C). In contrast, *Tgfbr3*$^{-/-}$ epicardial cells do not lose epithelial character or execute cell invasion in the presence of HMWHA (fig. 3.1D). Invaded epicardial cells were counted (fig. 3.1E). *Tgfbr3*$^{+/+}$ epicardial cells stimulated with HMWHA demonstrate a level of cell invasion that is 3 times the level seen in unstimulated *Tgfbr3*$^{+/+}$ cells (fig. 3.1E). Thus, consistent with our prior reports, TGFβR3 is required for HMWHA-stimulated epicardial cell invasion. In parallel experiments, *Tgfbr3*$^{+/+}$ epicardial cells were pre-incubated with 1μM PP2, a Src inhibitor, for 1 hour prior to HMWHA stimulation. The extent of cell invasion was visualized by phalloidin-detection of filamentous actin to observe invaded cell morphology and allow for counting of invaded cells into the collagen gel. *Tgfbr3*$^{+/+}$ epicardial cells executed EMT and invaded the collagen in response to HMWHA (fig. 3.1H). In contrast, *Tgfbr3*$^{+/+}$ cells stimulated with HMWHA in the presence of PP2 (fig. 3.1I) did not invade into the collagen matrix and retained an epithelial cell morphology. Invaded cells were quantified, and *Tgfbr3*$^{+/+}$ cells undergo a level of cell invasion that is 3 times higher in response to HMWHA when compared to unstimulated *Tgfbr3*$^{+/+}$ cells (fig. 3.1J) or cells pre-

incubated with PP2. Thus, Src kinase activity is required for HMWHA-stimulated epicardial cell invasion.

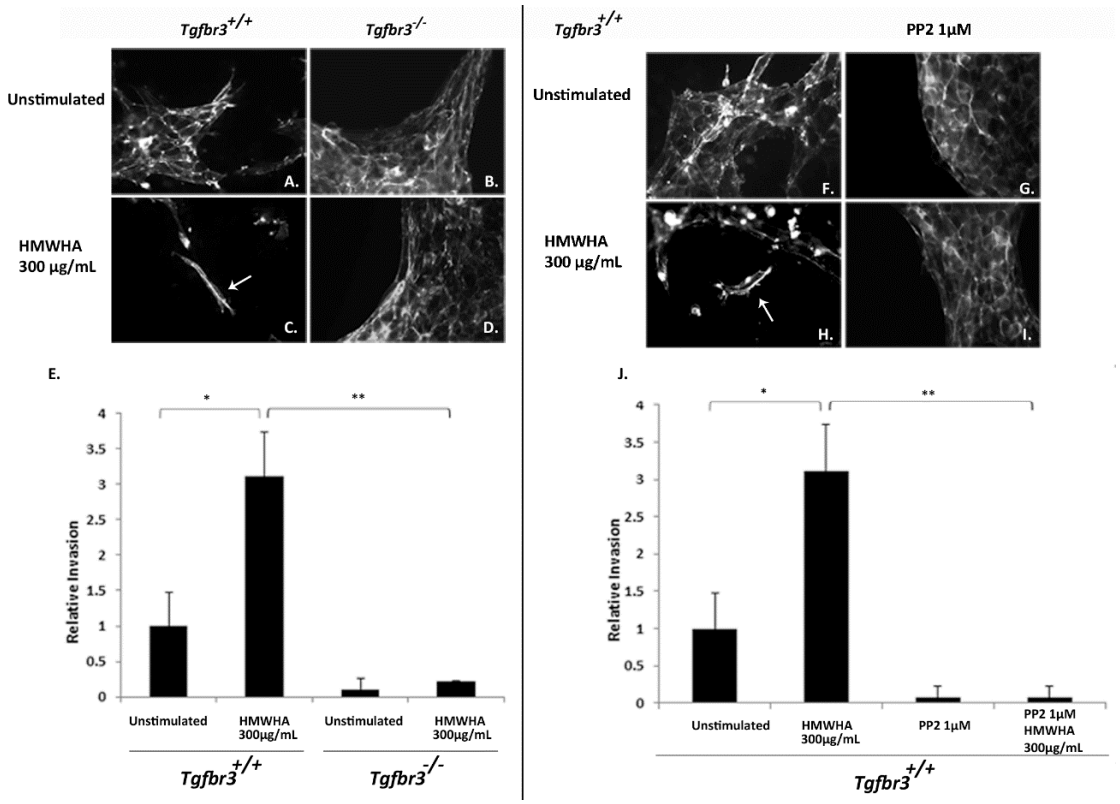


**Figure 3.1. TGFβR3 and Src are required for HMWHA-stimulated cell invasion.**

(A-D) *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells on collagen gels stimulated with 300 µg/mL HMWHA for 24 hours. Phalloidin staining used to visualize cell morphology (E) Counting of invaded epicardial cells (*p<0.05 **p<0.005). (F-I) *Tgfbr3*$^{+/+}$ epicardial cells on collagen gels stimulated with 300 µg/mL HMWHA after 24 hour pre-incubation with the Src kinase inhibitor PP2. (J) Counting of invaded epicardial cells (*p<0.05 **p<0.005).

### 3.3.2 TGFβR3 is required for HMWHA-stimulated f-actin polymerization.

Induction of filamentous actin stress fibers is essential for cell migration and invasion (105). Phalloidin staining was used to visualize filamentous actin polymerization (f-actin) in order to determine the role of TGFβR3 in HWMHA-stimulated cell motility. Unstimulated *Tgfbr3*$^{+/+}$ cells have low basal levels of organized stress fibers (fig. 3.2A). Upon stimulation with HMWHA, *Tgfbr3*$^{+/+}$ cells form f-actin into stress fibers compared to unstimulated *Tgfbr3*$^{+/+}$ cells (fig. 3.2B). In contrast, unstimulated *Tgfbr3*$^{-/-}$ cells have high basal levels of stress fibers (fig. 3.2C). Stimulation with HMWHA results in actin filament disassembly in *Tgfbr3*$^{-/-}$ cells (fig. 3.2D), but is not accompanied by the re-polymerization of actin filaments necessary for cell invasion. The failure of *Tgfbr3*$^{-/-}$ cells to mobilize filamentous actin stress fibers upon HA stimulation was independently confirmed using an f/g actin assay. A substantial deficiency in the ability of *Tgfbr3*$^{-/-}$ cells to mobilize actin was detected following stimulation with HMWHA (fig. 3.2E). Unstimulated *Tgfbr3*$^{+/+}$ cells have low levels of f-actin (fig. 3.2E, Lane 2), whereas *Tgfbr3*$^{-/-}$ cells have high levels of f-actin in the unstimulated conditions (fig. 3.2E, Lane 4). There is detection of increased f-actin in *Tgfbr3*$^{+/+}$ cells following HMWHA stimulation indicating normal f-actin polymerization (fig. 3.2E, Lane 6). In contrast, *Tgfbr3*$^{-/-}$ cells show a decrease in f-actin following HMWHA stimulation (fig. 3.2E, Lane 8). The f/g actin ratio was determined for *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells stimulated with or without HMWHA. Following HA-stimulation,

*Tgfbr3*$^{+/+}$ cells have an f/g ratio of 1.7 versus 0.47 for *Tgfbr3*$^{-/-}$ cells demonstrating a requirement for TGFβR3 to mediate HMWHA-stimulated f-actin polymerization.

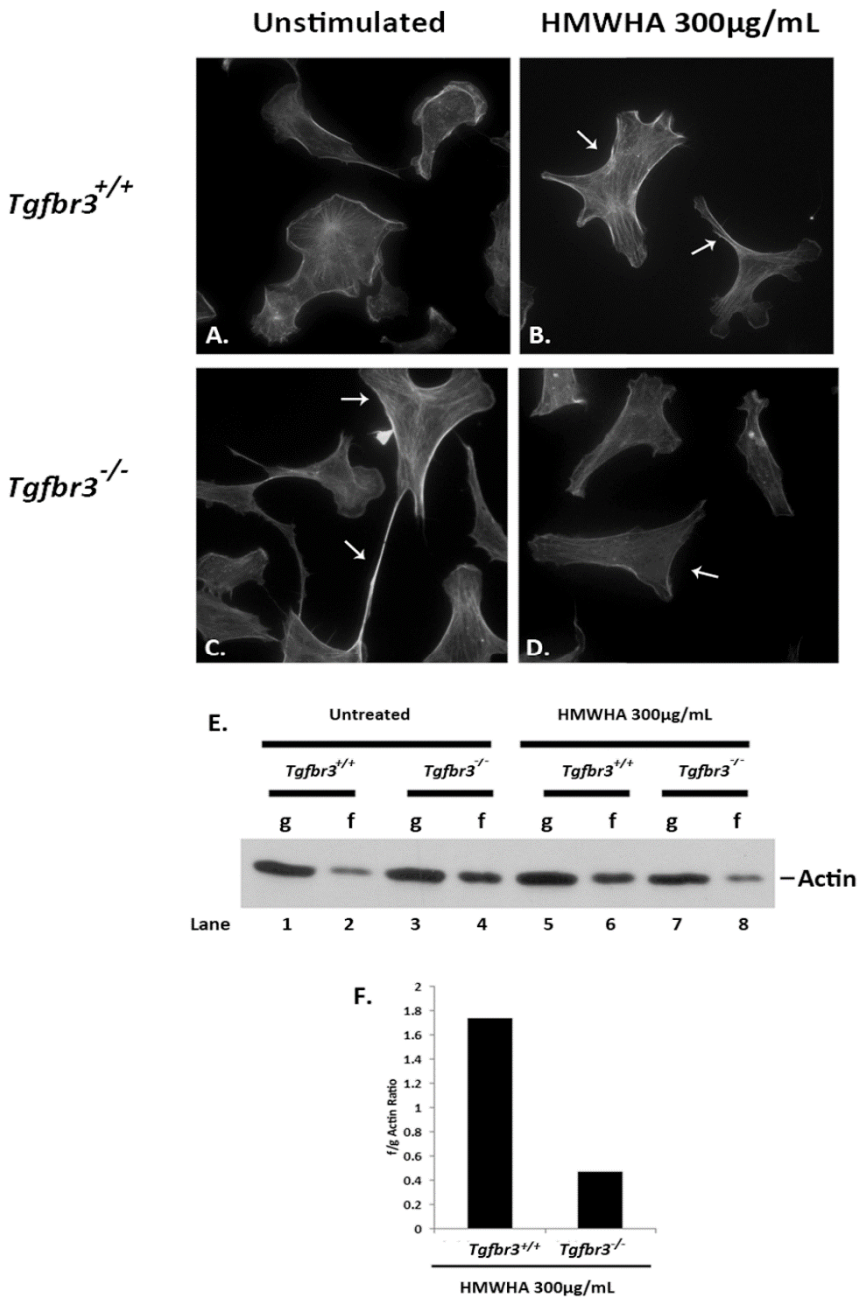


**Figure 3.2 Defective actin dynamics in *Tgfbr3*$^{-/-}$ epicardial cells.**

(A-D) Detection of filamentous actin in *Tgfbr3*$^{+/+}$ epicardial cells without (A) and with HMWHA 300 µg/mL stimulation for 60 minutes (B). Detection of filamentous actin in *Tgfbr3*$^{-/-}$ epicardial cells without (C) and with HMWHA 300 µg/mL stimulation (D) for 60 minutes. (E) Globular (g) or filamentous (f) actin detection by Western blot following ultracentrifugation of lysates from vehicle incubated and HMWHA-stimulated epicardial cells. (F) Representative graph of f/g actin ratios for HMWHA 300 µg/mL stimulated *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells.

### 3.3.3 HMWHA-stimulated f-actin polymerization is dependent on Src

The role of Src in HMWHA-stimulated f-actin polymerization was tested in *Tgfbr3*$^{+/+}$ epicardial cells. When stimulated with HMWHA, epicardial cells mobilize polymerization of f-actin as visualized by phalloidin staining (fig. 3.3B). In the presence of PP2, HMWHA-stimulated polymerization of f-actin is significantly inhibited (fig. 3.3D), a result also reflected in the f/g actin ratio calculated from performing the f/g actin assay (fig. 3.3E). *Tgfbr3*$^{+/+}$ cells incubated with increasing concentrations of PP2, and subsequently stimulated with HMWHA, exhibit concentration-dependent inhibition of f-actin polymerization (fig. 3.3E, Lanes 10, 12 compared to Lane 8). Cells stimulated with HMWHA have an f/g actin ratio of 2.1, whereas cells stimulated with HMWHA in the presence of 1 μM PP2 or 10 μM have f/g actin ratios of 1.2 and 0.8, respectively. These data suggest that Src kinase is required for HMWHA-stimulated filamentous actin polymerization and stress fiber formation in epicardial cells.

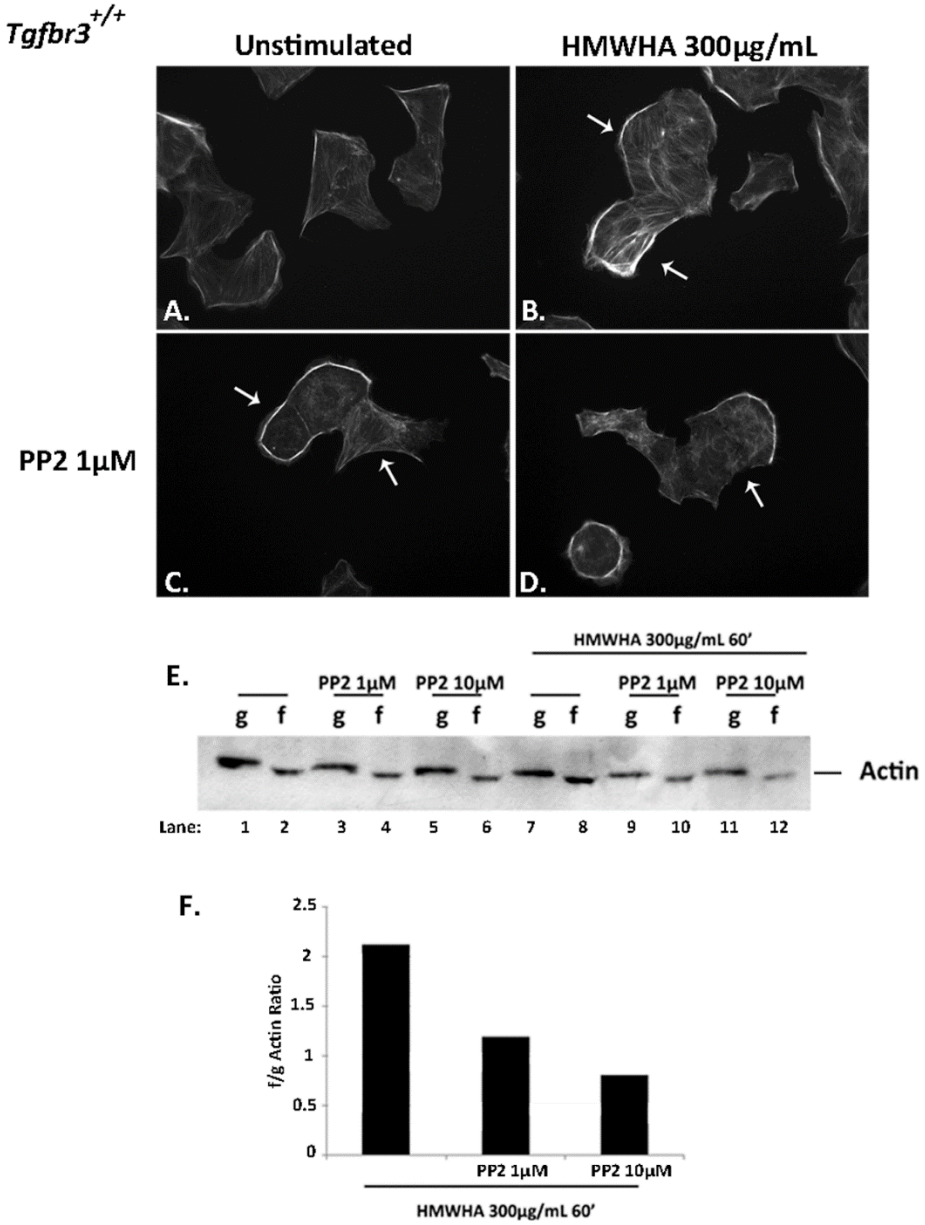


**Figure 3.3. HMWHA-induced filamentous actin polymerization dependent on Src.**

(A-D) *Tgfbr3*$^{+/+}$ epicardial cells were incubated without (A,B) or with Src kinase inhibitor (PP2 1 µM) (C,D) for 60 minutes, followed by 300 µg/mL HMWHA stimulation in the presence (D) or absence (B) of PP2 for 60 minutes, and subjected to phalloidin staining to visualize filamentous actin. (E) Globular (g) or filamentous (f) actin detection by Western blot following ultracentrifugation of lysates from vehicle incubated and HMWHA-stimulated epicardial cells with or without PP2. (F) Representative graph of the f/g actin ratio for HMWHA-stimulated vehicle incubated epicardial cells with or without PP2.

### 3.3.4 TGFβR3 is required for Src activation by HA.

Although these findings reveal TGFβR3 and Src are separately required for HMWHA-stimulated f-actin stress fiber formation and cell invasion, the data do not demonstrate that these effectors function in the same pathway. Therefore, experiments were performed to determine whether TGFβR3 is upstream of Src. HMWHA induces the phosphorylation of Src at tyrosine 416 (activating kinase activity) in *Tgfbr3*$^{+/+}$ cells 2.25 fold over unstimulated control (fig. 3.4A Lane 3), but not in *Tgfbr3*$^{-/-}$ epicardial cells (fig. 3.4A Lane 6). The phosphorylation of Src Y416 does not increase over basal levels in *Tgfbr3*$^{-/-}$ cells following HMWHA stimulation (fig. 3.4B). To address the requirement of TGFβR3 for HMWHA-stimulated Src activation, additional experiments were carried out with siRNA targeting TGFβR3 in *Tgfbr3*$^{+/+}$ epicardial cells to knockdown TGFβR3 expression. Src Y416 phosphorylation was induced 1.9 fold by HMWHA over the levels seen in *Tgfbr3*$^{+/+}$ cells transfected with control siRNA (fig. 3.4C Lane 3). *Tgfbr3*$^{+/+}$ cells transfected with siRNA targeting TGFβR3, and subsequently stimulated with HMWHA, did not induce Src Y416 phosphorylation above the levels seen in unstimulated *Tgfbr3*$^{+/+}$ cells transfected with the control siRNA (fig. 3.4C, D Lane 4). The knockdown of TGFβR3 by siRNA was confirmed by Western blot analysis (figs. 3.4E, 3.4F). These data suggest that TGFβR3 is required for HMWHA-dependent activation of Src kinase and establishes a novel role for TGFβR3 in the coordination of HMWHA specific signal transduction.

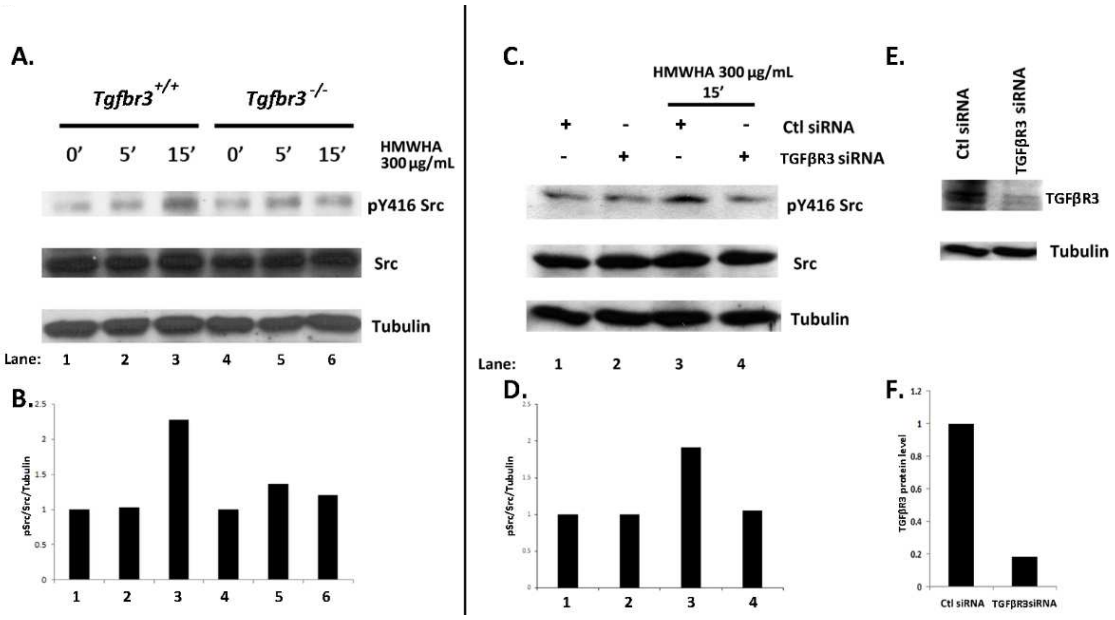


**Figure 3.4 Attenuated Src-activation in the absence of TGFβR3**

(A) *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$epicardial cells were stimulated with 300 µg/mL HMWHA for 5 and 15 minutes, whole cell lysates were analyzed by Western blot detection of pY416 Src. (B) Densitometry of pY46Src (C) Epicardial cells were transfected with siRNA to TGFβR3, stimulated with HMWHA for 15 minutes, whole cell lysates were subjected to Western blot detection of pY416 Src. (D) Densitometry of pY416Src (E-F) Confirmation of TGFβR3 knockdown by siRNA in epicardial cells validated by Western blot.

### 3.3.5 TGFβR3 regulates Rac1, RhoA, and cdc42.

The Rho Family of GTPases are modulators of the actin cytoskeleton and are directly upstream of f-actin polymerization and formation of stress fibers related to distinct cell membrane structures (108). RhoA has been implicated in both TGFβ and BMP2-stimulated epicardial EMT in a TGFβR3-dependent fashion (52). Since *Tgfbr3*$^{-/-}$ epicardial cells are deficient in forming filamentous actin stress fibers in the presence of HMWHA, the activation of the Rho family of GTPases was tested to determine if these are similarly dysregulated between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells. *Tgfbr3*$^{+/+}$ epicardial cells induce GTP-Rac1 (8 fold over unstimulated) and GTP-RhoA (5.5 fold over unstimulated) in response to HMWHA (fig. 3.5A, B Lane2, fig. 3.5C, D Lane 2). In contrast, *Tgfbr3*$^{-/-}$ epicardial cells fail to activate Rac1 or RhoA (fig. 3.5A, B Lane 4, fig. 3.5C, D Lanes 4-6). HMWHA stimulation of *Tgfbr3*$^{+/+}$ epicardial cells decrease GTP-cdc42 levels (fig. 3.5E, F Lane 2), whereas *Tgfbr3*$^{-/-}$ retain higher constitutive levels of GTP-cdc42 under unstimulated conditions relative to *Tgfbr3*$^{+/+}$ controls (fig. 3.5E, F lane 3). Furthermore, *Tgfbr3*$^{-/-}$ cells fail to decrease GTP-cdc42 following stimulation by HMWHA (fig. 3.5E, F lane 4). This constitutive and unregulated activation of cdc42 GTPase results in increased formation of filopodial structures in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$ as visualized by phase contrast microscopy (fig. 3.5G) and manual counting of filopodia (fig. 3.5H). Taken together these data demonstrate that TGFβR3 is required for HMWHA-stimulated activation of RhoA and Rac1 as well as the modulation of cdc42 activity.

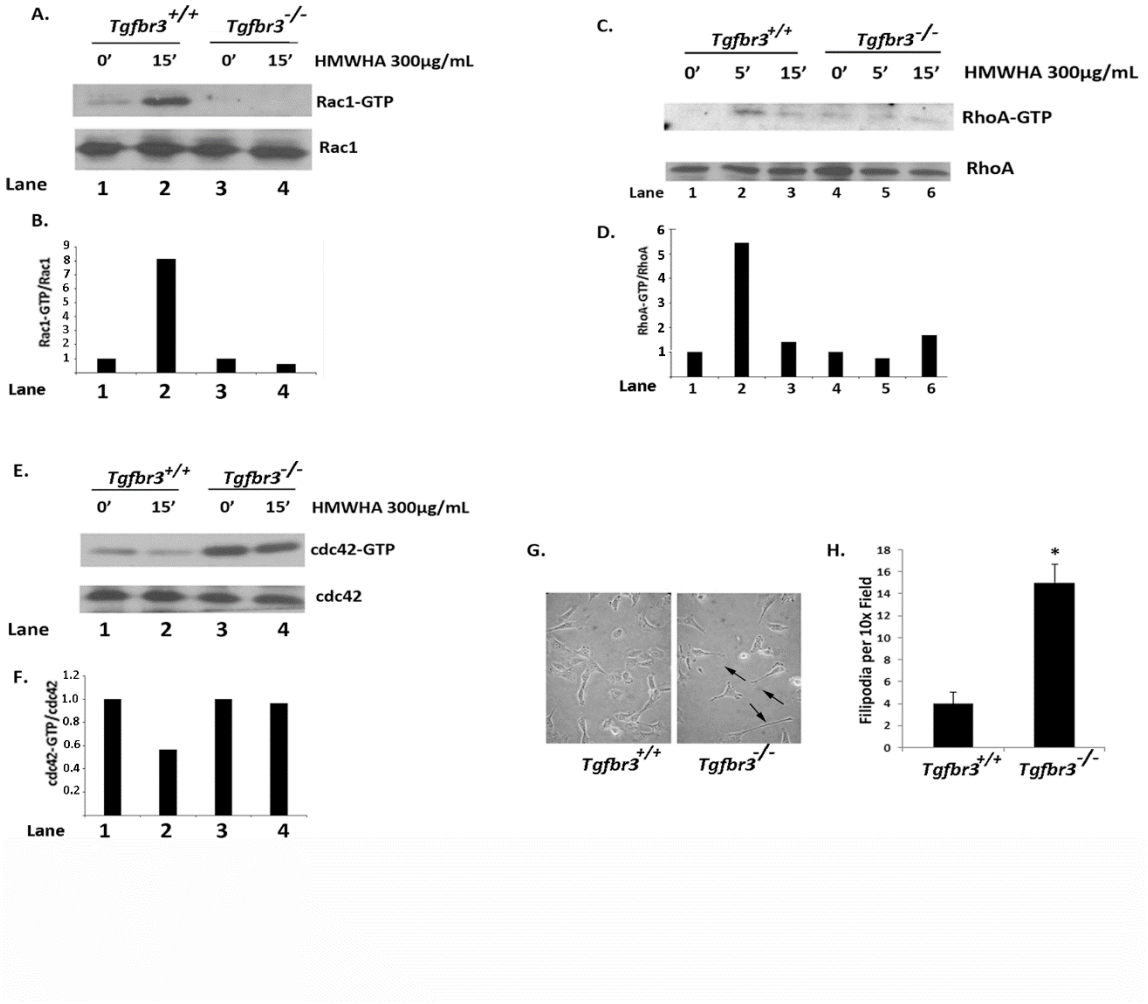


**Figure 3.5 Defective Rac1, RhoA and cdc42 activity in *Tgfbr3*$^{-/-}$ epicardial cells**.

(A-B) Detection of GTP-Rac1 in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$epicardial cells incubated with 300 µg/mL HMWHA for 15 minutes. (C-D) Detection of GTP-Rac1 in*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$epicardial cells incubated with 300 µg/mL HMWHA for 5 and 15 minutes. (E-F) Detection of GTP-cdc42 in) *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells incubated with 300 µg/mL HMWHA for 15 minutes. (G) Phase contrast microscopy visualizing filopodia of *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$epicardial cells. (H) Quantification of filopodia in *Tgfbr3*$^{+/+}$and *Tgfbr3*$^{-/-}$epicardial cells (*p= 5.90973E-08).

### 3.3.6 TGFβR3-T841A can drive Src activation

Given that TGFβR3 is required for HMWHA-stimulated activation of Src kinase and the formation of filamentous actin stress fibers, we investigated whether TGFβR3 activity could drive phosphorylation of Src. TGFβR3 (TGFβR3-T841A) has been shown to prevent β-Arrestin binding to TGFβR3 resulting in inhibited receptor internalization and continued signaling (70, 131) HEK 293 cells were transfected with pAdTrack vector or pAdTrack vector containing the TGFβR3-T841A. TGFβR3-T841A induces stimulus-independent phosphorylation of Y416 of Src to levels 2-fold over controls (fig. 3.6A-B, lane 2). Cells expressing TGFβR3-T841A also became elongated fibroblast-like consistent with the loss of epithelial character relative to control (fig. 3.6C). These data suggest that TGFβR3 is a direct upstream regulator of Src kinase activation.

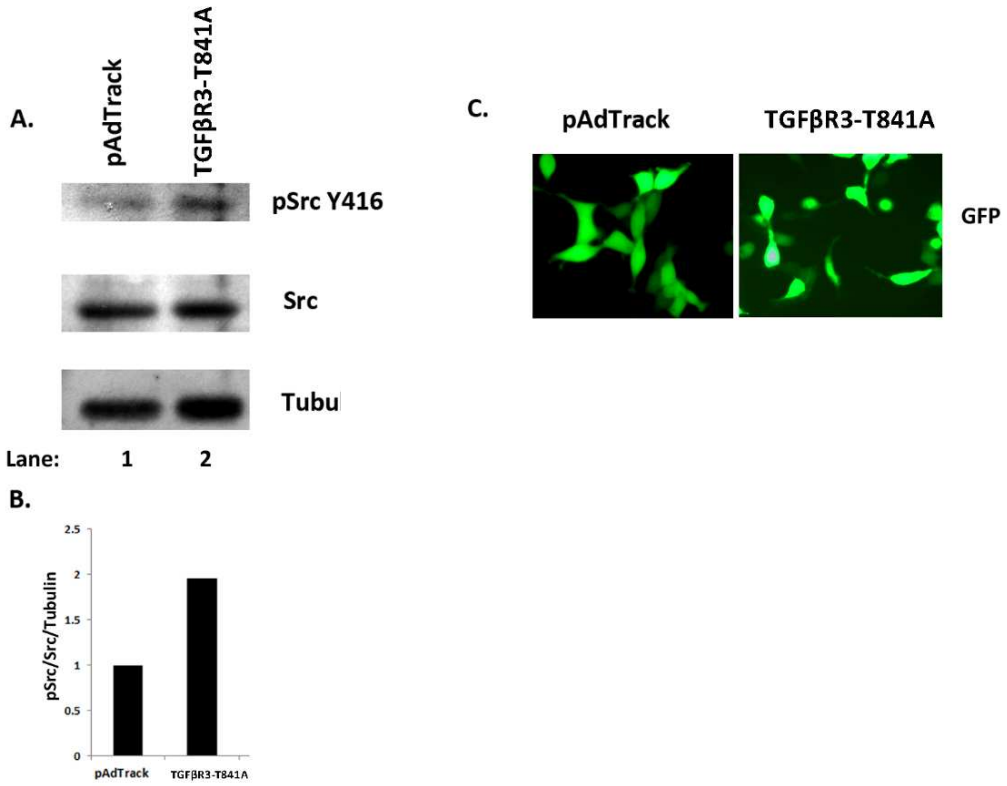


**Figure 3.6 TGFβR3-T841A drives Y416 Src phosphorylation.**

(A-B) Western blot detection of pY416 Src in whole cell lysates obtained from HEK293 cells expressing the constitutively active mutant (TGFβR3-T841A) of TGFβR3. (C) Confirmation of transfection of TGFβR3-T841A and pAdTrack vectors in HEK293 cells by fluorescence microscopy.

### 3.4 Discussion

Mobilization and polymerization of filamentous actin structures are the driving forces of cell migration and invasion (105). These events can be triggered by HMWHA which is a long chain glycosaminoglycan matrix molecule that serves structural and signaling functions in the developing heart (132). Previous work in our laboratory has shown that HMWHA can trigger epicardial cell invasion and differentiation (133) and that this occurs in a TGFβR3-dependent manner (41). A molecular explanation for this requirement has not been elucidated. In this study, the activation of Src kinase as a non-canonical effector downstream of TGFβR3 was investigated. Specifically, invasion assays and experiments assessing filamentous actin dynamics were performed in parallel to assess the roles of TGFβR3 and Src in HMWHA-stimulated cell invasion.

The loss of TGFβR3 expression in human ovarian cancer cells leads to enhanced cell migration (69), yet we show that *Tgfbr3*$^{-/-}$ epicardial cells are deficient in cell invasion when stimulated with HMWHA (fig.1). Similarly, TGFβR3 is required for chick endocardial invasion (134) during heart valve development where HMWHA is present and required (132), though a direct requirement for TGFβR3 in HMWHA-stimulated endocardial cell invasion has not been addressed. These observations may highlight a differential role for TGFβR3 in developing embryonic tissues versus terminally differentiated malignantly transformed tumors with respect to invasive cell motility. The HMWHA receptor CD44 has been shown to interact with Type I TGFβ receptors and enhance the canonical (Smad-dependent) TGFβ signaling (135). This supports the notion that

HMWHA promotes receptor complex formation for activation of multiple signaling pathways. A role for TGFβR3 in HMWHA-stimulated Src activity had not been demonstrated. Here we report that *Tgfbr3*$^{-/-}$ cells do not activate Src when stimulated with HMWHA, an observation confirmed with siRNA knockdown of TGFβR3 in *Tgfbr3*$^{+/+}$ cells which also failed to activate Src. We do not postulate that TGFβR3 directly binds HMWHA, but that TGFβR3 serves in a receptor complex with HA binding receptors to coordinate signal transduction. It should be noted that we have previously reported CD44 expression levels were not different between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells (63). Therefore CD44 expression is not sufficient to drive HA-stimulated Src, Rac1, or RhoA activation. CD44 may require TGFβR3 to initiate crosstalk with Src-activating receptors such as Type I TGFβ receptors (36) or receptor tyrosine kinases such as FGFR1 (136).

The data from this study establish that TGFβR3 and Src are required for HMWHA- stimulated filamentous actin polymerization in epicardial cells. We postulate that actin filament severing pathways stimulated by HMWHA in *Tgfbr3*$^{-/-}$ cells remain intact, but due to defective Src, Rac1, and RhoA activation (figs. 3.4, 3.5), these cells fail to repolymerize actin into stress fibers required for cell invasion. The activation of Rac1 and RhoA GTPases are detected in HMWHA-stimulated *Tgfbr3*$^{+/+}$ cells, concomitant with invasive cell motility. Activation of these GTPases in HMWHA-stimulated *Tgfbr3*$^{-/-}$ cells is strikingly absent, as is f-actin reorganization under identical conditions. Cdc42 activation has been reported to be activated by HMWHA in cancer cells (137), while our study shows

*Tgfbr3*$^{+/+}$ cells attenuating Cdc42 during HMWHA stimulation. In contrast, HMWHA-stimulated *Tgfbr3*$^{-/-}$ cells fail to decrease Cdc42 activity, an effect not observed in *Tgfbr3*$^{+/+}$ cells. This indicates that TGFβR3 modulates Cdc42 activity. Since *Tgfbr3*$^{-/-}$ cells lost the ability to regulate Rho, Rac1 and Cdc42, HMWHA stimulation causes a shift in the Rho family GTPase activation profile to one indicative of cell motility dependent on TGFβR3. Ligand dependent inhibition of RhoA was shown to be required for epicardial and endocardial EMT and cell invasion (51, 52). In these studies we find low induction of RhoA activity relative to Rac1 activation in *Tgfbr3*$^{+/+}$cells and postulate that Rac1 is the major GTPase in driving HMWHA-stimulated f-actin polymerization. It should be noted that Rac1 and cdc42 were not required or sufficient (via expression of dominant negative and constitutively active mutants, respectively) to drive endocardial or epicardial invasion (51, 52) suggesting that the activation of other signaling cascades upstream of Rho GTPases is required for cell invasion. Overexpression of TGFβR3 in ovarian cancer cells with reduced TGFβR3 leads to constitutive activation of cdc42 through β-Arrestin (63, 69) but we observe the opposite effect of cdc42 in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$ cells. We suggest the basis of TGFβR3 arrested motility is dependent on activation of cdc42, in agreement with our findings that *Tgfbr3*$^{-/-}$ cells fail to decrease cdc42 activation and remain non-invasive in response to HA.

Substitution of Threonine 841 for Alanine in TGFβR3 prevents β-Arrestin binding to TGFβR3, resulting in inhibited receptor internalization, relief of NFκB activity suppression, but no effect on ligand independent activation of the

canonical effector Smad2 (29, 30). This observation suggests that non-canonical TGFβR signal transduction may contribute to induction of cell motility which we examined in this study. As T841A-TGFβR3 can drive ligand independent cell invasion in epicardial cells (Barnett Unpublished), and previous work in our laboratory has shown NFkB activity to be required for HMWHA-directed cell invasion (80), T841A-TGFβR3 was tested to see if it can drive Src activation independent of stimulus. Indeed we detected Src activation and observed that T841A-TGFβR3 drives cell transformation (fig. 3.6) suggesting an important and previously unrecognized function of TGFβR3 signaling. These experiments constitute the first to report that TGFβR3 is upstream of HMWHA-stimulated Src tyrosine kinase activation. Collectively, these studies identify a novel TGFβR3-Src-Rac1-RhoA signaling axis that is required for HMWHA regulation of epicardial invasive cell motility.

# CHAPTER 4

# BMP2 RESCUES *Tgfbr3*$^{-/-}$ DELAYED WOUND HEALING AND REQUIRES SRC KINASE.

## 4.1 Introduction

The TGFβ family of growth factors and receptors are characterized in a wide range of cellular processes including regulating proliferation, migration and cell differentiation in both cardiovascular development and disease (138, 139). The Type III TGFβ receptor (TGFβR3) has no intrinsic catalytic activity and is most well characterized to function in TGFβ ligand presentation to Type I and Type II TGFβ receptors which drive epicardial cell invasion (41, 62). Upon TGFβR3 ligand binding, Type I and II receptors engage in canonical (Smad-dependent) signal transduction, but also activate several non-canonical (Smad-independent) signaling pathways such as MAP kinase, Rho GTPase, and Src tyrosine kinase cascades (31). *Tgfbr3*$^{-/-}$ phenotype is lethal at E14.5 as a result of inhibited development of the coronary vasculature (66). *Tgfbr3*$^{-/-}$ epicardial cells retain the ability to migrate to and attach to the myocardium, but fail to undergo cell invasion, leading to inhibited coronary vessel formation.

The bone morphogenic proteins (BMPs) are members of the TGFβ super family of ligands and receptors. BMPs have a wide variety of roles in embryonic tissues including inducing proliferation, migration and differentiation of developing tissues in organogenesis (140-142). The role of BMPs in endocardial cell transformation have been heavily investigated revealing a requirement for TGFβR3 in valve development (143, 144, 130) Recently it has been shown that BMPs function as directional signals in the attachment of the epicardium to the myocardium (53). In this regard, *Tgfbr3*$^{-/-}$ epicardial cells fail to undergo three-dimensional cell invasion *in vitro* when stimulated with BMP2 (41). *Tgfbr3*$^{-/-}$ cells exhibit delayed two-dimensional migration relative to *Tgfbr3*$^{+/+}$ under unstimulated conditions in a wound healing model of cell motility (63), though a deficit in random cell motility between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells as determined by live-cell tracking is not disparately observed.

Although, BMP2 and TGFβ receptor signaling in epicardial cells requires the canonical TGFβ signaling effector Smad4 (41), the requirement of non-canonical BMP effectors are not well studied in the epicardium. Src is a ubiquitously expressed non-receptor tyrosine kinase that is characterized as a driver of cell motility in many cell systems (91). It is unknown whether Src has is involved in TGFβR3 signal transduction, but it is suggested to be activated via Type I TGFβ receptor dependent pathway by TGFβ1 and BMP2 (36, 145). BMP2 is a modulator of the actin cytoskeleton and migration in many cell systems (55, 146), whether these pathways function in epicardial cell biology is unknown.

In order to investigate BMP2 in coronary progenitor cell biology, we used *Tgfbr3*$^{-/-}$ epicardial cells. These cells fail to express BMP2, and as such provides a unique cell model to decipher the function of BMP2 and TGFβR3. The addition of exogenous BMP2 to *Tgfbr3*$^{-/-}$ cells rescues delayed cell migration in the wound healing assay. We revealed that TGFβR3 is not required for BMP2-induced wound healing response. Finally, BMP2 stimulation induces filamentous actin polymerization in epicardial cells, and that Src kinase is required for BMP2 induced filamentous actin polymerization and epicardial cell migration.

## 4.2 Materials and Methods

### Cell lines and culture conditions

Conditionally immortal murine Epicardial cells were originally provided by Dr. Joey Barnett (Vanderbilt Medical University). Cell culture conditions were used as previously described (116). Epicardial cells used in this study were isolated from *wild-type* mouse embryos (*Tgfbr3*$^{+/+}$) and embryos lacking TGFβR3 (*Tgfbr3*$^{-/-}$). Recombinant human BMP2 and mouse Noggin was purchased from R&D Systems (#355-BM-10, #719-NG-050). The Src kinase inhibitor PP2 was purchased from EMD Millipore (#529573).

### RT-PCR

RNA from *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells was isolated and purified using Trizol RNA isolation reagent according to manufacturer's instructions (Invitrogen). cDNA was generated using first strand cDNA synthesis kit (Roche) from 1 µg RNA isolated from each experimental condition. Real-time PCR was

performed as previously described using TaqMan Master primer-probe system (Roche). The following primers were used to detect expression of indicated genes:

| | |
|---|---|
| *RPS7 (f)* | 5'-AGCACGTGGTCTTCATTGCT-3' |
| *RPS7 (r)* | 5'-CTGTCAGGGTACGGCTTCTG-3' |
| *Bmp2 (f)* | 5'-CGGACTGCGGTCTCCTAA-3' |
| *Bmp2 (r)* | 5'-GGGGAAGCAGCAACACTAGA-3' |
| *Has2 (f)* | 5'-GGCGGAGGACGAGTCTATG-3' |
| *Has2 (r)* | 5'-ACACATAGAAACCTCTCACAATGC-3' |
| *Tgfb1 (f)* | 5'-TGGAGCAACATGTGGAACTC-3' |
| *Tgfb1 (r)* | 5'-GTCAGCAGCCGGTTACCA-3' |
| *Tgfb2 (f)* | 5'-TGGAGTTCAGACACTCAACACA-3' |
| *Tgfb2 (r)* | 5'-AAGCTTCGGGATTTATGGTGT-3' |
| *Sm22α (f)* | 5'-CCTTCCAGTCCACAAACGAC-3' |
| *Sm22α (r)* | 5'-GTAGGATGGACCCTTGTTGG-3' |
| *Vim (f)* | 5'-TGCGCCAGCAGTATGAAA-3' |
| *Vim (r)* | 5'-GCCTCAGAGAGGTCAGCAA-3' |

**Wound Healing Assay**

Wound healing assays were performed in accordance with previously described methods (147). *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were seeded at 100,000 cells per well in a 12-well tissue culture dish and grown to confluence. Cells were serum starved for 4 hours (DMEM 0%FBS). A wound was made through the epithelial monolayer with a 200 µL pipette tip and washed twice with 1X phosphate buffered saline. Cells were then stimulated with 2 ng/mL BMP2 and allowed to undergo migration for 24 hours. For experiments using 1 µM PP2 or 200 ng/mL Noggin, cells were pretreated for one hour prior to wound, and replaced with media containing 1 µM PP2 or 200 ng/mL Noggin in the presence or absence of 2 ng/mL BMP2. 0 hour images were taken immediately after the

wound was made and immediately prior to BMP2 stimulation. Additional images were taken at 24 hours after BMP2 stimulation and compared to initial wound (0 hour) images for each condition. Image J was used to assess extent of wound healing by comparing the wound area at time 0 and 24 hours, and % closure of wound area was observed and reported. Each condition was performed in duplicate and repeated at least 3 times. Images were acquired using a Canon Powershot G5 and an Olympus CKX41 inverted microscope.

**Phalloidin staining**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were seeded on glass coverslips at equal and sub-confluent density (75,000 cells/coverslip in 12-well tissue culture plate) and allowed to adhere overnight. Cells were subject to overnight serum starvation (DMEM, 0% FBS), and stimulated with 2 ng/mL BMP2 for 60 minutes. Immunofluorescence visualization of filamentous actin was accomplished using AlexaFlour594 phalloidin (Life Technologies A12381) and a Leica DMLB microscope. Images were documented using a Retiga 200R camera and ImagePro Plus 5.1 software.

**Filamentous Actin Assay**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ *e*picardial cells were seeded at 800,000cells/10cm dish. After attachment, cells were serum starved overnight (DMEM, 0% FBS), subsequently stimulated with 2 ng/mL BMP2 for 60 minutes, and subjected to the G-Actin/F-Actin In Vivo Bioassay Biochem Kit (f/g actin assay) according to the manufacturer's instructions (Cytoskeleton BK037). Briefly, whole cell lysates

were ultracentrifuged (100,000xg) to fractionate filamentous (f) and globular (g) actin fractions. These fractions were resolved by SDS-PAGE and analyzed by Western blot detection of Actin to allow for relative quantitation of filamentous actin in each treatment. Densitometry of g and f actin bands was performed using Image J, and f/g actin ratio was obtained dividing f band density by g band density. f/g ratios under stimulated conditions were plotted relative to unstimulated f/g ratios from *Tgfbr3*$^{+/+}$ conditions. f/g ratio is used as a read out of relative amount of filamentous actin in whole cell lysates.

**Proliferation Assay**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were seeded at 10,000 cell/mL in a 96 well tissue culture dish and allowed to adhere overnight. Cells were serum starved (DMEM 0% FBS) for 4 hours, and subsequently stimulated with BMP2 (2 ng/mL) for 24 hours. Vybrant MTT Proliferation Assay (Promega) was performed according to manufacturer's instructions.

**Statistical Analysis**

All graphs represent mean values, all error bars represent the standard deviation of the mean, all experiments were performed in duplicate repeated at least 3 times. Statistical significance was assessed using a 2-way student's t-test, with p values below 0.05 considered significant.

## 4.3 Results

### 4.3.1 Differential expression of pro-EMT genes in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells

Detection of select genes known to promote epicardial cell invasion and motility was performed by RT-PCR analysis with RNA isolated from *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells. An up-regulation of molecules related to the Type III TGFβ receptor was detected in *Tgfbr3*$^{-/-}$ epicardial cells relative to *Tgfbr3*$^{+/+}$ expression (fig. 4.1). TGFβ2 binds TGFβR3 at picomolar affinity, making TGFβ2 its highest affinity ligand binding partner (120). Expression of TGFβ2, but not TGFβ1, is significantly up-regulated in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$. Hyaluronan Synthase 2 (Has2) synthesizes the glycosaminoglycan hyaluronan (HA), an important component of the extracellular matrix required for cardiac development and epicardial EMT (40, 76). mRNA expression of Has2 is 4.5-fold higher in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$. This outlines another TGFβR3 related compensation, as TGFβR3 is required for HMWHA stimulated epicardial cell invasion (148). Markers of cell differentiation, mesenchymal (vimentin) and vascular smooth muscle (SM22α) are both significantly reduced in *Tgfbr3*$^{-/-}$ cells. BMP2 mRNA is not detected in *Tgfbr3*$^{-/-}$ epicardial cells. Since *Tgfbr3*$^{-/-}$ epicardial cells lack BMP2, we wanted to understand the role of BMP2 in two-dimensional epicardial cell migration

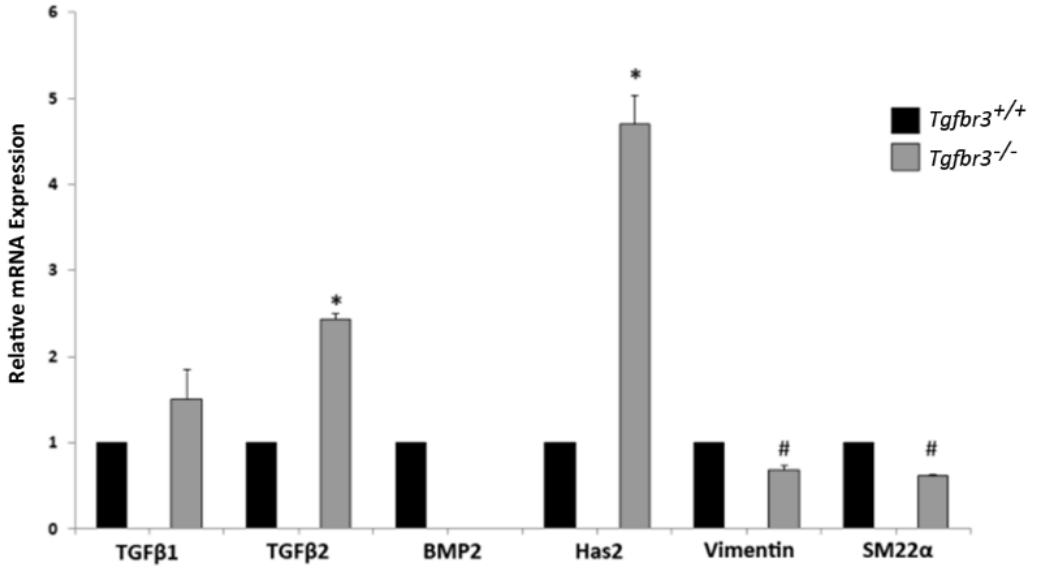


**Figure 4.1 – TGFβ2, BMP2, and Has2 are differentially expressed in *Tgfbr3*$^{-/-}$ cells.**

RNA isolated from *Tgfbr3*$^{+/+}$ (black bars) and *Tgfbr3*$^{-/-}$ (gray bars) epicardial cells was analyzed by RT-PCR for mRNA expression of molecules known to drive EMT. (#=p<0.005, *=p<0.0005).

### 4.3.2 BMP2 rescues *Tgfbr3*$^{-/-}$ delayed wound recovery.

*Tgfbr3*$^{-/-}$ epicardial cells are non-invasive in response to BMP2 in a three dimensional invasion assay (41), we evaluated the effect of BMP2 on cell migration *in vitro*. *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells grown to confluence were subjected to the wound healing assay for 24 hours in the presence, or absence of BMP2. Unstimulated *Tgfbr3*$^{+/+}$ epicardial cells at 24 hours fill in the wound by 82% (fig. 4.2 A-B, 2I). In contrast, there is only 55% wound closure by *Tgfbr3*$^{-/-}$ epicardial cells at 24 hours (fig. 4.2C-D, 4.2I). Thus, *Tgfbr3*$^{-/-}$ epicardial cells have a delay in two-dimensional cell migration as observed in the wound healing assay consistent with previously reported results (63). As BMP2 is shown to stimulate expansion and directional two-dimensional migration of the avian epicardium (53), and *Tgfbr3*$^{-/-}$ epicardial cells lack BMP2 mRNA, we investigated whether the addition of exogenous recombinant BMP2 could rescue delayed *Tgfbr3*$^{-/-}$ epicardial cell migration. The addition of BMP2 to in *Tgfbr3*$^{+/+}$ cells did not superimpose an increased wound healing effect (fig. 4.2E-F, 4.2I). In contrast, BMP2-induced wound healing of *Tgfbr3*$^{-/-}$ epicardial cells to a level comparable to unstimulated *Tgfbr3*$^{+/+}$ epicardial cells (fig. 4.2G-H, 4.2I). Wound healing of *Tgfbr3*$^{-/-}$ epicardial cells were not statistically significantly different from that of unstimulated or BMP2-stimulated *Tgfbr3*$^{+/+}$ epicardial cells (fig. 4.2I). Thus, TGFβR3 is required for epicardial migration in the wound healing model of motility, and BMP2 is sufficient to rescue the *Tgfbr3*$^{-/-}$ delay.

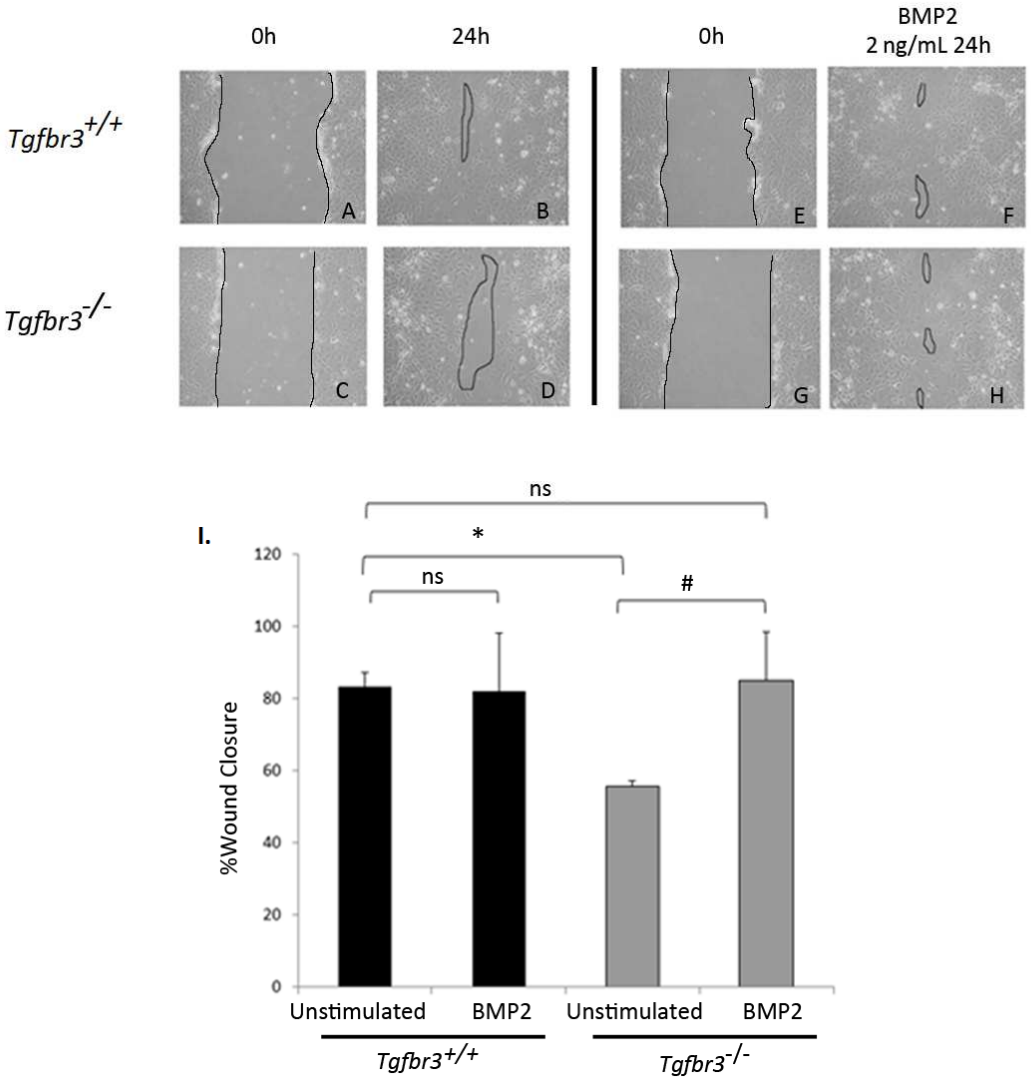


**Figure 4.2 BMP2 rescues *Tgfbr3*$^{-/-}$ delayed cell wound healing response.**

*Tgfbr3*$^{+/+}$ (A-B, E-F) and *Tgfbr3*$^{-/-}$ (C-D, G-H) epicardial cells allowed to migrate for 24 hours in the presence (E-H) or absence (A-D) of BMP2 (2 ng/mL) in the wound healing assay. (*=p<0.0005, #=p<0.05).

### 4.3.3 Epicardially secreted BMPs do not contribute to wound healing response.

We have shown that BMP2 can restore *Tgfbr3*$^{-/-}$ epicardial cell migration in the wound healing assay, which lack endogenous BMP2 expression. A role for epicardially secreted BMPs in driving epicardial wound healing response in *Tgfbr3*$^{+/+}$ cells was tested by neutralizing the action of epicardially secreted BMPs. *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were grown to confluence, and subjected to the wound healing assay for 24 hours in the presence or absence of 200 ng/mL Noggin, a potent sequester and inactivator of BMP ligands (149). As previously shown, unstimulated *Tgfbr3*$^{-/-}$ cells have a 20% delay in cell migration relative to *Tgfbr3*$^{+/+}$ in the wound healing assay (fig. 4.3A-D, 4.3I). Inactivation of BMP ligands by Noggin in this assay has no statistically significant effect on the wound healing response of *Tgfbr3*$^{+/+}$ or *Tgfbr3*$^{-/-}$ epicardial cells (Figure 4.3E-F, 4.3G-H, 4.3I*).* Thus, epicardially secreted BMPs *in vitro* do not contribute to epicardial migration in the wound healing assay.

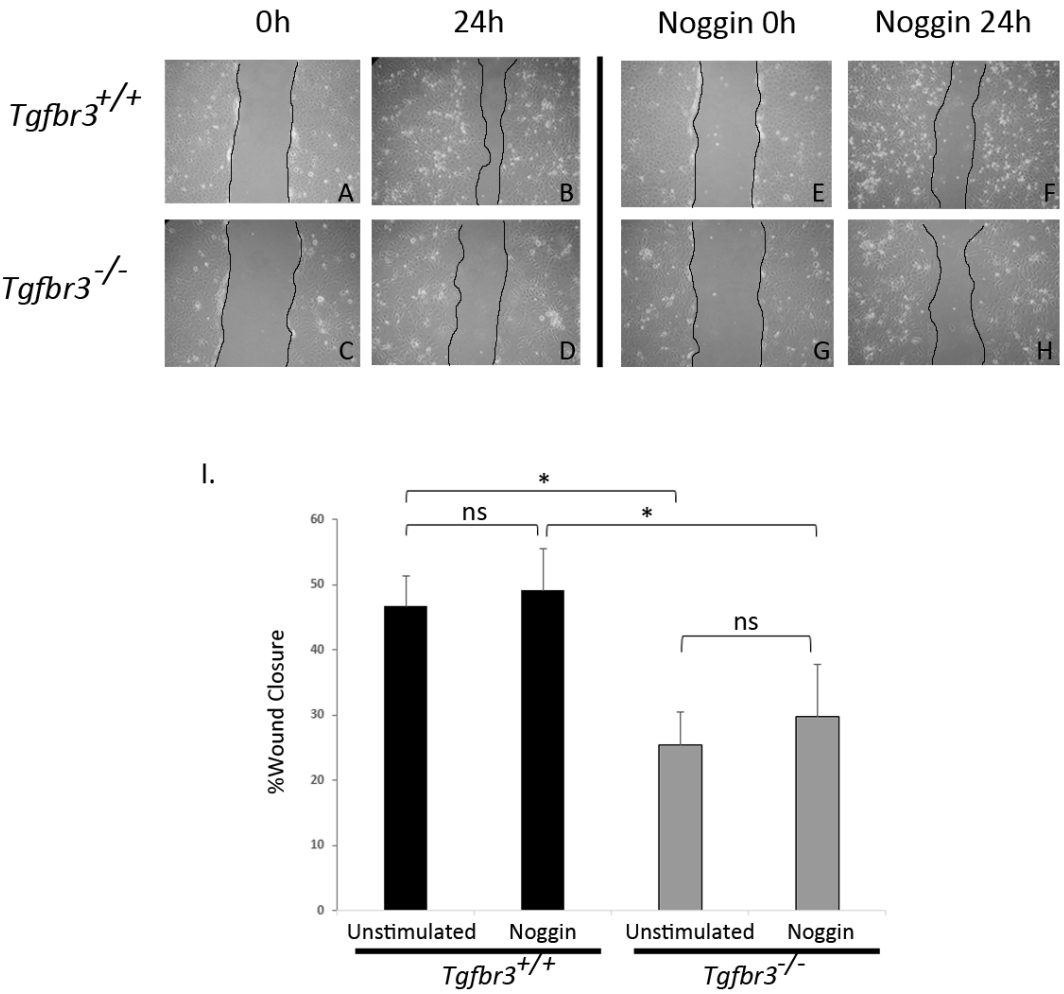


**Figure 4.3 – Endogenous BMPs are not required for epicardial cell wound healing response.**

*Tgfbr3*$^{+/+}$ (A-B, E-F) and *Tgfbr3*$^{-/-}$ (C-D, G-H) epicardial cells were subjected to the wound healing assay, and allowed to migrate for 24 hours in the presence (E-H) or absence (A-D) of Noggin (200 ng/mL). (*=$p<0.05$).

### 4.3.4 Src is required for BMP2 induction of *Tgfbr3*$^{-/-}$ epicardial cell migration in the wound healing assay.

In order to evaluate the role of Src kinase in BMP2-stimulated *Tgfbr3*$^{-/-}$ cell migration, an inhibitor of Src kinase activity, PP2, was used in the wound healing assay. *Tgfbr3*$^{-/-}$ epicardial cells were pre-incubated with PP2, the wound was made and cells were subsequently stimulated with BMP2 (2 ng/mL) for 24 hours in the continued presence of PP2. Unstimulated *Tgfbr3*$^{-/-}$ epicardial cells fill 40% of the wound area (fig. 4.4A-B, 4.4I). *Tgfbr3*$^{-/-}$ cells stimulated with BMP2 increase two-dimensional migration to fill 60% of the wound area (figure 4.4C-D, 4.4I). In the presence of PP2, BMP2-stimulated wound recovery of *Tgfbr3*$^{-/-}$ cells is completely blocked (Figure 4.4G-H, 4.4I). Wound recovery of *Tgfbr3*$^{-/-}$ cells in the presence of PP2 is slightly reduced from that of unstimulated control. These results indicate BMP2-stimulated epicardial cell wound recovery is dependent on Src.

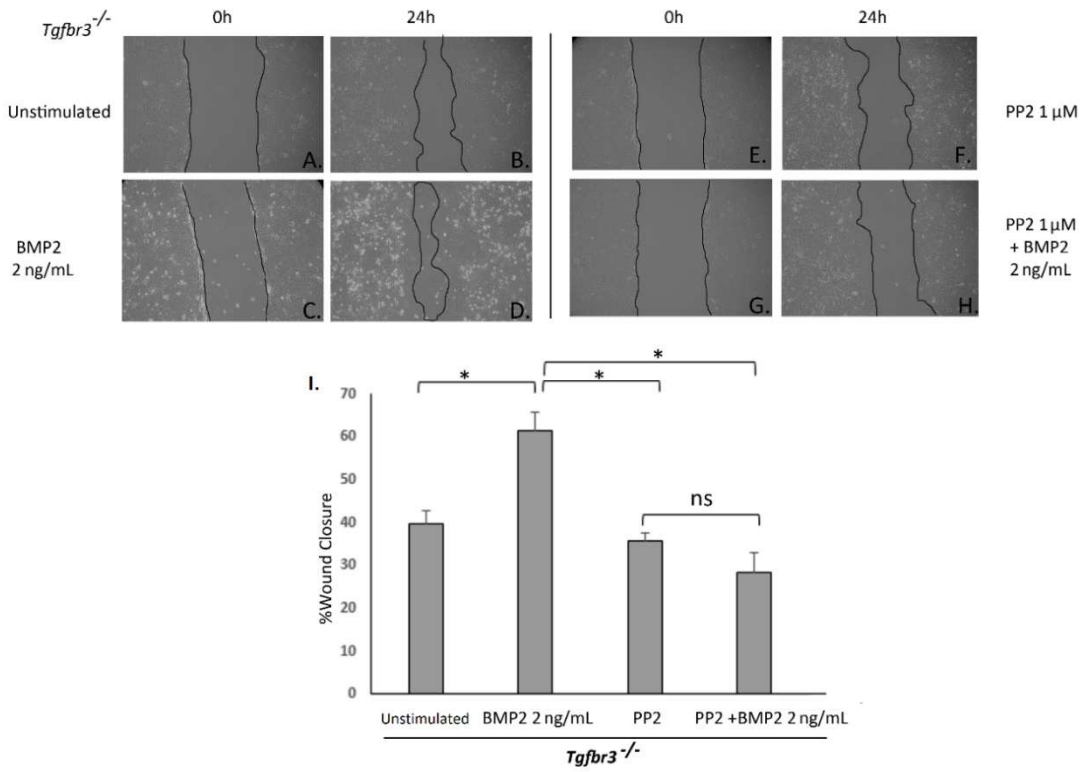


**Figure 4.4 – Src is required for BMP2-stimulated *Tgbr3*$^{-/-}$ wound healing response.**

*Tgfbr3*$^{-/-}$ epicardial cells were subjected to the wound healing assay and allowed to close the wound for 24 hours in the presence (C-D) or absence (A-B) of BMP2 (2 ng/mL). *Tgfbr3*$^{-/-}$ epicardial cells were subjected to the wound healing assay and allowed to migrate for 24 hours in the presence of 1 µM PP2 with (G-H) or without (E-F) BMP2 (2 ng/mL).( *=$p<0.05$).

### 4.3.5 Src is required for BMP2 induction of filamentous actin polymerization.

As the mobilization and reorganization of the actin cytoskeleton for the basis of cell motility (105), we investigated the roles of BMP2 and Src kinase functioning in filamentous actin dynamics. *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were stimulated with BMP2 (2 ng/mL) in the presence or absence of PP2 for 1 hour. Phalloidin staining to visualize actin fibers was performed, and the f/g actin assay for relative quantification of filamentous actin polymerization in whole cell lysates. *Tgfbr3*$^{+/+}$ epicardial cells stimulated with BMP2 induce formation of actin stress fibers and f/g actin ratios of 2.1 relative to untreated control (fig. 4.5A-B, 4.5I). *Tgfbr3*$^{-/-}$ cells under unstimulated conditions have a high basal levels of filamentous actin polymerization (fig. 4.5C-D), and under BMP2 stimulation induce polymerization of f-actin stress fibers and f/g ratios of 2.1 relative to untreated control (fig. 5I). When stimulated with BMP2 in the presence of PP2, formation of filamentous actin in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells is severely blocked (fig 5F, 5H). f/g actin ratios of cells stimulated with BMP2 and PP2 were reduced in *Tgfbr3*$^{+/+}$ (0.87) and *Tgfbr3*$^{-/-}$ (1.47) epicardial cells. *Tgfbr3*$^{-/-}$ cells are less affected, but this may be due to high basal f-actin levels in cells. These data show BMP2 is an inducer of f-actin polymerization in epicardial cells and dependent on Src kinase activity.

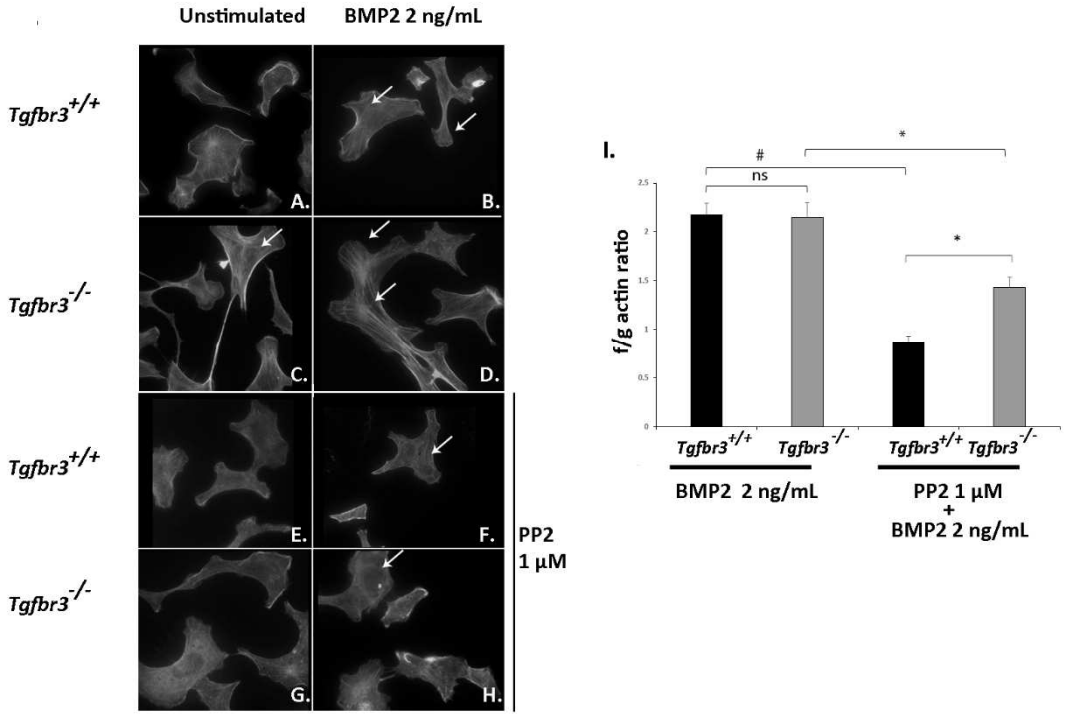


**Figure 4.5– BMP2 induces filamentous actin polymerization in *Tgfbr3*$^{-/-}$ epicardial cells and requires Src.**

(A-H) *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were stimulated with BMP2 (2 ng/mL) in the presence or absence of 1μM PP2 and subject to phalloidin staining (A-H) and the f/g actin assay (I). #=p<0.005, *=p<0.05.

### 4.3.6 BMP2 does not affect epicardial cell proliferation

BMP2 is sufficient to drive migration in a wound healing model of cell motility in *Tgfbr3*$^{-/-}$, but not *Tgfbr3*$^{+/+}$ epicardial cells (fig.4.2). In order to assess whether this was an artifact of enhanced cell proliferation rather than purely an effect of enhanced wound recovery, the MTT proliferation assay was performed under identical experimental conditions as wound healing assays. *Tgfbr3*$^{-/-}$ epicardial cells are hypo-proliferative relative to *Tgfbr3*$^{+/+}$ cells (fig. 4.6). Addition of BMP2 for 24 hours did not enhance or inhibit cell proliferation in *Tgfbr3*$^{+/+}$ or *Tgfbr3*$^{-/-}$ epicardial cells. Proliferation rate of *Tgfbr3*$^{-/-}$ cells in the presence of BMP2 was not statistically significantly different from that of untreated *Tgfbr3*$^{-/-}$ epicardial cells. This highlights an important aspect of this study in that BMP2 is a pro-migratory signal in mouse epicardial cells that has no effect on proliferation. Thus, we can say with confidence that BMP2 rescue of *Tgfbr3*$^{-/-}$ delay in wound recovery is not the result of enhanced proliferation.

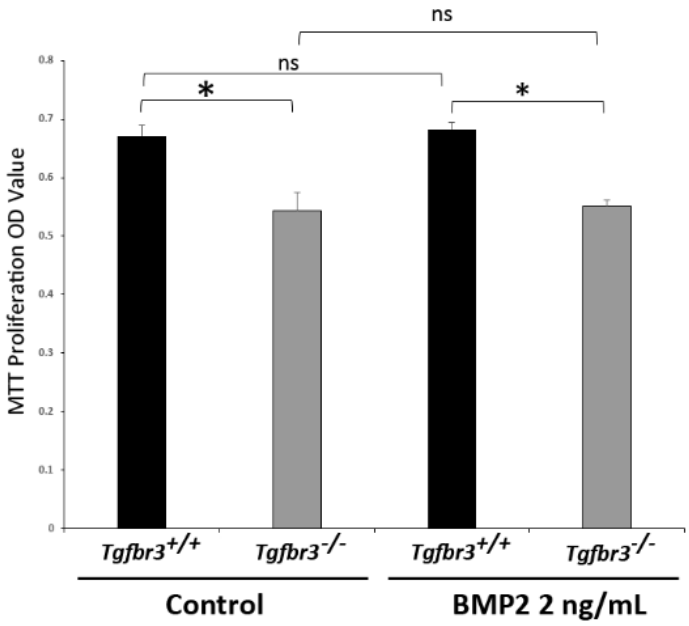


**Figure 4.6- BMP2 does not affect proliferation of epicardial cells.**

*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells were stimulated with BMP2 (2 ng/mL) for 24 hours. MTT Proliferation Assay was performed (*=p<0.005).

## 4.4 Discussion

The development of the coronary vasculature requires migration, invasion, and differentiation of the epicardium. This process is tightly regulated by growth factors influences from the neighboring myocardium to initiate epicardial contact and migration over the developing heart. In this study, we evaluate the role of TGFβR3, BMP2, and Src kinase as a novel pathway in epicardial cell migration required for early developmental events of coronary genesis.

The *Tgfbr3*$^{-/-}$ phenotype is lethal due to decreased epicardially derived cells resident in the myocardium and therefore inhibiting proper coronary vessel formation (66). In culture, *Tgfbr3*$^{-/-}$ cells are deficient in *in vitro* three-dimensional cell invasion in the presence of BMP2 relative to *Tgfbr3*$^{+/+}$. We demonstrate *Tgfbr3*$^{-/-}$ cells execute two-dimensional migration in a wound healing model of motility to levels of *Tgfbr3*$^{+/+}$ cells when stimulated with BMP2 without affecting cell proliferation (fig. 4.2, 4.6). *Tgfbr3*$^{+/+}$ epicardial wound recovery is not enhanced by addition of BMP2. We postulate TGFβR3 dependent signal transduction pathways required for the wound healing response are saturated in *Tgfbr3*$^{+/+}$ cells, and addition of BMP2 would not enhance this response. Conversely, *Tgfbr3*$^{-/-}$ cells are delayed in these TGFβR3 specific wound healing signaling pathways, but addition of BMP rescues this delay independent of TGFβR3. This is reflective of the *in vivo* phenotype in that the epicardium still migrates to, attaches, and covers the myocardium in *Tgfbr3*$^{-/-}$ embryos. Thus, BMP2 secretion from the myocardium is likely to be retained in *Tgfbr3*$^{-/-}$ embryos,

and be adequate to instruct epicardial migration, but not cell invasion, to *Tgfbr3*$^{-/-}$ epicardial cells. Analysis of mRNA expression of *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells demonstrate that types I and II TGFβ receptors, ALKs 1,2,3,4,5,6, expression is maintained at the same levels in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$ (41). Retention of these BMP2 responsive TGFβ superfamily receptors must be sufficient to drive epicardial wound healing in a TGFβR3-independent fashion. This highlights a non-redundant role for *Tgfbr3*$^{-/-}$ in directing cell invasion, but not migration, in coronary vessel development.

Exploring the role of BMP2 in epicardial cell migration was a logical avenue to address, as *Tgfbr3*$^{-/-}$ cells lack BMP2 mRNA (fig. 4.1), and delay in two-dimensional migration relative to *Tgfbr3*$^{+/+}$ in a wound healing model of cell motility (fig. 4.2). BMP2 directs epicardial cell motility towards the myocardium (53) and direct epicardial cell invasion in a TGFβR3 dependent manner (41). Since this phenotype is manifested in *Tgfbr3*$^{-/-}$ cells, we asked whether or not BMPs secreted from epicardial cells *in vitro* act in an autocrine manner were required for unstimulated wound recovery of *Tgfbr3*$^{+/+}$ cells. Using Noggin as a sequesterer and inhibitor of BMPs, we found that there was no change in unstimulated wound recovery of epicardial cells (fig. 4.3). Noggin can sequester other BMP ligands including BMP4, 5, 7, 13, and 14 (149), if these BMPs were involved, then an effect would have been observed. This is not all together surprising, as the myocardium is the major source of BMPs secreted to the epicardium *in vivo,* rather than the epicardium (53). Therefore, this further confirms no role for the action of autocrine BMP signaling in the epicardium.

Although exogenous BMP2 rescues the *Tgfbr3*$^{-/-}$ cell wound healing delay, down-regulated BMP2 expression in *Tgfbr3*$^{-/-}$ cells is not sufficient to fully explain the *in vivo* phenotype, and further study of disparately expressed genes (discussed in chapter 5) is required.

There are some considerations that must be addressed with respect to the model of cell migration used in these studies. We recapitulated a delay in two dimensional migration between *Tgfbr3*$^{-/-}$ epicardial cells relative to *Tgfbr3*$^{+/+}$ in a wound healing model of motility as previously described (63), though live-cell tracking between cell lines demonstrated no difference in random motility between cell lines. Results from the wound healing assay may be affected by several factors: migration, mesenchymal transformation, and proliferation. All three of these processes can contribute to wound closure that is used as the endpoint to assess cell migration. We ruled out proliferation as a confounding factor in our results, as we demonstrate that proliferation remains unaffected in epicardial cells stimulated with BMP2 (fig. 4.6). BMP2-stimulated *Tgfbr3*$^{-/-}$ cells retain the capacity to undergo EMT in two dimensions comparable to *Tgfbr3*$^{+/+}$cells (41). There is a detectable enhancement in the wound healing response in *Tgfbr3*$^{-/-}$ cells stimulated with BMP2, but this may be a result of increased EMT of *Tgfbr3*$^{-/-}$ cells relative to unstimulated *Tgfbr3*$^{-/-}$ cells. The Barnett laboratory does not detect an increase of random cell motility as observed by live cell tracking in *Tgfbr3*$^{-/-}$ cells stimulated with BMP2 (Unpublished). Although live cell tracking is a more sensitive assay for measuring cell motility compared to the wound healing assay, it lacks directionality of motility

toward the chemotactic stimulus being studied. To reconcile these findings, we propose that *Tgfbr3*$^{-/-}$ and *Tgfbr3*$^{+/+}$ epicardial cell migration toward sepharose beads soaked with BMP2 be assessed as similarly executed in avian proepicardial explants by Ishii et al (53). This method not only provides directionality, but is also more replicative of *in vivo* processes than either live cell tracking or the wound healing assay.

Although some previously described non-canonical BMP signaling pathways such as PI3-kinase and cdc42 have been investigated in BMP2-dependent cell migration (55), we assessed the role of Src kinase in BMP2-stimulated two-dimensional cell migration in the wound healing assay. Blocking Src attenuates BMP2-stimulated migration in *Tgfbr3*$^{-/-}$ epicardial cells in the wound healing assay (fig. 4.4). Although BMP2 is known to stimulate cell migration and reorganization of the actin cytoskeleton, a connection to Src kinase in this process has not yet been described. BMP2-induced f-actin polymerization in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells (fig. 4.5) is blocked by PP2. It appears that BMP2 has a more profound effect on formation of dense f-actin structures in *Tgfbr3*$^{-/-}$ cells than *Tgfbr3*$^{+/+}$ as visualized by phalloidin staining (fig. 4.5), although f/g actin ratios are not different between BMP2-stimulated *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells. This is most likely due to high unstimulated levels of f-actin in *Tgfbr3*$^{-/-}$ cells. This study highlights a unique signaling pathway required for migration of epicardial cells in coronary vessel development. We show that TGFβR3 is required for epicardial cell migration in the wound healing model of cell motility, but not BMP2-stimulated migration, in a Src dependent manner.

# CHAPTER 5

# SUMMARY, CONCLUSIONS AND SIGNIFIGANCE, FUTURE DIRECTIONS

## 5.1 SUMMARY

The underlying molecular mechanisms of cardiovascular development and repair have yet to be fully elucidated. The experiments described in this dissertation reveal a unique integration of ECM and growth factor signaling pathways that directs commitment of coronary progenitor cells to constituents of the coronary vasculature. The study of pathways common to HMWHA and TGFβ modulation of epicardial cell behavior remain largely unexplored. We identified Src as a critical regulator of TGFβ2 and HMWHA-stimulated cell invasion, and BMP2-stimulated cell migration. We defined TGFβR3 as a coordinator of HMWHA-stimulated signaling through Src, Rac1, and Rho GTPases and subsequent filamentous actin polymerization, *this is a novel role for TGFβR3*. These insights reveal novel target pathways in the treatment of cardiovascular disease and cancer.

## 5.2 CONCLUSIONS AND SIGNIFIGANCE

The following conclusions are the major contributions of this work to the field of epicardial cell biology and the study of TGFβ and HA signal transduction pathways.

### 5.2.1 Src is required for TGFβ induced invasion and differentiation

The importance of the non-canonical TGFβ signaling pathway is not well studied in the epicardium. We show that TGFβ2 induces HA synthesis and CD44 expression, when Src activity is blocked, this induction is inhibited. Src is required for TGFβ2-induced EMT, cell invasion, filamentous actin polymerization, and smooth muscle differentiation. This requirement for Src is all in the context of TGFβ2. Src activity is required and sufficient to induce EMT, but not smooth muscle differentiation (fig. 5.1, Aim 1).

*What does this mean for improving cardiovascular health?* Fibrotic wound healing was once thought to be the only repair pathway in the heart following myocardial infarction (MI). Following infarction, activated fibroblasts invade the infarct, undergo proliferation, and synthesize collagen and fibrotic matrix to replace infarcted myocardium. This results in a mosaic pattern of scar tissue in the diseased heart (150). Scarring is irreversible, and fibrotic tissue impedes myocardial contractility resulting in decreased heart function and progressive heart failure. The goal of regenerative therapy is to replace infarcted tissue with functional myocardium using cell and molecular strategies. Recent studies have demonstrated that the adult heart does have a regenerative capacity. Following

amputation of the apex of the heart muscle in Zebrafish, 80% of lost myocardium is regenerated from EPDCs (3). This regenerative capacity also exists in mammalian models of MI, wherein mouse EPDCs contribute to neovascularization of the infarct and replacement of cardiomyocytes (4). The T-box 18 (*tbx18*) transcription factor, normally expressed only in coronary vessel development, is re-expressed in the adult epicardium during this regenerative response. TGFβ2 and Has2 are target genes transcribed by *Tbx18,* and therefore may be present and required in this repair (151). This capacity is more limited in mammals than lower vertebrates. Signaling pathways known to be required for coronary vessel development such as Wnt (152, 153) and Notch (154) are reactivated in the epicardium during MI in mouse models. Therefore, examining novel pathways in coronary vessel development may give new insights into molecular regulation of endogenous repair mechanisms.

This intrinsic repair process is not sufficient to fully repair infarcted myocardium in mammals. Work by Riley et al (155, 156) shows that adult epicardium may be reactivated by Thymosin Beta 4 (Tβ4) and enhances intrinsic epicardial contribution to myocardial repair in a mouse model of MI. These studies remain controversial as Pu et al (157) have failed to reproduce these effects. It is more likely that multiple, rather than single factors, are required to reactivate adult epicardium in repair. We propose that TGFβ2 is an attractive candidate for aiding in myocardial replacement by EPDCs post MI. Local injection of TGFβ2 in the subepicardial space adjacent to an infarct could conceivably contribute to myocardial repair and neovascularization of the adult

heart. Adult human epicardial cells are responsive to TGFβ ligands by undergoing EMT and smooth muscle differentiation *in vitro* (158), but the role of the TGFβ pathway in an *in vivo* model of mammalian heart regeneration post MI has not yet been addressed.

Inhibitors to Src are attractive candidates in cancer therapy with several in preclinical stages of development used as single or as a combination therapy with existing drugs (159). We show Src to be required for TGFβ2-induced epicardial EMT and cell invasion. As Src inhibitors are potential cancer therapies, patients undergoing future treatment who experience myocardial infarctions may have inhibited endogenous Src-dependent repair pathways that could negatively affect outcomes post MI. These small molecule inhibitors to Src may prove to be cardio toxic like most tyrosine kinase inhibitors (82). Preserving the regenerative capacity of the heart will become a key therapeutic area in precision medicine. Conversely, temporary administration of Src activators (such as TGFβ2, HMWHA, and BMP2 as presented in this work) to the heart following myocardial infarction may help improve cardiovascular outcomes.

This study addresses the role of Src in TGFβ2-dependent developmental EMT and cell invasion, but it does have broader implications for cancer biology. TGFβ activity is dysregulated in many human cancers including breast, ovarian and prostate (160). Diagnostic tools assessing molecular basis of individual tumors are becoming more widely used, and identifying TGFβ dependent tumors will lead to better targeted therapies in the clinic. We show Src to be required for TGFβ2-induced EMT and invasion, conceivably, it could be recommended that

drugs targeting Src be used as therapies in TGFβ dependent cancers to improve patient outcomes.

5.2.2 TGFβR3 is an HMWHA-responsive, Src activating receptor.

This study we identify that TGFβR3 is required for HMWHA-stimulated epicardial cell invasion and activation of Src kinase. This is a significant and novel finding identifying TGFβR3 as an HMWHA responsive Src activating receptor. TGFβR3 is required for HMWHA-stimulated of Rac1 and RhoA GTPase activation. It is known that HA stimulates Src, Rac1 and RhoA activation, though the requirement of TGFβR3 for this activation constitutes a *novel* finding in the fields of HA signal transduction and heart development. TGFβR3 activity is sufficient to drive Src kinase activation, establishing a novel role for TGFβR3 as being directly upstream of Src signaling (fig. 5.1 Aim 2).

*How can these new insights be applied to human health?* The capacity for the adult epicardium to contribute to myocardial repair in response to infarct is a rapidly growing area of research. Our work demonstrates TGFβ2 and HA share a common mediator in Src for directing epicardial cell invasion. As such, Src may serve as a central node directing the development of the coronary vasculature. As cardiovascular development and repair are regulated by integrating multiple growth factor and extracellular matrix signals, it is likely that therapies to enhance repair will consist of multiple agents. For example, a cocktail of Src-activating growth factors and ECM may induce epicardial repair of infarcted myocardium.

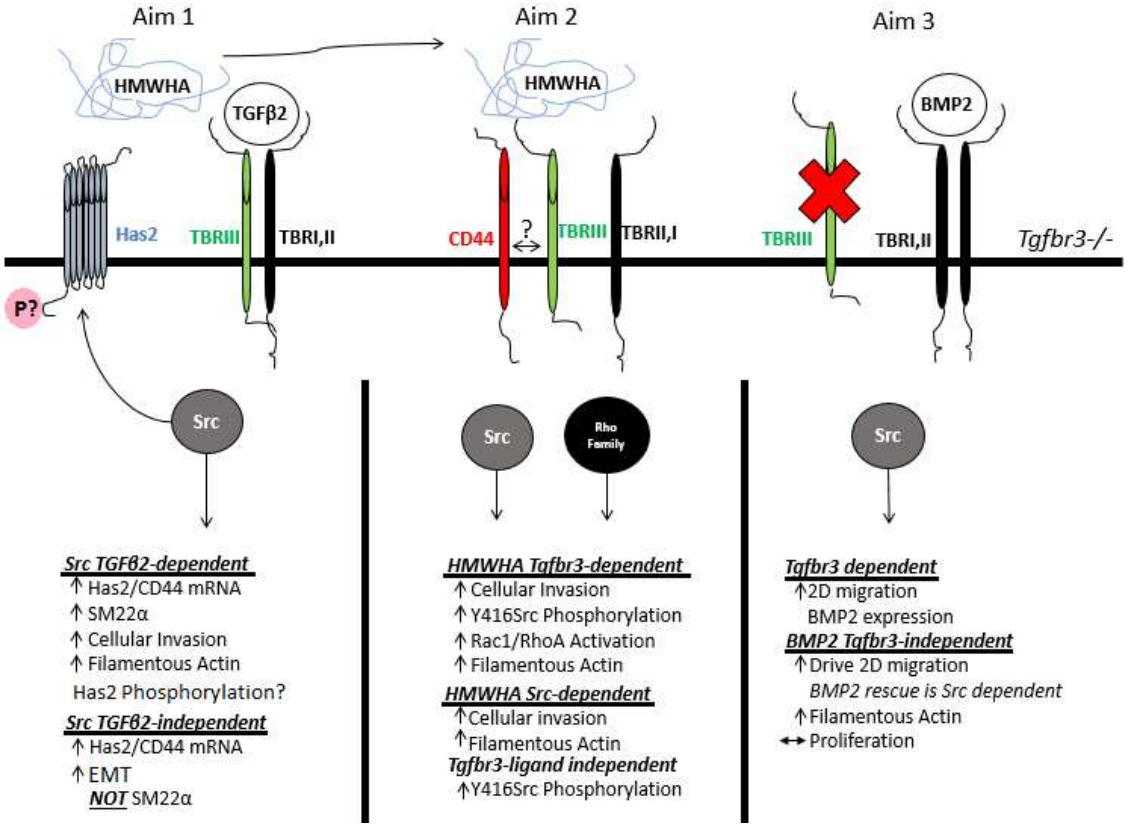


**Figure 5.1 –Src is a critical regulator of TGFβ and HMWHA-stimulated epicardial cell invasion, migration, and differentiation.**

**(Aim 1)** Src is required for TGFβ2-stimulated HA synthesis, EMT, invasion, smooth muscle differentiation, and filamentous actin polymerization. **(Aim 2)** TGFβR3 and Src are required for HMWHA-stimulated epicardial EMT, invasion and filamentous actin polymerization. TGFβR3 is directly upstream of HMWHA-induced Src, Rac1, and RhoA activation. **(Aim 3)** BMP2 directs cell migration in the wound healing assay independent of TGFβR3 without affecting cell proliferation in a Src dependent manner.

Given our studies described in this dissertation, a TGFβ2 and HMWHA combination agent would make for an attractive therapy in reactivating the epicardium in repair post-myocardial infarction.

TGFβR3 functions in coronary vessel development and has a role in cancer cell motility and metastasis. TGFβR3 has been studied in the context of TGFβ and FGF growth factor ligand signal transduction almost exclusively. We discovered TGFβR3 is required for HMWHA-stimulated signal transduction and cell invasion. HA overproduction is observed in several cancer cell lines and contributes to cell invasion of metastatic cancers including breast, ovarian, and prostate (161). As TGFβR3 plays a role in HMWHA-directed cell invasion during developmental EMT and cell invasion, these pathways may be similarly observed in tumor progression. We propose TGFβR3 to be a possible therapeutic target in HA-dependent cancers. As TGFβR3 lacks a catalytic domain, therapeutic strategies including the development of monoclonal antibodies targeting TGFβR3 to block HMWHA responsiveness could be effective against metastasizing tumors in a similar way as Trastuzumab (Herceptin) indicated in HER2+ breast cancer treatment (162). Given our findings that TGFβR3 is required for HMWHA-stimulated Src, Rac1 and RhoA activation, we postulate that blocking TGFβR3 in HMWHA-dependent tumors would decrease activation of these effectors ultimately leading to decreased cell motility and cell invasion, leading to improved patient outcomes.

### 5.2.3 BMP2 drives epicardial cell wound healing response independent of TGFβR3

It has previously been shown that *Tgfbr3*$^{-/-}$ epicardial cells are non-responsive to TGFβ ligands in cell invasion assays, as well as exhibit delayed two-dimensional migration in the wound healing assay. The delay of *Tgfbr3*$^{-/-}$ cells in cell migration relative to *Tgfbr3*$^{+/+}$ cells was recapitulated in our studies, a molecular explanation for this phenotype has not yet been addressed. We discovered that *Tgfbr3*$^{-/-}$ epicardial cells do not express BMP2. We tested whether exogenous BMP2 could rescue delayed wound recovery in *Tgfbr3*$^{-/-}$ cells. BMP2 is sufficient to drive the wound healing response of *Tgfbr3*$^{-/-}$ cells without affecting proliferation, and requires Src kinase (fig. 5.1 Aim 3). Since this rescue effect occurs in the absence of TGFβR3, we must conclude this effect was independent of the receptor.

*How can these novel findings contribute to improved cardiovascular outcomes and other diseases?* Src-dependent epicardial cell motility stimulated by BMP2 reveals a novel pathway in the epicardium. BMP2 could be used to initiate adult epicardial migration into the site of a myocardial infarct. BMP2 is currently approved for use in the clinic to facilitate bone fracture healing and recovery from orthopedic surgery, though complications have included increased incidence of cancers, and long-term benefits are still rather unclear (163, 164). BMP2 could be repurposed for cardiac repair and regeneration. More investigation is required to circumvent these risks and evaluate long-term outcomes of BMP2 as a regenerative therapy. This study defines BMP2-

stimulated epicardial cell migration and filamentous actin polymerization to be Src dependent, the use of Src inhibitors could be indicated in BMP-dependent cancers such as osteosarcoma (165).

We have defined a common pathway between TGFβ2, HMWHA, and BMP2 required for epicardial EMT, cell invasion, and migration. HMWHA, TGFβ2, and BMP2 stimulate epicardial EMT and cell invasion. TGFβ2 is an inducer of smooth muscle differentiation, whereas HMWHA is not inductive of a smooth muscle phenotype (Barnett Unpublished), and BMP2 inhibits TGFβ2-induced smooth muscle differentiation. BMP2 stimulates two dimensional migration in the wound healing assay, whereas HMWHA and TGFβ2 block this process (Allison Unpublished). It is the temporal and spatial regulation of these factors, and others, in the subepicardial space that directs coronary vessel development. With regards to cardiovascular repair, identifying the ability of these three factors to enhance epicardial repair of the adult infarcted heart will require subtle manipulation of concentration and timing of introduction to the subepicardial space proximal to myocardial infarct. We have defined a shared Src-dependent signaling network that represents a new target pathway for therapies against cardiovascular disease.

## 5.3 FUTURE DIRECTIONS

The present study has identified novel signaling pathways required for TGFβ and HMWHA stimulated epicardial cell differentiation, invasion and migration required for development of the coronary vasculature; however, these pathways described require further elucidation.

### 5.3.1 What is the role of CD44 in TGFβR3 dependent HA-mediated cell invasion?

We have demonstrated that *Tgfbr3*$^{-/-}$ cells fail to activate Src, Rac1, and RhoA activity under HMWHA stimulation, and are deficient in HMWHA-induced cell invasion (Ch. 3). It is clear that TGFβR3 is required for HMWHA responsiveness in epicardial cells, but the molecular interactions governing this at the receptor level is unclear. We postulate that TGFβR3 acts as a co-receptor with HMWHA-binding receptors rather than directly binding HMWHA. This has yet to be demonstrated, but reverse IP of TGFβR3 and HA could rule-out a TGFβR3-HMWHA interaction. Do CD44 and TGFβR3 interact upon HA-stimulation?

CD44 is not differentially expressed between *Tgfbr3*$^{-/-}$ and *Tgfbr3*$^{+/+}$ epicardial cells (63), so it is clear CD44 expression is not sufficient for HA-stimulated cell invasion in *Tgfbr3*$^{-/-}$ cells. HA binding to CD44 extracellular domain is regulated by specific glycosylation sites (166), is it possible that CD44 glycosylation status in *Tgfbr3*$^{-/-}$ cells is less favorable for HMWHA binding than in

*Tgfbr3*$^{+/+}$ cells? This could explain the lack of HA-responsiveness while CD44 expression remains equal between cell lines. Mass spectroscopy techniques would be required to assess glycosylation status of CD44 between *Tgfbr3*$^{-/-}$ and *Tgfbr3*$^{+/+}$ cells.

### 5.3.2 What other disparately expressed genes between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells could contribute to phenotype?

We have demonstrated that several genes, including *Has2* and *Bmp2* that are required for epicardial cell invasion (40, 41), are disparately expressed between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells (fig. 4.1). Although exogenous BMP2 is sufficient to drive *Tgfbr3*$^{-/-}$ epicardial cell migration in two dimensions, it is not sufficient to drive three dimensional cell invasion into a collagen matrix (41). Integrin receptors mediate cell-matrix interactions including Fibronectin, Collagens, and VCAM (96). The α4 integrin subunit is required for epicardial attachment and invasion into the myocardium (167, 168). RT-PCR analysis of α4 and β1 integrin mRNA expression in*Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells was performed (fig. 5.2A). α4 integrin mRNA, but not β1 integrin mRNA, is down-regulated in *Tgfbr3*$^{-/-}$ epicardial cells relative to *Tgfbr3*$^{+/+}$. Protein levels of α4 integrin was assessed by Western blot analysis in whole cell lysates obtained from *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells (fig. 5.2B). Down-regulation of α4 integrin mRNA in *Tgfbr3*$^{-/-}$ cells results in down-regulation of α4 integrin protein in *Tgfbr3*$^{-/-}$ cells.

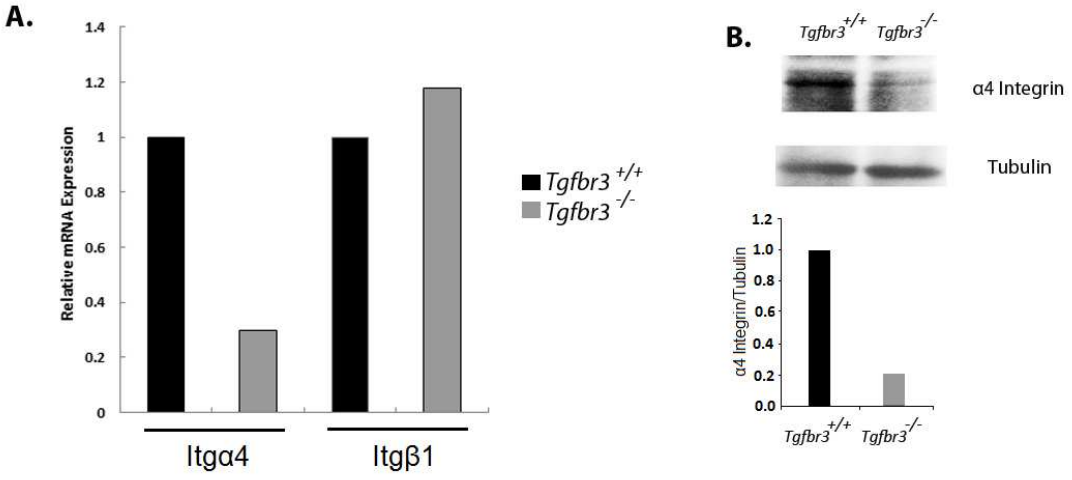


**Figure 5.2 – Expression of Integrins in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells.**

(A) mRNA expression of Itgα4 and Itgβ1 in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells. (B) Western blot detection of α4 Integrin protein levels in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ epicardial cells.

*What do these findings mean for the observed Tgfbr3$^{-/-}$ epicardial phenotype?* Conceivably, a down-regulation of α4 integrin could result in decreased epicardial invasion into the myocardium and invasion into collagen matrix in *in vitro* assays. Although we do not speculate this deficit contributes to insensitivity to TGFβ ligands or HA matrix, a basally low level of α4 integrin expression could prime *Tgfbr3*$^{-/-}$cells to be deficient in cell invasion into the collagen matrix. Could re-expressing α4 integrin in *Tgfbr3*$^{-/-}$epicardial cells to equivalent *Tgfbr3*$^{+/+}$ levels rescue defective cell invasion into collagen matrix in the presence of TGFβs or HMWHA? What effect would re-expression of α4 integrin have on Src/Rac1/RhoA activation and f-actin polymerization in the presence of HMWHA or TGFβ ligands? Is expression of α4 integrin stimulated by TGFβR3? If re-expressing TGFβR3 in *Tgfbr3*$^{-/-}$ cells could restore α4 integrin expression, this would constitute a novel link between TGFβR3 and α4 integrin.

Experiments evaluating the activity and expression of critical transcription factors in EMT may be investigated. ZEB1, Twist, and Snail are transcriptional regulators of EMT (16). Is expression or activation of these transcription factors disparate between *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ cells under TGFβ or HMWHA induction of EMT or cell invasion? Is there a connection between TGFβR3 or Src and these transcription factors?

In our laboratory, we are currently conducting *in situ* hybridization studies in *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ E11.5-13.5 embryos to visualize expression of *Bmp2*, *Has2*, and *Itgα4* to validate our *in vitro* expression data presented in this study (fig. 4.1). We are developing *in vitro* assays to assess immortalized epicardial

cell invasion into primary embryonic myocardium. This may provide a more relevant *in vitro* model than the collagen gel invasion assay, as myocardial attachment and invasion into the myocardium can be assessed with all the advantages of an *in vitro* assay.

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