Published in final edited form as:

Cell Signal. 2012 May ; 24(5): 1012–1022. doi:10.1016/j.cellsig.2011.12.022.

BMP2 signals loss of epithelial character in epicardial cells but requires the Type III TGF β receptor to promote invasion

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Abstract

Coronary vessel development depends on a subpopulation of epicardial cells that undergo epithelial to mesenchymal transformation (EMT) and invade the subepicardial space and myocardium. These cells form the smooth muscle of the vessels and fibroblasts, but the mechanisms that regulate these processes are poorly understood. Mice lacking the Type III Transforming Growth Factor β Receptor (TGF β R3) die by E14.5 due to failed coronary vessel development accompanied by reduced epicardial cell invasion. BMP2 signals via TGF β R3 emphasizing the importance of determining the relative contributions of the canonical BMP signaling pathway and TGF β R3-dependent signaling to BMP2 responsiveness. Here we examined the role of TGF β R3 in BMP2 signaling in epicardial cells. Whereas TGF β induced loss of epithelial character and smooth muscle differentiation, BMP2 induced an ALK3-dependent loss of epithelial character and modestly inhibited TGF β -stimulated differentiation. *Tgfr3*^{-/-} cells respond to BMP2 indicating that TGF β R3 is not required. However, *Tgfr3*^{-/-} cells show decreased invasion in response to BMP2 and overexpression of TGF β R3 in *Tgfr3*^{-/-} cells rescued invasion. Invasion was dependent on ALK5, ALK2, ALK3, and Smad4. Expression of TGF β R3 lacking the 3 C-terminal amino acids required to interact with the scaffolding protein GIPC (GAIP-interacting protein, C terminus) did not rescue. Knockdown of GIPC in *Tgfr3*^{+/+} or *Tgfr3*^{-/-} cells rescued with TGF β R3 decreased BMP2-stimulated invasion confirming a requirement for TGF β R3/GIPC interaction. Our results reveal the relative roles of TGF β R3-dependent and TGF β R3-independent signaling in the actions of BMP2 on epicardial cell behavior and demonstrate the critical role of TGF β R3 in mediating BMP2-stimulated invasion.

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Keywords

epithelial to mesenchymal transformation; transforming growth factor beta; bone morphogenic protein; epicardium; invasion; differentiation; coronary vessels

1.0 Introduction

Transforming Growth Factor β (TGF β) plays a critical role in regulating epithelial to mesenchymal transformation (EMT), an important process in the regulation of both embryonic development and disease progression [1–5]. The regulation of EMT by members of the TGF β family is a major component of heart valve [6, 7] and coronary vessel [8, 9] development. Formation of the coronary vessels begins when cells of the proepicardium attach to the heart to form the epicardium [8, 10–12]. A fraction of the cells of the epithelial epicardium undergo EMT and the resulting mesenchymal cells populate the subepicardial space with some cells invading further into the myocardium [13]. Mesenchymal cells in the subepicardial space and myocardium contribute to several lineages that include cardiac fibroblasts and vascular smooth muscle [14, 15].

Despite the recognition of the importance of TGF β in regulating EMT, the mechanism of TGF β signaling is not well understood. The roles of the serine-threonine kinase containing Type I (TGF β R1 or ALK5) and Type II (TGF β R2) receptors in TGF β signaling are well described [16, 17], but the contributions of the Type III receptor (TGF β R3) are poorly understood. However a significant role for TGF β R3 is demonstrated by the observation that targeting of *Tgfb3* in mice causes embryonic lethality due to failed coronary vessel development [18] associated with dysregulated epicardial cell invasion [19]. TGF β R3 binds multiple members of the TGF β family. In addition to binding TGF β 1 and TGF β 3, TGF β R3 is required for the high affinity binding of TGF β 2 [20]. TGF β R3 has also been identified as the inhibin receptor [21] and binds BMP2 [22].

Studies of epicardial cells have shown that TGF β stimulates the loss of epithelial cell character and smooth muscle differentiation [23]. Although loss of epithelial character and smooth muscle differentiation does not require TGF β R3, TGF β -mediated epicardial cell invasion was shown to be dependent on specific cytoplasmic residues of TGF β R3 and the interaction of these residues with the scaffolding protein GIPC [19]. TGF β -stimulated epicardial cell invasion also requires TGF β R3 to access the Par6/Smurf1/RhoA pathway which is necessary for cell invasion [24]. The role of TGF β R3 in BMP2 signaling is less well described. BMP2 binds TGF β R3 and is required for endothelial cell transformation *in vitro* [22]. In endothelial cells, both TGF β and BMP2 share a common, TGF β R3-dependent pathway to signal transformation that includes activation of the Par6/Smurf1/RhoA pathway [25, 26] and a requirement for specific cytoplasmic residues of TGF β R3 and the interaction of these residues with the scaffolding protein GIPC [27]. In epicardial cells, BMP2 is known to induce invasion *in vitro* that is dependent on the Par6/Smurf1/RhoA pathway [24].

Here we show that TGF β R3 is required for BMP2-stimulated epicardial cell invasion *in vitro* although TGF β R3 is not required for BMP2-stimulated loss of epithelial character as measured by the redistribution of ZO1. BMP2-stimulated invasion was shown to require specific cytoplasmic residues in TGF β R3 that are known to interact with the scaffolding protein GIPC. Deletion of these residues, or the targeting of GIPC, demonstrated a requirement for each in BMP2-stimulated invasion. These data suggest that loss of BMP2 responsiveness, as well as the previously recognized loss of TGF β responsiveness, may underlie the epicardial defects associated with failed coronary vessel development in *Tgfb3*^{−/−} mice [18].

2.0 Methods

2.1 Immortalized Epicardial Explant Culture

Immortalized epicardial cell lines from *Tgfb β 3*^{+/+} and *Tgfb β 3*^{-/-} mice were generated as described previously [23]. To sustain the cell's immortalized state, they were grown at 33°C in immortal media: 10% fetal bovine serum (FBS), 100U/ml Penicillin/Streptomycin (P/S), 1X Insulin-Transferrin-Selenium (ITS; 1 µg/ml insulin, 5.5×10⁻⁴ µg/ml transferrin, 0.677 µg/ml selenium), and 10U/ml interferon γ (INFγ). For growth factor addition, cells were transferred to standard DMEM media (5% FBS and 100U/ml P/S) and cultured at 37°C for 24 hours prior to growth factor addition. Growth factors (TGFβ1, TGFβ2, or BMP2) or small molecule inhibitors were added to the cell medium and assayed after 24, 48, or 72 hours. Multiple immortalized epicardial cell lines (E11.5) were generated from *Tgfb β 3*^{+/+} and *Tgfb β 3*^{-/-} littermate pairs and used in experiments.

2.2 Growth Factors and Inhibitors

Reagents were obtained from the following sources: TGFβ1, TGFβ2, and BMP2 were purchased from R&D Systems; SB431542, from Sigma-Aldrich. DMH1 was a generous gift from Dr. Charles Hong.

2.3 Immunohistochemistry

Tgfb β 3^{+/+} and *Tgfb β 3*^{-/-} epicardial cells (E11.5) were plated in 4-well collagen coated chamber slides (BD Biosciences) at a density of 50,000 cells per well. Cells for ZO-1 staining were fixed in 70% methanol for 10 min at room temperature, then blocked with 2% bovine serum albumin in PBS for 1 hr and incubated with diluted primary antibody (ZO-1, 2 µg/ml) overnight at 4°C. For SM22α (Abcam) staining, cells were fixed with 2% paraformaldehyde (PFA) for 30 min at room temperature and permeabilized with PBS and 0.1% Triton X-100 for 5 min. Cells for SM22α staining were blocked with 5% horse serum, and incubated with primary antibody (SM22α, 1:200) overnight at 4°C. Primary antibody detection was with goat anti-rabbit cy3 (ZO-1) or donkey anti-goat cy3 (SM22α) secondary antibody (1:800; Jackson ImmunoResearch). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; Sigma). Photomicrographs were captured with Nikon Eclipse E800 microscope and SPOT imaging software.

2.4 qRT-PCR

To determine changes in gene expression, we used qRT-PCR as previously described [19]. Total RNA was isolated using the TRIzol reagent (Invitrogen). cDNA was generated from 1 µg total RNA using oligo-dT primers and Superscript III polymerase (Invitrogen). Primer pairs are shown in the table below. Real-time PCR analysis was done with iQ SYBR green supermix (Bio-Rad) in the Bio-Rad iCycler for 40 cycles. The expression levels are calculated using the $\Delta\Delta C_T$ method. The threshold cycle (C_T) represents the PCR cycle at which an increase of the reporter fluorescence above the baseline is first detected. The fold change in expression levels, R, is calculated as follows: $R = 2^{-\Delta\Delta C_T}$ (where $R = 2^{(\Delta C_T \text{ treated} - \Delta C_T \text{ control})}$) to normalize the abundance of all transcripts to the level of *GAPDH* RNA expression.

Gene	Sense Primer (5' → 3')	Anti-Sense Primer (5' → 3')
<i>GAPDH</i>	ATGACAATGAATACGGCTACAG	TCTCTTGCTCAGTGCCTTG
<i>SmaA</i>	GAGAAGCCAGCCAGTCG	CTCTTGCTCTGGGCTCA

Gene	Sense Primer (5' → 3')	Anti-Sense Primer (5' → 3')
<i>Sm22α</i>	AGCCAGTGAAGGTGCTGAGAAC	TGCCCCAAGCCATTAGAGTCCTC
<i>Calponin</i>	GAAGGCAGGAACATCATTGGACTG	CTCAAAGATCTGCCGCTTGGTGCC
<i>Snai1</i>	GTGAAGAGATACCAGTGCCAG	AAGATGCCAGCGAGGATG
<i>SKIL</i>	GAAATGCACCTGTGACTCAAC	TTCATCTTGGAGTTCCTGCC
<i>FXYD5</i>	TCCTACATTGAACATCCACTGG	GACAACTGCCTACACTTCCC
<i>Flk1</i>	TGCGGGCTCCTGACTACACTA	TTCCCAAATGCTCCACCAACTCTG
<i>Tie2</i>	TTGGATTGTCACGAGGTCAAGAAG	CAATACACCATAGGACCAGACATCAC
<i>VE Cadherin</i>	CAGCACTTCAGGCAAAAACA	TTCTGGTTTCTTGGCAGCTT
<i>Cx43</i>	TTCCTTTGACTTCAGCCTCC	CGTGGAGTAGGCTTGGAC
<i>Nkx2.5</i>	AAGTGTCTCTCTGCTTTC	CGTCTCGGCTTTGTCCAG
<i>cTnT</i>	AGGAGCTGATTTCCCTCAAAG	TTTCCTTCTCCCGCTCATTG
<i>GIPC1</i>	TTGGATTGTCACGAGGTCAAGAAG	CAATACACCATAGGACCAGACATCAC
<i>ALK2</i>	AGAGGGTCGATATTGGGC	AACTGGGTCATTGGGAAC
<i>ALK3</i>	ACCATTTCAGCCCTACA	TCACTGGGCACCATGTT
<i>ALK5</i>	CCTTCTGATCCATCGGTTGA	CCATTGGCATACCAGCAT
<i>Smad4</i>	CGTGGCAGGGAACATC	GCCCTTCACAAAGCTCAT

2.5 Measurement of lacZ Staining and Activity

Immortalized E11.5 *Sm22α-lacZ*:Immorto epicardial cells were assayed as previously described [23] with the Galacto-Light Plus system (Applied Biosystems). Luminescence was quantified on a Turner 20/20 Luminometer.

2.6 Western Blot

Tgfb^{3+/+} epicardial cells were incubated with vehicle, TGFβ1, TGFβ2, or BMP2 for 72 h prior to Western blot analysis. Cells were lysed and diluted in NP-40 lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 20mM NaF). Total cellular lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking in 5% non-fat milk, membranes were incubated with primary antibody (SM22α (abcam – ab10135 goat polyclonal to SM22 alpha); 0.5 µg/mL) diluted in 1% blocking solution for 1hr at 4°C. After 3 washes for 10 min each in TBST, the blot was incubated with the secondary antibody (abcam – ab6885 donkey polyclonal to goat IgG – H&L (HRP conjugated) for one hour. Beta tubulin (sigma T7816; 1:10000) was used as a loading control. Following 3 additional washes, detection was performed using ECL Western lightning chemiluminescent reagent (Perkin Elmer).

2.7 Transfections and Adenovirus Infections

Plasmids—Cells were plated at a density of 50,000 per well of a 6-well plate in immorto media without pen/strep and allowed to adhere overnight at 33°C. The cells were transfected the following day with 2 µg of pAdTrackCMV vector alone or expressing constitutively active (ca) ALK2, caALK3, or caALK5 and 3 µl of X-treme gene DNA transfection reagent (Roche). The media was changed 24 h after transfection to 5% FBS/DMEM and cells were incubated at 37°C. 48 h after transfection, cells were fixed for immunohistochemistry, harvested for qRT-PCR analysis, or used for invasion assays.

siRNA—Cells were plated at a density of 200,000 per well of a 6-well plate in immorto media without pen/strep and allowed to adhere overnight at 33°C. The following day the cells were transfected with 2 µg siRNA (Ambion) and 8 µl Xtreme siRNA transfection reagent (Roche). The media was changed to 5% FBS/DMEM 16 h after transfection and cells were incubated at 37°C. 24 h later cells were harvested for qRT-PCR analysis to confirm knockdown, or used directly for invasion assays. Where overexpression of TGFβR3 was done in conjunction with knockdown of GIPC1, cells were transfected with siRNA, then infected with adenovirus 24 h after, and plated for invasion assays 24 h after that.

siRNA	Sense (5' → 3')	Anti-Sense (5' → 3')
<i>Scramble</i>	CUCCTUGTCAATUUACCGCTT	AAGCGGTAAATTGACAAGGAG
<i>ALK2-a</i>	GAGACGGAAUUGUACAACATT	UGUUGUACAAUCCGUCUCCC
<i>ALK2-b</i>	GAGGCAUGAAAAUAUCUUATT	UAAGAUUUUUCAUGCCUCA
<i>ALK3-a</i>	GGCUCGUCGUUGUAUUACATT	UGUAAUACAACGACGAGCCAT
<i>ALK3-b</i>	GGCUGACAUCUAUAGCUUUTT	AAAGCUAUGAUGUCAGCCAT
<i>ALK5-a</i>	CAAACGCGCUGACAUCUAUTT	AUAGAUGUCAGCGGUUGAA
<i>ALK5-b</i>	GAGUAGGCACUAAAAGGUATT	UACCUUUUAGUGCCUACUCTG
<i>GIPC1-a</i>	GCAGUGUGAUUGACCACAUTT	AUGUGGUCAAUCACACUGCCT
<i>GIPC1-b</i>	AGGACAAAAGGAACCCGATT	UCCGGGUCCUUUUGUCCUTT
<i>Smad4-a</i>	CGGCGAUUGUGCAUUCUCATT	UGAGAAUGCACAACGCCGGA
<i>Smad4-b</i>	GGAGAAGGUUUUGUAUAAATT	UUUAUACAAAACCUUCUCCCT

Adenovirus—Adenoviruses were generated using the pAdEasy system [28]. Viruses were tittered by performing serial dilutions of the concentrated virus and counting the number of GFP-expressing HEK293 cells after 18–24 h. The following adenoviruses co-expressing GFP were used: full length TGFβR3 (FL), TGFβR3 lacking the cytoplasmic domain (CYTO), TGFβR3 lacking the last 3 amino acids (Δ3), Smad1, and Smad3. Cells were plated at a density of 200,000 per well in immorto media and allowed to adhere overnight at 33°C. The following day, virus was added directly to the cells at a final concentration of 10⁸ PFU/ml. 24 h later, the cells were plated for invasion as described below.

2.8 Invasion Assay

Invasion assays were performed using a modified Boyden chamber assay as previously described [19]. Briefly, cells were fluorescently labeled with CalceinAM (BD Biosciences) and plated at 12,000 cells per well in 0.5% FBS/DMEM on collagen gels [29] in the top chamber. Cells were allowed to adhere overnight at 37°C. 24 h later, vehicle, growth factors, or growth factors plus inhibitors were added in 20% FBS/DMEM to the bottom chamber. In addition, cells also received inhibitor in the top well in 0.5% FBS/DMEM. After 24 h, the top insert was removed and placed in 0.25% Trypsin/2.21 mM-EDTA in HBSS (CellGro). Cells that invaded the collagen and crossed the membrane detached from the membrane into the trypsin containing plate, which was then read using SpectraMax 96-well plate reader (Ex: 485, Em: 538, Cutoff: 530; sensitivity: 30).

2.9 Proliferation Assay

Proliferation was scored using a MTS assay as previously described [24], which relies on the *in vivo* reduction of MTS tetrazolium to a colored formazan product by NADPH in metabolically active cells. The product formed is read at 490 nm and is directly proportional

to the number of living cells in culture. Briefly, cells were plated at a density of 5,000 cells/well in 100 μ l of 10 % FBS/DMEM in triplicate in a 96 well plate and incubated at 37°C. After 48 h, 20 μ l of substrate (Promega: Cell Titer 96 Aqueous Solution) was added to each well and incubated for 30 min at 37°C. Absorbance of the colorimetric reaction was read at 490 nm using SpectraMax 96-well plate reader.

2.10 Statistical Analysis

Paired students t-test was performed to establish significance. Data are presented as the mean of three experiments $\pm$ SEM for one littermate pair, unless otherwise specified. P-values of <0.05 were considered significant.

3.0 Results

3.1 BMP2 induces loss of epithelial character but not smooth muscle differentiation

We have previously shown that TGF β 1 or TGF β 2 induces loss of epithelial character and smooth muscle differentiation in epicardial cells [23]. The loss of TGF β 3 does not alter these responses to ligand but does result in decreased invasion in response to ligand [19]. Since TGF β 3 also binds BMP2 [22], and BMP2 has been reported to induce the migration of proepicardial cells [30] we characterized BMP2 responsiveness in *Tgfb3*^{+/+} epicardial cells. The addition of 5 nM BMP2 induced redistribution of ZO-1 (Fig. 1A,D) but failed to increase SM22 α (Fig. 1 E,H) while the addition of 250 pM TGF β 1 or TGF β 2 both induced redistribution of ZO1 and increased SM22 α (Fig. 1A–C & E–G). We examined the expression by qRT-PCR of additional markers associated with smooth muscle differentiation and the loss of epithelial character to confirm the effects of BMP2. To confirm that BMP2 does not induce smooth muscle differentiation, the smooth muscle markers SM α -actin and calponin, in addition to SM22 α , were examined. TGF β 1 or TGF β 2 but not BMP2 significantly induced the expression of SM22 α , SM α -actin, and calponin (Fig. 1I). To confirm the loss of epithelial cell character we examined the expression of Snai1 [31–33], SKIL [34, 35], and FXYD5 [36, 37]. The expression of each of these markers was increased by 24 hours after the addition of BMP2 (Fig. 1J). These data demonstrate that although BMP2 induced loss of epithelial cell character, unlike TGF β 1 or TGF β 2, BMP2 does not induce smooth muscle differentiation. Given that epicardial cells have been suggested to contribute to other lineages in the heart [38–42], we examined the endothelial markers Flk 1, Tie 2, and VE Cadherin as well as the myocardial markers Nkx2.5, Connexin 43, and cardiac troponin T(cTnT) by qRT-PCR. The addition of BMP2 did not alter the expression of any markers associated with the endothelial or myocardial lineages (Fig. 1K,L). These data demonstrate that BMP2 induced loss of epithelial cell character but not smooth muscle, endothelial, or myocardial differentiation.

3.2 BMP2 can partially inhibit smooth muscle differentiation

To further elucidate the actions of BMP2, we next took advantage of *Tgfb3*^{+/+} epicardial cells isolated from SM22 α -*lacZ* mice. The addition of TGF β 1 or TGF β 2, but not BMP2, induces *lacZ* expression (Fig. 2A–D) with an EC₅₀ of 12.5 pM (Fig. 2E). BMP2 at concentrations from 0.1 to 5000 pM failed to induce *lacZ* expression (Fig. 2E). Similarly, Western blot analysis revealed significantly increased expression of SM22 α protein in response to 250 pM TGF β 1 or TGF β 2 but not 5000 pM BMP2 (Fig. 2F). The concentration response data suggested that BMP2 inhibits SM22 α expression. To address this question, cells with the SM22 α -*lacZ* transgene were preincubated with either vehicle, 5, 50, 500, or 5000 pM BMP2. After 1 hour 12.5 pM TGF β 1 or TGF β 2 was added and the cells assayed after an additional 72 hours of incubation. Cells preincubated with concentrations of BMP2 of 50 pM and greater showed an approximate 35% decrease in *lacZ* expression (Fig. 2G). The inhibition of TGF β -mediated smooth muscle differentiation by BMP2 was confirmed

when the smooth muscle markers SM α -actin and calponin as well as SM22 α were examined by qRT-PCR. In *Tgfb β 3*^{+/+} cells, BMP2 significantly inhibited the expression of SM22 α , SM α -actin, and calponin in response to TGF β 1 (45%, 64%, and 48% respectively) or TGF β 2 (28%, 57%, 34% respectively) (Fig. 2H). These data demonstrate that BMP2 can partially inhibit TGF β -mediated smooth muscle differentiation in epicardial cells.

3.3 TGF β 3 is not required for loss of epithelial character to TGF β 1, TGF β 2, or BMP2

Given that TGF β 3 binds TGF β 1, TGF β 2, and BMP2 we used *Tgfb β 3*^{-/-} epicardial cells to determine if TGF β 3 was required for the actions of these ligands. Immunostaining revealed that *Tgfb β 3*^{-/-} cells have abundant ZO-1 at the cell borders (Fig. 3A). The addition of 250 pM TGF β 1 or TGF β 2 resulted in a significant loss of ZO-1 from cell borders (Fig. 3A–C) and expression of the smooth muscle markers SM22 α , SM α -actin, and calponin (Fig. 3E) as previously reported [19]. However, the addition of 5 nM BMP2 to *Tgfb β 3*^{-/-} cells resulted in the loss of ZO-1 from cell borders (Fig. 3D) without induction of SM22 α , SM α -actin, or calponin (Fig. 3F). These data demonstrate that TGF β 3 is not required for the BMP2-stimulated loss of epithelial character in epicardial cells.

3.4 BMP2 signals loss of epithelial character via ALK3

The data above suggests that TGF β and BMP2 are signaling through their respective canonical pathways via ALK signaling [43] to mediate the loss of epithelial character. Since BMP2 may signal through several ALK's, including ALK2, ALK3, or ALK6, we next addressed the relative role of each of these ALKs. Analysis of ALK expression in epicardial cells by RT-PCR revealed that ALKs 1–5 are abundantly expressed, ALK6 is barely detectable, and 7 is absent (Fig. 4A). RNAseq analysis confirmed abundant expression of ALKs 1–5 and the lack of ALK6 and ALK7 expression (data not shown). We have previously shown that both the loss of epithelial character and smooth muscle differentiation in response to 250 pM TGF β 1 or TGF β 2 in epicardial cells requires ALK5 kinase activity [23]. Here we sought to determine the ALKs required for the effects of BMP2 on epicardial cells. Consistent with prior results [23], the addition of 2.5 μ M of the ALK5 kinase inhibitor SB431542 blocked the actions of TGF β 1 or TGF β 2 in *Tgfb β 3*^{+/+} cells (Fig. 4D, F). However, SB431542 had no effect on the actions of BMP2 (Fig. 4H). DMH1 is a recently identified highly selective and potent BMP inhibitor with selectivity for ALK2 and ALK3 [44]. The addition of 2 μ M DMH1 blocked BMP2 induced loss of epithelial character, but not the actions of TGF β 1 or TGF β 2 in *Tgfb β 3*^{+/+} cells. Cells co-incubated with DMH1 and TGF β 1 or TGF β 2 following pre-incubation with DMH1 showed redistribution of ZO-1 staining characteristic of the loss of epithelial character (Fig. 4E, G) while cells co-incubated with DMH1 and BMP2 following pre-incubation with DMH1 retained ZO-1 at cell borders (Fig. 4I).

We next addressed the potential roles of ALK2, ALK3, and ALK5 by expressing constitutively active (ca) forms in epicardial cells. All 3 caALKs induced loss of epithelial character as determined by the redistribution of ZO-1 (Fig. 5A–E). Cells expressing caALKs were generally elongated and found to lie in a plane beneath the uninfected epithelial cells. However examination of smooth muscle markers by qRT-PCR reveals that only caALK5 and caALK2 induce smooth muscle differentiation while caALK3 does not (Fig. 5F). None of the caALK's tested induced endothelial markers (Fig. 5G). Taken together, these data are consistent with ALK3 activation downstream of BMP2 being responsible for the loss of epithelial character in epicardial cells.

3.5 TGF β 3 and GIPC interaction is required for BMP2 induced invasion

We recently reported that TGF β 3 is required for TGF β -stimulated epicardial cell invasion *in vitro* and *in vivo* [19] and that BMP2 can access the Par6/Smurf1/RhoA pathway to

mediate invasion [24]. Here we report that BMP2-mediated epicardial cell invasion requires TGF β R3 and interaction with GIPC. BMP2 stimulates the invasion of *Tgfb β 3*^{+/+} epicardial cells while *Tgfb β 3*^{-/-} epicardial cells display decreased invasion in response to BMP2 (Fig. 6A). Overexpression of TGF β R3 (TGF β R3-FL) in *Tgfb β 3*^{-/-} epicardial cells rescues invasion in response to BMP2 (Fig. 6B). Consistent with results obtained for TGF β [19], receptor constructs lacking the entire cytoplasmic domain (TGF β R3-CYTO) or the 3 C-terminal amino acids required for GIPC binding (TGF β R3- Δ 3) failed to rescue invasion in *Tgfb β 3*^{-/-} cells (Fig. 6B). Targeting of GIPC by siRNA revealed that GIPC was required for BMP2-stimulated cell invasion in *Tgfb β 3*^{+/+} cells (Fig. 6C) and in *Tgfb β 3*^{-/-} cells rescued with TGF β R3-FL (Fig. 6D). Knockdown of GIPC is confirmed in Fig. 6D and F, respectively. These data demonstrate that BMP2-stimulated invasion requires TGF β R3 interaction with GIPC.

3.6 BMP2 induced invasion requires ALK activation and is Smad dependent

We have reported that TGF β R3-dependent invasion of endocardial cells requires ALK2, ALK3, and ALK5 while ALK4 and ALK6 are dispensable [25, 27]. To address the role of ALK signaling in TGF β R3-dependent invasion of epicardial cells we determined invasion in *Tgfb β 3*^{+/+} cells incubated with 2.5 μ M of the ALK5 kinase inhibitor SB431542 or 2 μ M of the BMP inhibitor DMH1. Both inhibitors blocked invasion in response to 250 pM TGF β 1 or TGF β 2 or 5 nM BMP2 (Fig. 7A) consistent with our results in endocardial cells where multiple ALKs are required for invasion [25] but distinct from that seen for the loss of epithelial character in epicardial cells reported here. As a second, independent method to address the role of ALKs in mediating TGF β R3-dependent invasion we used siRNA to target ALK2, ALK3, or ALK5 in *Tgfb β 3*^{+/+} cells. The targeting of either of these ALKs inhibited invasion to all ligands (Fig. 7B) which is compatible with our results using small molecule inhibitors. Knockdown was confirmed for each of 2 independent constructs used to target each ALK (Fig. 7C). However, the use of constitutively active forms of ALK2, ALK3, and ALK5 revealed that each was sufficient to increase epicardial cell invasion (Fig. 7D) without any effects on proliferation (Fig. 7E). These data suggest that although each of these constitutively active ALK's is sufficient for invasion, the activity of the native forms of these receptors is coordinately regulated and interdependent when stimulated by ligand. This conclusion is also consistent with the ability of small molecule inhibitors of ALK5 (SB431542) or ALK2, ALK3 (DMH1) kinase activity to block invasion of *Tgfb β 3*^{+/+} epicardial cells (Fig. 7A).

ALK activation results in stimulation of the Smad signaling pathway [43]. ALK5 activates Smad2 and 3 while ALK2 or ALK3 activates Smad1, 5, and 8. Each of these activated Smads binds to Smad4 and the complex is translocated to the nucleus to alter gene transcription [43]. To determine if Smads are required for epicardial cell invasion, we took advantage of the described role of Smad4 and used siRNA targeted to Smad4 to prevent the translocation of all Smads. Targeting of Smad4 significantly inhibited invasion in response to 5nM BMP2 demonstrating that invasion requires Smad signaling (Fig. 8A). Knockdown of Smad4 was confirmed for each of 2 independent siRNA constructs (Fig. 8B). We next investigated whether Smad activation is sufficient to induce EMT in epicardial cells. Since overexpression of Smads results in constitutive activity [25, 45] we chose to overexpress representative Smads downstream of specific ALKs, Smad3 (downstream of ALK5) or Smad1 (downstream of ALK2, ALK3), in *Tgfb β 3*^{+/+} epicardial cells and score for invasion. Overexpression of Smad1 resulted in a marginally significant increase in epicardial cell invasion while Smad3 overexpression had no effect on invasion (Fig. 8C). Neither Smad altered proliferation (Fig. 8D). Overexpression of Smad1 or Smad3 in *Tgfb β 3*^{-/-} cells failed to alter invasion (data not shown). These data demonstrate that Smad activation alone is not sufficient to induce epicardial cell invasion.

4.0 Discussion

Our experiments reveal that BMP2 induces the loss of epithelial character in epicardial cells but, unlike TGF β , BMP2 does not induce smooth muscle differentiation of epicardial cells. The use of small molecule inhibitors and constitutively active ALKs suggest that BMP2-stimulates loss of epicardial cell character via activation of ALK3 while TGF β -stimulated loss of epithelial character and smooth muscle differentiation requires ALK5. These actions of BMP2 or TGF β do not require TGF β R3. However, BMP2- or TGF β -stimulated invasion of epicardial cells does require TGF β R3 (Fig. 9). These results and the observation that *Tgfb3*^{-/-} embryos have failed coronary vessel development [18] associated with decreased invasion of epicardial cells into the subepicardial space and myocardium [19] suggests that the loss of BMP2 responsiveness, as well as TGF β responsiveness, may underlie the defects seen in *Tgfb3*^{-/-} mice. Recently BMP2 has been identified as an important signal in directing the migration of proepicardial cells to the myocardium [30]. Analysis of *Tgfb3*^{-/-} mice did not reveal any defects in proepicardial cell migration to the myocardium [18] suggesting that proepicardial cell migration is independent of TGF β R3. However, our observation that BMP2 at least partially inhibits TGF β -stimulated smooth muscle differentiation suggests that this may be a mechanism by which BMP2 attenuates responsiveness to TGF β -induced differentiation so that cells may remain motile while interacting with the myocardium.

Invasion stimulated by BMP2 is dependent upon the presence of the cytoplasmic domain of TGF β R3, specifically the 3 C-terminal amino acids that are required for interaction with the scaffolding protein GIPC. The finding that siRNAs targeting GIPC inhibit invasion stimulated by BMP2 in *Tgfb3*^{+/+} cells, and that siRNA to GIPC blocks rescue by TGF β R3 in *Tgfb3*^{-/-} cells, confirms that interaction of GIPC with TGF β R3 is critical to invasion. We have previously shown that TGF β -stimulated, TGF β R3-mediated invasion of epicardial cells [19], as well as TGF β -stimulated, TGF β R3-mediated endocardial cell transformation [27], is dependent on GIPC interaction with TGF β R3. However, the role of TGF β R3/GIPC in regulating cell behavior is not clearly understood. In cancer cell lines the regulation of proliferation, migration, and adhesion is regulated by TGF β R3 [46]. In contrast to results in endocardial cell transformation [25, 27, 47] or epicardial cell invasion described here that implicates TGF β R3 in mediating invasion, TGF β R3 has been shown to inhibit cell proliferation, migration, and adhesion in myeloma cells [48] and suppress breast cancer progression through GIPC-mediated inhibition of TGF β signaling [49]. TGF β R3 expression is reported to be decreased in human breast, ovarian, pancreatic, prostate, and non-small lung cell cancers [50–54]. Although TGF β R3 regulation of Cdc42 has been shown to regulate cancer cell motility [55], Cdc42 does not play a role in regulating endocardial cell [26] or epicardial cell migration or invasion [24]. Overall these data demonstrate that TGF β R3 and GIPC have critical roles in development and disease but also underscore our lack of understanding of the mechanisms that regulate context- and cell-specific responses.

Our finding that several ALKs are required for TGF β R3-dependent epicardial cell invasion is consistent with prior findings for TGF β R3-dependent endocardial cell transformation where ALK2, ALK3, and ALK5 are all required [27]. Epicardial cell invasion also requires ALK2, ALK3, and ALK5. However, whereas ALK2, ALK3, and ALK5 are sufficient for invasion in epicardial cells, in endocardial cells ALK2 and ALK3 are sufficient for transformation and ALK5 is not [56, 57]. A requirement for ALK5 in endocardial and epicardial cells is consistent with the described role of ALK5 in activating the Par6/Smurf1/RhoA pathway that aids in the maintenance of epithelial cell polarity [58]. In addition, ALK5 is also required for the full activation of ALK1 downstream of endoglin in endothelial cells [59]. Endoglin, although unable to bind TGF β directly, functions as a co-receptor for TGF β in the presence of TGF β R2 [60]. The cytoplasmic domain of endoglin is

identical to that of TGF β R3 [20, 61] and endoglin has been shown to regulate the activation of ALK5 and ALK1 to regulate endothelial cell proliferation and migration [62, 63]. ALK1 downstream of endoglin is not fully active without ALK5 activity [59] demonstrating the interdependence of ALK activity in response to ligand. Therefore, although several lines of evidence support roles for ALK5 in regulating transformation or cell invasion, the mechanisms that underlie the lack of sufficiency in endocardial cells or sufficiency in epicardial cells is not understood. In both endocardial [25, 64] and epicardial cells TGF β R3-dependent invasion is Smad-dependent. In endocardial cells this dependency has been shown both by the targeting of the common mediator Smad, Smad4 [25, 64], and using siRNA to target individual receptor Smads [25], Smads1, 2, 3, and 5, each of which were shown to be required for endocardial cell transformation. The requirement of multiple Smads that are known to be downstream of several ALKs is consistent with the finding that numerous ALKs are also required. However, the overexpression of Smad1 or Smad3 in endocardial cells, Smads primarily associated with the BMP or TGF β pathways respectively, failed to induce transformation. Consistent with these data, experiments reported here demonstrate that Smad overexpression is not sufficient to drive epicardial cell invasion. Taken together these data suggest that cell transformation or invasion requires the coordinated activation of ALKs and Smads associated with both the canonical TGF β and BMP signaling pathways, and this coordinated activation is dependent on TGF β R3 (Fig. 9)

Recent findings have revealed that BMP2 can signal via TGF β R3 which has highlighted the importance of determining the relative contributions of the canonical BMP signaling pathway and TGF β R3-dependent signaling in mediating BMP2 responsiveness. Our results reveal the relative roles of TGF β R3-dependent and TGF β R3-independent signaling in the actions of BMP2 on epicardial cell behavior. TGF β R3 is required for BMP2-stimulated epicardial cell invasion *in vitro* which suggests that the decreased epicardial cell invasion seen in *Tgfbr3*^{-/-} mice [18] may be at least partially due to loss of BMP2 responsiveness.

Acknowledgments

The authors thank members of the Barnett laboratory for helpful discussions and comments and George Davis for adenoviral constructs. Overall project support by National Institutes of Health, HL085708. N.S.S. and J.D.L. were supported by supplements to HL085708. J.A.A. was supported by R25 HL96223 short term training for minority students. J.V.B. acknowledges the support of the Vanderbilt-Ingram Cancer Center.

Abbreviations

ALK	Activin Receptor-Like Kinases
BMP	Bone Morphogenetic Protein
Cdc42	Cell division control protein 42 homolog
EMT	Epithelial-Mesenchymal Transformation
GFP	Green Fluorescent Protein
GIPC	GAIP-interacting protein, C terminus
Par6	Par-6 partitioning defective 6 homolog gamma
RhoA	Ras homolog gene family member A
Smurf1	Smad ubiquitination regulatory factor1
TGFβ	Transforming Growth Factor Beta
TGFβR1	Type I TGF β receptor

TGFβR2	Type II TGFβ receptor
TGFβR3	Type III TGFβ receptor
TGFβR3-FL	Type III TGFβ receptor-full length
TGFβR3-CYTO	Type III TGFβ receptor-lacking the entire cytoplasmic domain
TGFβR3-Δ3	Type III TGFβ receptor-lacking the 3 C-terminal amino acids

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Highlights

- BMP2 signals the ALK3-dependent loss of epithelial character in epicardial cells
- BMP2-stimulated invasion is dependent on TGF β R3 interaction with GIPC
- Invasion also requires activation of ALK2, ALK3, ALK5, and Smad4

Fig. 1. TGFβ and BMP2 have distinct effects on epicardial cells

Immortalized epicardial cells (E11.5) were incubated with vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2 for 24 or 72 h. **A–H:** Immunohistochemistry: epicardial cells were stained with an epithelial marker, zonula occludens-1 (ZO-1), or smooth muscle marker, smooth muscle 22 alpha (SM22α). Cells incubated for 72 h with vehicle formed a compact monolayer and displayed a round epithelial phenotype (A). Cells incubated with TGFβ1 (B), TGFβ2 (C) or BMP2 (D) for 72 h are elongated and separated from one another and display a redistribution of ZO-1 expression. Cells incubated with vehicle (E) lacked SM22α expression. Cells incubated with TGFβ1 (F), or TGFβ2 (G) are elongated and separated from one another and express SM22α in organized fibers consistent with a smooth muscle phenotype. Cells incubated with BMP2 (H) are elongate with no up-regulation of SM22α expression (compared to vehicle). (n=3, results shown for one littermate pair) **I–L:** qRT-PCR: TGFβ1 and TGFβ2, but not BMP2, increase the expression of SM22α, SMαA, and calponin after 72 h (I). TGFβ1, TGFβ2, and BMP2 induce expression of mesenchymal markers (Snail, SKIL, and FXYD5) after 24 h (J). BMP2 did not induce expression of endothelial (K) or myocardial (L) markers after 72 h. *Columns*, median fold change obtained from 5 separate experiments; *bars*, SEM. **P*<0.05.

Fig. 2. TGFβ1 or TGFβ2, but not BMP2, induces smooth muscle 22 alpha (SM22α) expression
SM22α-lacZ:Immortalized epicardial cells (E11.5) were incubated with vehicle, TGFβ1 (250 pM), TGFβ2 (250 pM), or BMP2 (5000 pM) for 72 h and assayed for *lacZ* activity. **A–D:** Cells incubated with vehicle retained an epithelial phenotype and did not express *lacZ* (A). Cells incubated with TGFβ1 (B) and TGFβ2 (C) elongated and expressed *lacZ*. Cells incubated with BMP2 (D) are elongate but did not express *lacZ*. (n=3, results shown for one littermate pair) **E:** Dose response: cells were incubated with vehicle or the following concentrations of ligand: TGFβ1 or TGFβ2 - 0.125, 0.25, 0.5, 1.25, 12.5, 125, 250, and 500 pM; BMP2 - 0.01, 0.05, 0.125, 0.25, 0.5, 1250, 2500, and 5000 pM. Cells incubated with increasing concentrations of TGFβ1 (squares) or TGFβ2 (triangles) show an increase in *lacZ* expression. There was no up-regulation of *lacZ* expression at any concentration of BMP2 (diamonds). (n=3) **F:** Immortalized epicardial cells (E11.5) were incubated with vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2 for 72 h prior to harvest, SDS-PAGE, and immunoblot analysis for SM22α. TGFβ1 and TGFβ2 increased SM22α expression while BMP2, did not. β-tubulin was used as a loading control. Chicken gizzard was used as a positive control (not shown). (n=3, results shown for one littermate pair) **G:** Immortalized epicardial cells (E11.5) carrying the SM22α-lacZ transgene were preincubated with either vehicle, 5, 50, 500, or 5000 pM BMP2. After 1 h 12.5 pM TGFβ1 or TGFβ2 was added and the cells assayed after an additional 72 h of incubation. Cells preincubated with concentrations of BMP2 of 50 pM and greater showed an approximate 35% decrease in *lacZ* expression (n=3, *=p<0.05) (G). Immortalized epicardial cells (E11.5) were preincubated with either vehicle or BMP2 (5000 pM) for 1 h followed by incubation with either vehicle, TGFβ1 (250pM), TGFβ2 (250pM), BMP2 (5000pM), TGFβ1+BMP2, or TGFβ2+BMP2 for 72 h prior to qRT-PCR. Pre and Co-incubation of BMP2 significantly inhibited the expression of SM22α, Sma-actin, and calponin in response to TGFβ1 (45 %, 64 %, and 48

% respectively) or TGF β 2 (28 %, 57 %, 34 % respectively). *Columns*, median fold change obtained from 5 separate experiments; *bars*, SE. $\ast=p<0.05$.

Fig. 3. *Tgfb3*^{-/-} epicardial cells lose epithelial character in response to BMP2
Tgfb3^{-/-} immortalized epicardial cells (E11.5) were incubated with vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2 for 72h. **A–D:** Immunohistochemistry: *Tgfb3*^{-/-} cells were stained with an epithelial marker, zonula occludens-1 (ZO-1). Cells incubated with vehicle express ZO-1 along the cell-cell borders consistent with an epithelial phenotype (A). Cells incubated with TGFβ1 (B), TGFβ2 (C) or BMP2 (D) are elongated and have decreased cell-cell contacts and redistributed ZO-1 expression. (n=3, results shown for one littermate pair). TGFβ1 and TGFβ2, but not BMP2, increase the expression of SM22α, SMαA, and calponin after 72 h as shown by qRT-PCR analysis (E). *Columns*, median fold change obtained from 5 separate experiments; *bars*, SEM. *=p<0.05.

Fig. 4. Distinct ALK activity is required for the effects of TGFβ and BMP2

RT-PCR analysis confirmed expression of ALK1, ALK2, ALK3, ALK4, and ALK5 in *Tgfr3*^{+/+} and *Tgfr3*^{-/-} epicardial cells. (n=3, data shown for one littermate pair).

Immortalized *Tgfr3*^{+/+} epicardial cells (E11.5) were incubated with vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2 in the presence or absence of 2.5 μM of the ALK5 kinase inhibitor SB431542 or 2 μM DMH1 (ALK2/3 kinase inhibitor) for 72 h prior to fixation. **A-H:** Immunohistochemistry: Cells were stained with an epithelial marker, zonula occludens-1 (ZO-1). **A-D:** Cells incubated with vehicle and ALK5 inhibitor formed a compact monolayer and displayed an epithelial phenotype (A). Cells incubated with TGFβ1 (D), or TGFβ2 (F) in the presence of ALK5 inhibitor retained zonula occludens-1 (ZO-1) expression at cell-cell borders consistent with an epithelial phenotype. Cells incubated with BMP2 (H) in the presence of ALK5 inhibitor are elongated and separated from one another with a loss or decrease of ZO-1 expression. **E-H:** Cells incubated with vehicle and ALK2/3 inhibitor express ZO-1 along the cell-cell borders consistent with an epithelial phenotype (C). Cells incubated with TGFβ1 (E), or TGFβ2 (G), in the presence of ALK2/3 inhibitor are elongated and have decreased cell-cell contacts and ZO-1 expression. BMP2 and ALK2/3 inhibitor incubated cells (I) retained the cell-cell contacts and ZO-1 expression. (n=3, data shown for one littermate pair).

Fig. 5. ALKs are sufficient to induce loss of epithelial character or smooth muscle differentiation in epicardial cells

Tgfb β 3^{+/+} immortalized epicardial cells (E11.5) were transfected with plasmid encoding green fluorescent protein (GFP) alone or GFP and constitutively active (ca) ALK2, caALK3, and caALK5. **A–D**: Immunohistochemistry: Cells were stained with an epithelial marker, zonula occludens-1 (ZO-1). GFP alone expressing cells form an epithelial sheet and express ZO-1 at cell-cell borders (A). Cells expressing caALK2 (B), caALK3 (C), or caALK5 (D) appear elongated, separated from their neighbor, and have discontinuous ZO-1 expression. (n=3, results shown for one littermate pair) **E**: Quantification of the percent of cells that loose epithelial character after transfection with plasmids. Data are plotted as the mean percent $\pm$ SD of three independent experiments (*=p<0.05) **F–G**: qRT-PCR analysis of cells transfected with plasmids. Cells expressing caALK2 and caALK5, but not caALK3, undergo smooth muscle differentiation as shown by induction of SM22 α , SM α A, and Calponin (F). Cells transfected with plasmids do not induce endothelial marker expression (G). *Columns*, mean fold change obtained from 5 separate experiments; *bars*, SD. *=p<0.05.

Fig. 6. TGF β R3 interaction with GIPC is required for BMP2 induced epicardial cell invasion
 Quantification of invasion using a modified boyden chamber assay of *Tgfr3*^{+/+} and *Tgfr3*^{-/-} immortalized epicardial cells (E11.5, littermate pairs). (A) *Tgfr3*^{-/-} epicardial cells have a reduced ability to invade in response to 5000 pM BMP2 when compared to *Tgfr3*^{+/+} epicardial cells. (n=3, replicates of 6, *p<0.05). (B) *Tgfr3*^{-/-} epicardial cells were infected with adenovirus co-expressing GFP and either FL, CYTO, or Δ3 receptor and incubated with 5000 pM BMP2. Cells expressing the full length receptor were restored in their ability to invade. (n=3, replicates of 6, *p<0.05). (C) *Tgfr3*^{+/+} epicardial cells were transfected with control siRNA or siRNA to GIPC1 and incubated with 5000 pM BMP2. Knockdown of GIPC results in reduced invasion potential. (n=3, replicates of 6, *p<0.05). (D) Relative expression levels following transfection with siRNA targeting GIPC1 (n=3; *p<0.05 [FL+Scramble+BMP2] vs. [GFP+Scramble+BMP2] §p<0.05 [FL+Scramble+BMP2] vs. [FL+GIPCsRNA+BMP2]). (E) *Tgfr3*^{-/-} cells infected with adenovirus expressing GFP or FL receptor, transfected with siRNA to GIPC1, and incubated with

vehicle or 5000 pM BMP2. GIPC knockdown in the cells inhibited rescue of invasion by FL receptor. (n=3, replicates of 6, $*=p<0.05$). (F) Relative expression levels following transfection with siRNA targeting GIPC1 (n=3; $*=p<0.05$).

Fig. 7. ALK activity is required and sufficient for TGFβ and BMP2 induced invasion
 Invasion was analyzed as described in Fig. 6. (A) *Tgfr3*^{+/+} immortalized epicardial cells (E11.5) were incubated with either vehicle, 2.5 μM of the ALK5 kinase inhibitor SB431452, or 2 μM DMH1 (ALK2/3 kinase inhibitor) and scored for invasion in response to vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2. Inhibition of ALK2/3 or ALK5 activity results in the cells inability to invade in response to all ligands. (n=3, replicates of 6, **p*<0.05). (B) *Tgfr3*^{+/+} immortalized epicardial cells (E11.5) were transfected with control siRNA or siRNA to ALK2, ALK3, or ALK5 and incubated with vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2. Knockdown of any ALKs resulted in a decrease in invasion potential in response to all ligands. (n=3, replicates of 6, **p*<0.05 [Scramble+TGFβ1] vs. [ALKsiRNA+TGFβ1] \$*p*<0.05 [Scramble+TGFβ2] vs. [ALKsiRNA+TGFβ2] #*p*<0.05 [Scramble+BMP2] vs. [ALKsiRNA+BMP2]). (C) Relative expression levels following transfection with siRNA targeting ALK2, ALK3, and ALK5

(n=3; *p<0.05). (D) *Tgfb β 3*^{-/-} epicardial cells were transfected with plasmids expressing either green fluorescent protein alone (GFP) or GFP and constitutively active (ca) ALK2, caALK3, and caALK5 and scored for invasion. Overexpression of all constitutively active receptors was sufficient to rescue invasion. (n=3, replicates of 6, *p<0.05). (E) Quantification of cell number using MTS assay in *Tgfb β 3*^{+/+} cells transfected with plasmids coding for GFP, GFP + caALK2, GFP + caALK3, and GFP + caALK5. (n=3)

Fig. 8. Smads are required, but not sufficient for invasion of epicardial cells

Invasion was analyzed as described in Fig. 6. (A) *Tgfbr3*^{+/+} immortalized epicardial cells (E11.5) were transfected with control siRNA or siRNA to Smad4 and incubated with vehicle, 250 pM TGFβ1, 250 pM TGFβ2, or 5000 pM BMP2. Knockdown of Smad4 resulted in a decrease in invasion potential in response to all ligands. (n=3, replicates of 6, *p<0.05 [Scramble+TGFβ1] vs. [Smad4siRNA+TGFβ1] \$p<0.05 [Scramble+TGFβ2] vs. [Smad4siRNA+TGFβ2] #p<0.05 [Scramble+BMP2] vs. [Smad4siRNA+BMP2]). (B) Relative expression levels following transfection with siRNA targeting Smad4 (n=3; *p<0.05). (C) *Tgfbr3*^{-/-} epicardial cells were infected with adenovirus expressing either GFP alone, GFP + Smad1, or GFP + Smad3 and scored for invasion. Overexpression of Smad1 was not sufficient to rescue invasion, whereas overexpression of Smad3 was barely significantly different from control infected cells. (n=3, replicates of 6, *p<0.05). (D) Quantification of cell number using MTS assay in *Tgfbr3*^{+/+} cells infected with adenovirus expressing either GFP alone, GFP + Smad1, or GFP + Smad3. (n=3)

Fig. 9. Canonical and non-canonical BMP2 signaling regulates epicardial cell behavior
 The loss of epithelial character in response to TGFβ or BMP2 is mediated by canonical signaling via ALK5 or ALK3, respectively. Both TGFβ- and BMP2-stimulated invasion requires TGFβR3 and contribution from ALK5, ALK2, and ALK3 signaling. We propose that TGFβR3, through interaction with GIPC, is required for the coordinate activation of these ALKs in order to mediate invasion.