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RESEARCH PAPER

BMP2 rescues deficient cell migration in *Tgfbr3*$^{-/-}$ epicardial cells and requires Src kinase

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ABSTRACT

During embryogenesis, the epicardium undergoes proliferation, migration, and differentiation into several cardiac cell types which contribute to the coronary vessels. The type III transforming growth factor-β receptor (TGFβR3) is required for epicardial cell invasion and development of coronary vasculature in vivo. Bone Morphogenic Protein-2 (BMP2) is a driver of epicardial cell migration. Utilizing a primary epicardial cell line derived from *Tgfbr3*$^{+/+}$ and *Tgfbr3*$^{-/-}$ mouse embryos, we show that *Tgfbr3*$^{-/-}$ epicardial cells are deficient in BMP2 mRNA expression. *Tgfbr3*$^{-/-}$ epicardial cells are deficient in 2-dimensional migration relative to *Tgfbr3*$^{+/+}$ cells; BMP2 induces cellular migration to *Tgfbr3*$^{+/+}$ levels without affecting proliferation. We further demonstrate that Src kinase activity is required for BMP2 driven *Tgfbr3*$^{-/-}$ migration. BMP2 also requires Src for filamentous actin polymerization in *Tgfbr3*$^{-/-}$ epicardial cells. Taken together, our data identifies a novel pathway in epicardial cell migration required for development of the coronary vessels.

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BMP2; epicardium; migration; Src; TGFβR3

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.[1] Cells from the proepicardium are transferred to the surface of the heart to form the epicardium that contribute to the coronary vessels. 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.[2,3] Epicardially derived cells differentiate into cardiac fibroblasts resident in the myocardium that contribute to repair, and vascular smooth muscle cells that constitute the mature coronary vessels.[4] As cell migration 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.[5]

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.[6,7] TGFβ2 binds TGFβR3 at picomolar affinity,[28] and TGFβR3 required for TGFβ2 and TGFβ1 induced epicardial cell invasion.[19] 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.[8,9] 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.[10] *Tgfbr3*$^{-/-}$ phenotype is lethal at E14.5 as a result of inhibited development of the coronary vasculature.[11] *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. *In vitro*, *Tgfbr3*$^{-/-}$ epicardial cells fail to undergo cellular invasion (migration into, and residence in a 3D matrix), and exhibit delayed migration (migration in 2-dimensions) relative to wild-type.[19]

The Bone Morphogenic Proteins (BMPs) are members of the TGFβ super family of ligands and receptors.

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BMPs have a wide variety of roles in embryonic tissues including inducing proliferation, migration and differentiation of developing tissues in organogenesis.[12-14] The role of BMPs in endocardial cell transformation have been heavily investigated revealing a requirement for TGFβR3 in valve development.[15-17] TGFβ1, BMP7 and BMP2, directly bind to TGFβR3.[34] TGFβR3 is required for BMP2 activation of Smad1 (canonical signaling) and BMP2-dependent endocardial cell transformation.[34] It has been shown that BMPs function as directional signals in the attachment of the epicardium to the myocardium.[18] In this regard, *Tgfbr3*$^{-/-}$ epicardial cells fail to undergo 3-dimensional cell invasion *in vitro* when stimulated with BMP2.[8] *Tgfbr3*$^{-/-}$ cells exhibit deficient 2-dimensional migration relative to *Tgfbr3*$^{+/+}$ under unstimulated conditions in a wound healing model of cell motility.[19]

Although, BMP2 and TGFβ receptor signaling in epicardial cells requires the canonical TGFβ signaling effector Smad4,[8] 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.[20] Src is activated via Type I TGFβ receptor dependent pathway by TGFβ1 and BMP2.[21,22] The Type III TGFβ receptor is directly upstream of Src activity [23] BMP2 is a modulator of migration in many cell systems,[24,25] whether BMP2 and Src are required for epicardial cell migration 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 deficient cell migration in the wound healing assay. We revealed that TGFβR3 is not required for BMP2-induced migration. 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.

Results

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

Detection of select genes known to promote epicardial cell invasion and migration 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. 1). TGFβ2 binds TGFβR3 at picomolar affinity, making TGFβ2 its highest affinity ligand binding partner.[28] Expression of TGFβ2, but not TGFβ1, is significantly up-regulated in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$; enhanced expression of TGFβ2 in vitro is likely compensatory for loss of TGFβR3, though not significant to direct cell behavior. Hyaluronan Synthase 2 (Has2) synthesizes the glycosaminoglycan hyaluronan (HA), an important component of the extracellular matrix required for cardiac development and epicardial migration and invasion.[29,30] mRNA expression of Has2 is 4.5-fold higher in *Tgfbr3*$^{-/-}$ cells relative to *Tgfbr3*$^{+/+}$. This further outlines TGFβR3 related compensation, as TGFβR3 is required for both TGFβ2 and HMWHA stimulated epicardial cell invasion.[31] 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, though it is not known if TGFβR3 drives BMP2 expression in epicardial cells. Deficient expression of these genes (SM22α, vimentin, and BMP2) could pre-dispose *Tgfbr3*$^{-/-}$ epicardial cells to be less sensitive to differentiation and cell migration. Since *Tgfbr3*$^{-/-}$ epicardial cells lack BMP2, we wanted to understand the role of BMP2 in 2-dimensional epicardial cell migration

BMP2 rescues *Tgfbr3*$^{-/-}$ deficient migration

Tgfbr3$^{-/-}$ epicardial cells are non-invasive in response to BMP2 in a 3 dimensional invasion assay,[8] we evaluated the effect of BMP2 on 2-dimensional migration. *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% (Figs. 2A, B, I). In contrast, there is only 55% wound closure by *Tgfbr3*$^{-/-}$ epicardial cells at 24 hours (Figs. 2C, D, I). Thus, *Tgfbr3*$^{-/-}$ epicardial cells are deficient in 2-dimensional cell migration as observed in the wound healing assay consistent with previously reported results.[19] As BMP2 is shown to stimulate expansion and directional 2-dimensional migration of the avian epicardium,[18] and *Tgfbr3*$^{-/-}$ epicardial cells lack BMP2 mRNA, we investigated whether the addition of exogenous recombinant BMP2 could rescue deficient *Tgfbr3*$^{-/-}$ epicardial cell migration. BMP2 did not superimpose an increased migration effect in *Tgfbr3*$^{+/+}$ cells (Figs. 2E, F, I). In contrast, BMP2-induced migration of *Tgfbr3*$^{-/-}$ epicardial cells to a level comparable to unstimulated *Tgfbr3*$^{+/+}$ epicardial cells (Figs. 2G, H, I). Migration of *Tgfbr3*$^{-/-}$ epicardial cells were not statistically significantly different from that of unstimulated or BMP2-stimulated *Tgfbr3*$^{+/+}$ epicardial cells (Fig. 2I). Thus,

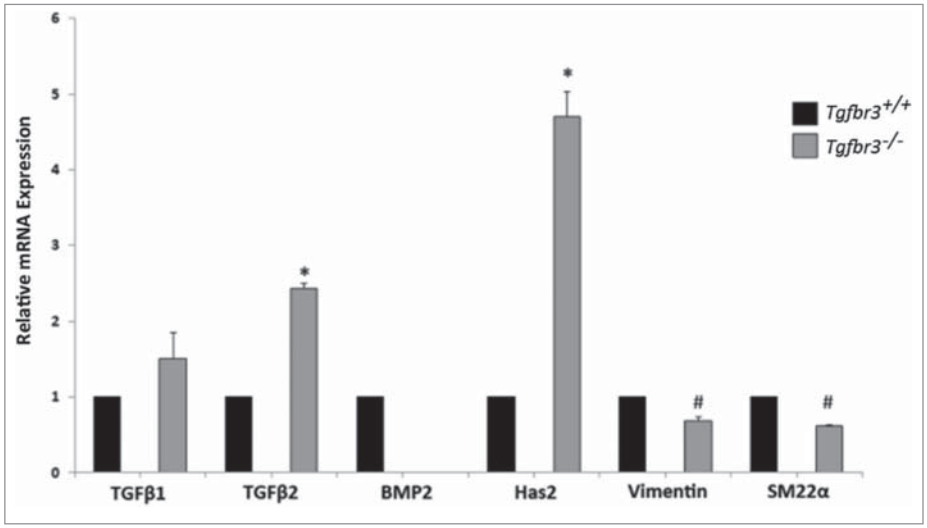

Figure 1. TGFβ2, BMP2, and Has2 are differentially expressed in *Tgfbr3*$^{-/-}$ cells. RNA isolated from *Wild-type* (black bars) and *Tgfbr3*$^{-/-}$ (gray bars) epicardial cells was analyzed by RT-PCR for mRNA expression of molecules known to drive migration. (#=p < 0.005, *=p < 0.0005).

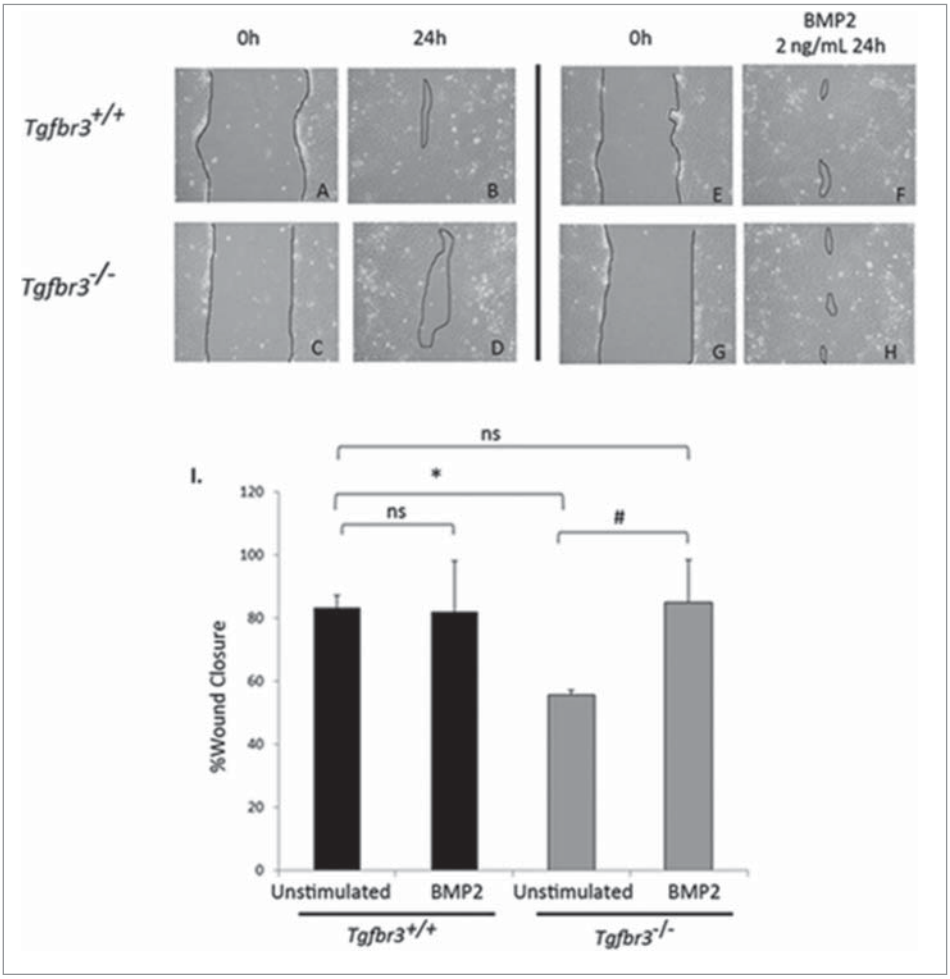

Figure 2. BMP2 rescues *Tgfbr3*$^{-/-}$ deficient cell migration. *Wild-type* (A-B, E-F) and *Tgfbr3*$^{-/-}$ (C-D, G-H) epicardial cells allowed to migrate for 24 hours in the presence (E-H) or absence (A-D) of BMP2 (2 ng/mL) in the wound healing assay. (*=p < 0.0005, #=p < 0.05).

TGFβR3 is required for epicardial migration in the wound healing model of motility, and BMP2 is sufficient to rescue the *Tgfbr3*$^{-/-}$ deficit.

Epicardially secreted BMPs do not drive migration

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.[32] As previously shown, unstimulated *Tgfbr3*$^{-/-}$ cells have a 20% deficiency in cell migration relative to *Tgfbr3*$^{+/+}$ (Figs. 3A, D, I). Inactivation of BMP ligands by Noggin in this assay has no statistically significant effect on migration of *Tgfbr3*$^{+/+}$ or *Tgfbr3*$^{-/-}$ epicardial cells (Figs. 3E–I). Thus, epicardially secreted BMPs *in vitro* do not contribute to epicardial migration.

Src is required for BMP2 induction of Tgfbr3$^{-/-}$ epicardial cell migration

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 (Figs. 4A, B, I). *Tgfbr3*$^{-/-}$ cells stimulated with BMP2 increase 2-dimensional migration to fill 60% of the wound area (Figs. 4C, D, I). In the presence of PP2, BMP2-stimulated migration of *Tgfbr3*$^{-/-}$ cells is completely blocked (Figs. 4G, H, I). Migration of *Tgfbr3*$^{-/-}$ cells in the presence of PP2 is slightly reduced from that of unstimulated

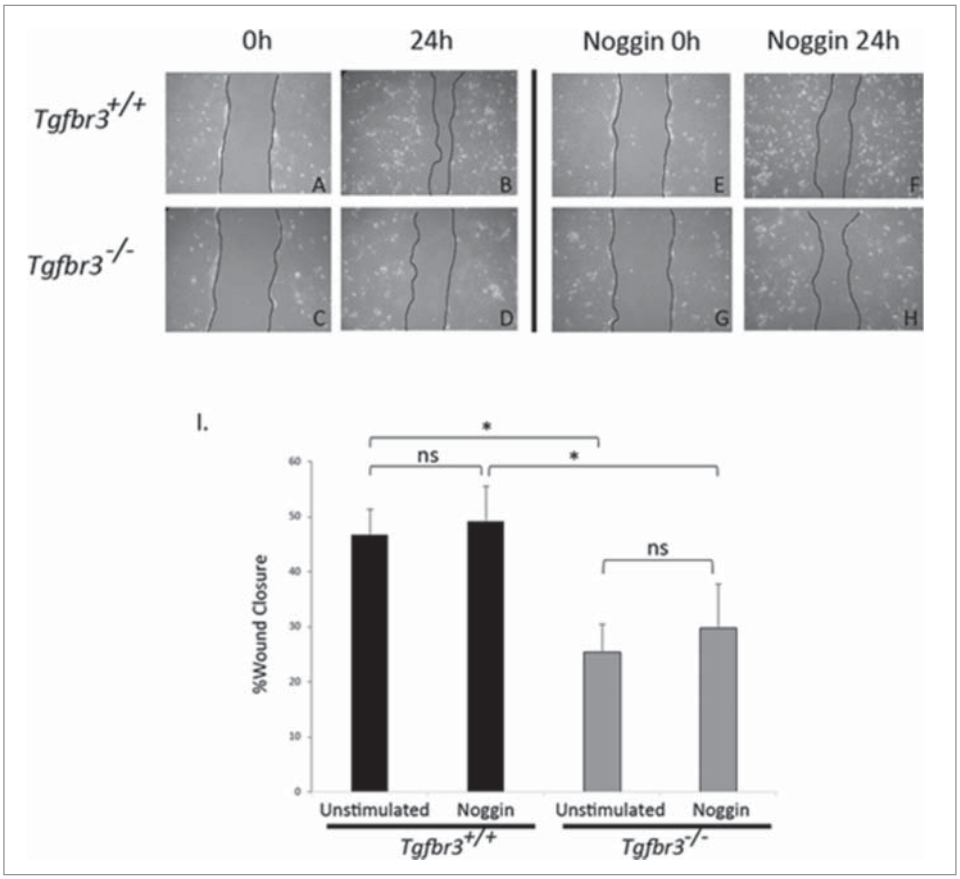

Figure 3. Endogenous BMPs are not required for epicardial cell migration. *Wild-type* (A-B, E-F) and *Tgfbr3*$^{-/-}$ (C-D, G-H) epicardial cells were subjected to the wound healing assay, and allowed to migrate for 24 hours in the presence (E-H) or absence (A-D) of Noggin (200 ng/mL). (*=$p < 0.05$).

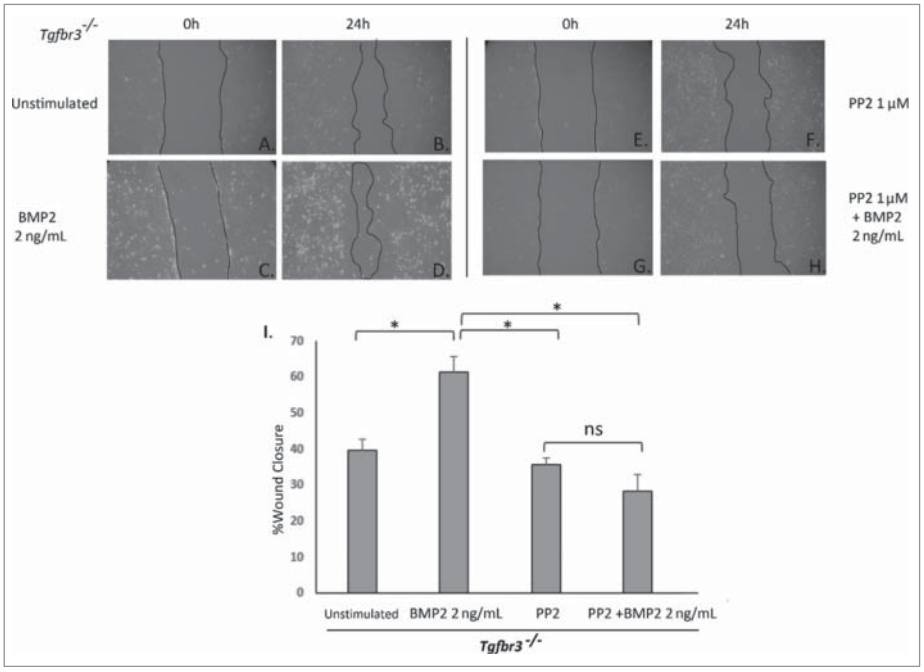

Figure 4. Src is required for BMP2-stimulated *Tgbr3*$^{-/-}$ epicardial cell migration. *Tgfbr3*$^{-/-}$ epicardial cells were subjected to the wound healing assay and allowed to close the wound for 24 hours in the presence (C-D) or absence (A-B) of BMP2 (2 ng/mL). *Tgfbr3*$^{-/-}$ epicardial cells 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).(*=$p < 0.05$).

control. These results indicate BMP2-stimulated epicardial cell migration is dependent on Src.

Src is required for BMP2 induction of filamentous actin polymerization

We investigated the roles of BMP2 and Src kinase functioning in filamentous actin dynamics required for cell migration.[33] *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 filamentous actin polymerization, and f/g actin ratios of 2.1 relative to untreated control (Figs. 5A, B, I). *Tgfbr3*$^{-/-}$ cells under unstimulated conditions have a high basal levels of filamentous actin polymerization (Figs. 5C and 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 (Figs. 5F, H, I). 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, independent of TGFβR3 and dependent on Src kinase activity.

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. 2). In order to assess whether this was an artifact of enhanced cell proliferation rather than purely an effect of enhanced migration, 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. 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*$^{-/-}$ deficient migration is not the result of enhanced proliferation.

Discussion

The development of the coronary vasculature requires migration, invasion, and differentiation of the

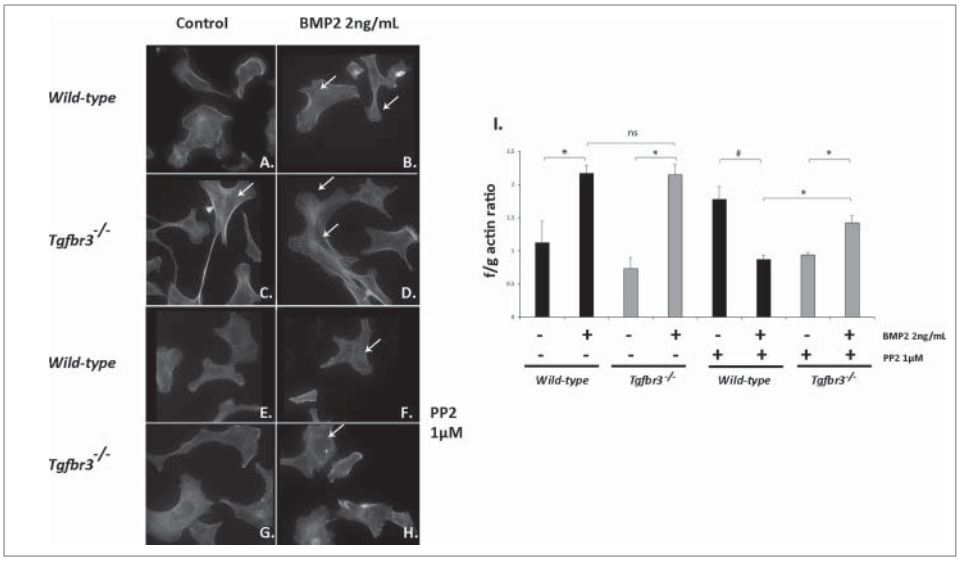

Figure 5. BMP2 induces filamentous actin polymerization in *Tgfbr3*$^{-/-}$ epicardial cells and requires Src. (A-H) *Wild-type* and *Tgfbr3*$^{-/-}$ epicardial cells were stimulated with BMP2 (2 ng/mL) in the presence or absence of 1μM PP2 and subject to phalloidin staining (A-H) and the f/g actin assay (I). #=$p < 0.005$, *=$p < 0.05$.

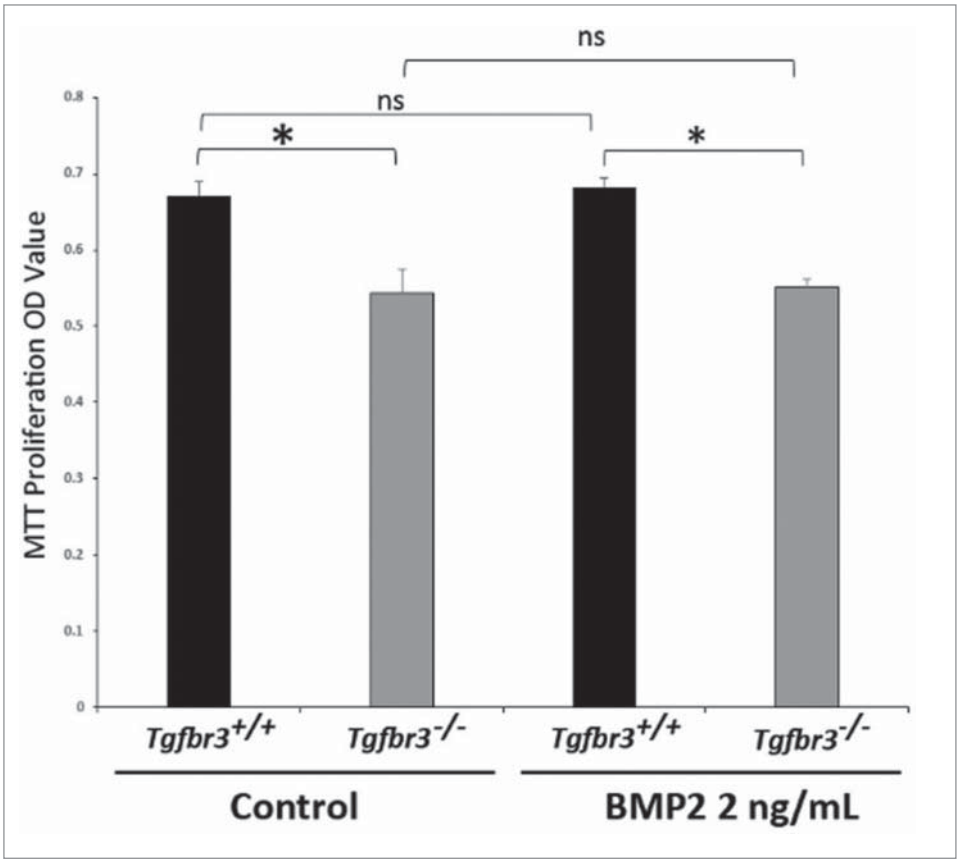

Figure 6. BMP2 does not affect proliferation of epicardial cells. *Wild-type* and *Tgfbr3*$^{-/-}$ epicardial cells were stimulated with BMP2 (2 ng/mL) for 24 hours. MTT Proliferation Assay was performed (*=$p < 0.005$).

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.[11] In culture, *Tgfbr3*$^{-/-}$ cells are deficient in *in vitro* 3-dimensional cell invasion in the presence of BMP2 relative to *Tgfbr3*$^{+/+}$. We demonstrate *Tgfbr3*$^{-/-}$ cells execute 2-dimensional migration in a wound healing model of motility to levels of *Tgfbr3*$^{+/+}$ cells when stimulated with BMP2 without affecting cell proliferation (Figs. 2 and 6). Though delayed of unstimulated migration of *Tgfbr3*$^{-/-}$ cells may partly be due to low proliferation, enhancing migration of *Tgfbr3*$^{-/-}$ cells by BMP2 *in vitro* is not through increased proliferation (Figs. 2 and 6). *Tgfbr3*$^{+/+}$ epicardial migration 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 deficient in these TGFβR3 specific wound healing signaling pathways, but addition of BMP rescues this deficiency 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*$^{+/+}$.[8] Retention of these BMP2 responsive TGFβ superfamily receptors must be sufficient to drive epicardial migration 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. 1). *Tgfbr3*$^{-/-}$ cells also revealed increased expression of TGFβ2 and Has2 relative to wild-type, inducers of epicardial cell invasion in a TGFβR3 dependent fashion. Furthermore, *Tgfbr3*$^{-/-}$ cells and decreased unstimulated SM22α and Vimentin expression. The importance of these expression disparities in *Tgfbr3*$^{-/-}$ cell behavior are unclear, and global gene expression analysis is required to fully understand this phenotype. *Tgfbr3*$^{-/-}$ cells are deficient in 2-dimensional migration relative to *Tgfbr3*$^{+/+}$ in a wound healing model of cell motility (Fig. 2). BMP2 directs epicardial cell motility toward the myocardium [18] and direct epicardial cell invasion in a TGFβR3 dependent manner.[8] We asked whether or not BMPs secreted from epicardial cells *in vitro* signal in an autocrine manner were required for unstimulated migration of *Wild-type* cells. Using Noggin to sequester and inhibit BMPs, we found that there was no change in unstimulated migration of epicardial cells (Fig. 3). Noggin can sequester other BMP ligands including BMP4, 5, 7, 13, and 14,[32] 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.[18] Therefore, this further confirms no role for the action of autocrine BMP signaling in the epicardium. Although exogenous BMP2 rescues *Tgfbr3*$^{-/-}$ deficient migration, down-regulated BMP2 expression in *Tgfbr3*$^{-/-}$ cells is not sufficient to fully explain the *in vivo* phenotype, and further study of disparately expressed genes is required.

Although some previously described non-canonical BMP signaling pathways such as PI3-kinase and cdc42 have been investigated in BMP2-dependent cell migration,[25] we assessed the role of Src kinase in BMP2-stimulated 2-dimensional cell migration in the wound healing assay. Y416 phosphorylation of Src is robustly induced by BMP2 in both *Tgfbr3*$^{-/-}$ and *Wild-type* epicardial cells (Supplemental Fig. 1). Blocking Src attenuates BMP2-stimulated migration in *Tgfbr3*$^{-/-}$ epicardial cells in the wound healing assay (Fig. 4).

BMP2 is known to stimulate cell migration and reorganization of the actin cytoskeleton, though a connection to Src kinase in this process has not yet been described. BMP2-induced f-actin polymerization in *Wild-type* and *Tgfbr3*$^{-/-}$ epicardial cells (Fig. 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 *Wild-type* as visualized by phalloidin staining (Fig. 5), although f/g actin ratios are not different between BMP2-stimulated *Wild-type* and *Tgfbr3*$^{-/-}$ cells. This would suggest that TGFβR3 is not required for BMP2 induced filamentous actin polymerization, and that this occurs in a Src dependent manner through other receptors.

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, but not

BMP2-stimulated migration, in a Src dependent manner.

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.[26] Epicardial cells used in this study were isolated from *wild-type* mouse embryos (*Wild-type*) and embryos lacking TGFβR3 (*Tgfbr3*$^{-/-}$) as previously described.[8] 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 *Wild-type* 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). Genes and primer sets in Table 1.

Wound healing assay

Wound healing assays were performed in accordance with previously described methods.[27] *Wild-type* 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.

Table 1. Genes and primer sets.

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′

Phalloidin staining

Wild-type 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

Wild-type and *Tgfbr3*$^{-/-}$ epicardial cells were seeded at 800,000 cells/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 ultra centrifuged (100,000xg) to fractionate filamentous (f) and globular (g) actin fractions. f/g ratios under stimulated conditions were plotted relative to unstimulated f/g ratios from *Wild-type* conditions. f/g ratio is used as a read out of relative amount of filamentous actin in whole cell lysates.

Proliferation assay

Wild-type 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.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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