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TGF β and BMP-2 regulate epicardial cell invasion via TGF β R3 activation of the Par6/Smurf1/RhoA pathway

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Abstract

Coronary vessel development requires transfer of mesothelial cells to the heart surface to form the epicardium where some cells subsequently undergo epithelial-mesenchymal transformation (EMT) and invade the subepicardial matrix. *Tgfb β 3*^{-/-} mice die due to failed coronary vessel formation associated with decreased epicardial cell invasion but the mediators downstream of TGF β R3 are not well described. TGF β R3-dependent endocardial EMT stimulated by either TGF β 2 or BMP-2 requires activation of the Par6/Smurf1/RhoA pathway where Activin Receptor Like Kinase (ALK5) signals Par6 to act downstream of TGF β to recruit Smurf1 to target RhoA for degradation to regulate apical-basal polarity and tight junction dissolution. Here we asked if this pathway was operant in epicardial cells and if TGF β R3 was required to access this pathway. Targeting of ALK5 in *Tgfb β 3*^{+/-} cells inhibited loss of epithelial character and invasion. Overexpression of wild-type (wt) Par6, but not dominant negative (dn) Par6, induced EMT and invasion while targeting Par6 by siRNA inhibited EMT and invasion. Overexpression of Smurf1 and dnRhoA induced loss of epithelial character and invasion. Targeting of Smurf1 by siRNA or overexpression of constitutively active (ca) RhoA inhibited EMT and invasion. In *Tgfb β 3*^{-/-} epicardial cells which have a decreased ability to invade collagen gels in response to TGF β 2, overexpression of wtPar6, Smurf1, or dnRhoA had a diminished ability to induce invasion. Overexpression of TGF β R3 in *Tgfb β 3*^{-/-} cells, followed by siRNA targeting of Par6 or Smurf1, diminished the ability of TGF β R3 to rescue invasion demonstrating that the Par6/Smurf1/RhoA pathway is activated downstream of TGF β R3 in epicardial cells.

1.0 Introduction

Epithelial to mesenchymal transformation (EMT) is an important process during embryonic development and disease progression [1-4]. Transforming Growth Factor β (TGF β) plays a critical role in regulating EMT in both of these settings [2, 5] and is particularly relevant in cardiovascular development during heart valve formation and coronary vessel development [6-8]. Coronary vessel development is initiated when a group of mesothelial cells termed the proepicardium are transferred to the surface of the heart to form the epicardium [8-11]. A subset of these cells undergoes epithelial EMT and invades the subepicardial space with

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some cells continuing into the myocardium. Cells may then differentiate into one of several cell lineages including vascular smooth muscle cells and cardiac fibroblasts [12, 13].

Targeting of *Tgfb β 3* in mice is embryonic lethal at E14.5 due to failed coronary vessel development [14]. Although 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 understood [15] there remain significant gaps in our understanding of how the wide array of TGF β -induced responses are signaled and regulated, particularly with respect to the contributions of TGF β R3. TGF β R3 binds TGF β 1 and TGF β 3 and is required for the high affinity binding of TGF β 2 [16]. TGF β R3 also binds and signals in response to BMP-2 [17] and functions as an inhibin receptor [18]. After TGF β binding to TGF β R3, TGF β R3 presents ligand to the Type I (TGF β R1) and Type II (TGF β R2) TGF β receptors to augment signaling through the canonical signaling pathway that requires the phosphorylation and nuclear translocation of the Smads [19]. TGF β R3 contains a highly conserved, 43 amino acid intracellular domain with no identified catalytic activity [20, 21]. The cytoplasmic domain is not required for the ability of TGF β R3 to present ligand to TGF β R1 and TGF β R2 and augment canonical signaling [22]. TGF β R3 is postulated to play a unique and non-redundant role in TGF β signaling in addition to ligand presentation based on the results of targeting TGF β R3 in cardiac cushion explants [23] and mice [14]. Regulation of the migration and invasion of several cancer cell lines [24, 25], as well as in epicardial [26] and endothelial [27] cells, has been shown to require the cytoplasmic domain of TGF β R3 suggesting the presence of a non-canonical signaling pathway activated by TGF β R3.

Our laboratory has recently reported that the Par6/Smurf1/RhoA pathway mediates EMT in the atrioventricular (AV) cushion endocardial cells which contribute to the heart valves [28, 29]. The Par6/Smurf1/RhoA pathway aids in the maintenance of epithelial cell polarity and is activated in a Smad-independent manner to induce loss of epithelial character and EMT [30]. This pathway has also been demonstrated to play a key role in the control of morphological transformation in breast cancer metastasis [31, 32]. Par6 was initially described in *C.elegans* as a mediator of apical-basal polarity [33] and is localized with TGF β R1 or ALK5 at the tight junctions by occludin [34]. Upon TGF β stimulation, TGF β R2 phosphorylates Par6 which then recruits the E3 ubiquitin ligase, Smurf1 [35]. Smurf1 in turn targets RhoA for degradation allowing dissolution of tight junctions and subsequently EMT [36]. We established that BMP-2 binds and signals via TGF β R3 in endocardial cells [17] and that BMP-2 activates the Par6/Smurf1/RhoA pathway in a TGF β R3-dependent manner [28].

TGF β R3 is also expressed in epicardial cells which are major contributors to the coronary vessels, and *Tgfb β 3*^{-/-} mice die at E14.5 due to failed coronary vessel development [14] associated with decreased invasion by the epicardial-derived mesenchymal cells [26]. *In vitro*, *Tgfb β 3*^{-/-} epicardial cells lose epithelial character in response to TGF β but have a diminished ability to undergo TGF β -induced invasion into a collagen gel *in vitro* [26]. Given the role of the Par6/Smurf1/RhoA pathway in mediating TGF β and BMP-2-induced EMT in endocardial cushions and the requirement of TGF β R3 for this process, we investigated the role of Par6/Smurf1/RhoA in epicardial cell invasion *in vitro* and demonstrate that the Par6/Smurf1/RhoA pathway is operant in epicardial cells. Our results demonstrate that access to the Par6/Smurf1/RhoA pathway by both TGF β and BMP-2 is regulated by TGF β R3.

2.0 Materials and Methods

2.1 Cell Culture

Immortalized epicardial cell lines were obtained as previously described [37]. To maintain the immortalized state, cells were grown in immorto media: DMEM containing 10% FBS (fetal bovine serum), 100 U/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) INF γ at 33°C. For experiments, the T antigen was silenced by culturing at 37°C in the absence of ITS and INF γ . Epicardial cells isolated from E11.5 embryos were used in all experiments.

2.2 Growth Factors and Inhibitors

TGF β 1, TGF β 2, and BMP-2 were purchased from R&D Systems and were reconstituted in 4mM HCl/0.01% BSA. TGF β 1 and TGF β 2 were used at 250 pM. SB431452 was purchased from Sigma and Cal 616451 was purchased from Calbiochem and used at 2.5 µM and 150 nM, respectively.

2.3 Adenovirus Infections

Adenoviruses were generated using the pAdEasy system [38]. All concentrated viruses were titered by performing serial dilutions of the concentrated virus and counting the number of GFP-expressing 293 cells after 18–24 h. The following adenoviruses co-expressing GFP were used: full length TGF β R3 (FL), constitutively active Alk5 (caAlk5), wild-type Par6 (WTPar6), dominant negative Par6 (dnPar6), Smurf1, constitutively active RhoA (caRhoA), dominant negative RhoA (dnRhoA), constitutively active cdc42 (cacdc42), dominant negative cdc42 (dncdc42), constitutively active Rac1 (caRac1), dominant negative Rac1 (dnRac1). Epicardial cells were plated in collagen coated 6 well dishes at a density of 200,000 cells per well in immorto media overnight at 33°C. The following day, virus was added directly to the cells at a final concentration of 10^8 PFU/ml and allowed to incubate for an additional 24 hours. The next day cells were plated for invasion, proliferation or qRT-PCR analysis as described below.

2.4 Transfections

Cells were plated at a density of 200,000 per well of 6-well plate. The following day cells were transfected with 2 µg siRNA (Ambion) and 8 µl Xtreme siRNA Transfection Reagent (Roche). Cells were incubated for an additional 48 hours before proceeding with experiments. Sequences for siRNA used are as follows:

siRNA	Sense 5'→3'	Anti-Sense 5'→3'
<i>Scramble</i>	CUCCTUGTCAATUUACCGCtt	AAGCGGTAAATTGACAAGGag
<i>ALK5-A</i>	CAAACGCGCUGACAUCUAUtt	AUAGAUGUCAGCGGUUUGaa
<i>ALK5-B</i>	GAGUAGGCACUAAAAGGUAtt	UACCUUUUAGUGCCUACUCtg
<i>PAR6-A</i>	GUAUCUUCAUUUCUGAAUtt	AUUCGAGAAAUGAAGAUACct
<i>PAR6-B</i>	CUCUGUCACGGAAGAAAAAtt	UUUUUCUUCGUGACAGAGtg
<i>SMURF1-A</i>	GGAACUAGAGCUGAUAAUAtt	UAUUAUCAGCUCUAGUUCct
<i>SMURF1-B</i>	GAUCAACCCAGAUUCUUCUtt	AGAAGAAUCUGGGUUGAUCtg
<i>RhoA-A</i>	AGCCCUGAUAGUUUAGAAAtt	UUUCUAAACUAUCAGGGCUgt

siRNA	Sense 5'→3'	Anti-Sense 5'→3'
<i>RhoA-B</i>	CAGCCUGAUAGUUUAGAAAtt	UUCUAAACUAUCAGGGCUGtc

2.5 Immunohistochemistry

Tgfb β 3^{+/+} cells were plated at a density of 25,000 cells per well in one well of a 4-well collagen coated chamber slide and allowed to adhere overnight at 37°C. The following day virus was added to each well and 24 hours later the media was replaced with DMEM containing 5% FBS. After a 72 hour incubation period at 37°C, cells were fixed in 2% paraformaldehyde (PFA) for 30 min and permeabilized with PBS and 0.2% Triton X-100 for 5 min at room temperature. Cells immunostained for ZO-1 were blocked with 2% bovine serum albumin (BSA) in PBS for 1 hr and incubated with dilute primary antibody (ZO-1, 2µg/ml, Zymed) overnight at 4°C. Primary antibody was detected with goat anti-rabbit cy3 secondary antibody (1:800; Jackson ImmunoResearch). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma). Photomicrographs were captured with Nikon Eclipse TE2000-E microscope and QED imaging software.

2.6 Invasion Assay

To determine the invasive potential of immortalized epicardial cells, a modified Boyden chamber assay was employed. Collagen gels were prepared as described [26, 39]. Briefly, cells were fluorescently labeled with CalceinAM (BD Biosciences) and then plated at 12,000 cells per well in DMEM containing 0.5% FBS (fetal bovine serum) in the top chamber (4-6 replicates/treatment group). Cells were then allowed to settle overnight at 37°C. For wells receiving growth factor treatment the following day DMEM containing 20% FBS +/- 250 pM TGFβ1 or TGFβ2) was added to the bottom chamber. Wells receiving inhibitor treatment, growth factor plus inhibitor were added to bottom well as described above. In addition, cells were treated with inhibitor in the top well in 0.5% DMEM + FBS. All treatment groups were incubated for an additional 24 hours at 37°C. The top insert was then removed and placed in a plate containing 0.25% Trypsin/2.21 mM-EDTA in HBSS (CellGro). Cells were allowed to detach 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)

2.7 Proliferation Assay

Proliferation was determined using and MTS assay, as described previously [26], 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 490nm and is directly proportional to the number of living cells in culture. Cells were plated in triplicate in a 96-well plate at a density of 5,000 cells/well in 100 µl of DMEM containing 10% FBS and 100U/ml P/S overnight at 37°C. At 48h post-plating, 20 µl of substrate (Promega: Cell Titer 96 Aqueous Solution) was added to each well. Colorimetric reaction was allowed to proceed for 30 minutes at 37°C, followed by reading at 490nm.

2.8 qRT-PCR

To confirm gene knockdown we used qRT-PCR as previously described [26]. For siRNA studies, RNA was collected 48 hours after transfection. Total RNA was isolated using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNA was generated from 1ug total RNA using oligo-dT primers and Superscript III polymerase (Invitrogen). 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 [40]. 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: $R = 2^{-\Delta\Delta CT}$ (where $R = 2^{(\Delta CT_{treated} - \Delta CT_{control})}$). This method normalizes the abundance of all transcripts to the constitutive expression level of *GAPDH* RNA. Primer pairs to confirm gene knockdown are as follows:

Gene	Sense primer (5'→3')	Anti-sense primer (5'→3')
<i>Alk5</i>	CCTTCTGATCCATCGGTTGA	CCATTGGCATACCAGCAT
<i>Par6g</i>	CCTTGCTTCGAGTCTTCAT	GCTGATGTTGAGGTGGG
<i>Smurf1</i>	CTTCAAGGCTCTGCAAGG	GCTTCTCATAGGACTCATAGGG
<i>RhoA</i>	GACAGGAAGATTATGACCGC	TGGACAGAAATGCTTGACTTC

2.9 Statistical analysis

Paired student t-test was used to establish significance. Data are presented as the average of experiments $\pm$ SEM. P-values of < 0.05 were considered significant.

3.0 Results

3.1 ALK5 is sufficient to drive epicardial EMT and invasion

Par6 and ALK5 are co-localized at tight junctions by occludin [30] where ligand binding induces ALK5 to phosphorylate Par6 initiating the activation of the Par6/Smurf1/RhoA pathway [30]. We have previously shown that ALK5 is required and sufficient for the loss of epithelial character in epicardial cells [37] (Figure 1A). Here, we sought to determine whether ALK5 is required for TGF β -induced invasion into a collagen gel using a modified Boyden Chamber Assay. Cells were incubated with vehicle or vehicle plus one of two structurally dissimilar small molecule inhibitors of ALK5 kinase activity, 2.5 μ M SB431452 or 150 nM Calbiochem 616451 (Figure 1B). Incubation with 250 pM TGF β 2 induced invasion 2.7-fold over vehicle incubated cells. The ability of TGF β 2 to induce invasion was abrogated with the addition of either inhibitor (Figure 1B). To confirm these results we used two independent siRNA constructs targeting ALK5 with GC-matched siRNA as a control. Cells transfected with control siRNA and incubated with either 250 pM TGF β 1 or TGF β 2 induced a 2.7 and 2.2-fold invasion relative to control siRNA + vehicle incubated cells. Addition of either siRNA targeting ALK5 inhibited the ability of TGF β 1 or TGF β 2 to induce invasion (Figure 1C). Together these data demonstrate that ALK5 is required for TGF β -induced invasion in epicardial cells.

3.2 Par6 is sufficient and required to drive epicardial EMT and invasion

We next used overexpression of wildtype Par6 (wtPar6) or dominant negative Par6 (dnPar6) to determine whether Par6 activity regulates epithelial character and invasion in epicardial cells. Immunohistochemistry was used to score for the loss of epithelial character by analyzing the redistribution of the tight junction protein, zonula occludens-1 (ZO-1). Cells were infected with adenovirus co-expressing GFP alone or GFP and either wtPar6 or dnPar6. GFP expressing cells demonstrated ZO-1 at cell-cell junctions (Figure 2A). Overexpression of wtPar6 induced loss of ZO-1 from cell-cell junctions in the absence of ligand to an extent similar to that seen in cells infected with caALK5 (Figure 1A and 2A). In contrast, cells expressing dnPar6 retained expression of ZO-1 at cell-cell junctions (Figure 2A). These data indicate that Par6 is sufficient and required for epicardial EMT. To address the role Par6 in epicardial invasion cells were infected with adenovirus co-expressing GFP

alone or GFP and wtPar6 or dnPar6. GFP expressing cells incubated with 250 pM TGF β 2 showed a 3-fold change in invasion relative to vehicle incubated cells (Figure 2B). Expression of wtPar6 alone was sufficient to induce ~5.8-fold change in invasion relative to GFP infected, vehicle incubated cells. Addition of TGF β 2 to wtPar6 expressing cells did not further stimulate invasion. Expression of dnPar6 inhibited the ability of TGF β 2 to induce invasion (Figure 2B). To address the possibility that the increased number of cells invading seen in wtPar6 infected cells was due to altered cell proliferation, we used the MTS assay to measure cell number in GFP, WTPar6, and dnPar6 infected cells (Figure 2C). No difference in cell number was noted in any of the groups. To further confirm the role of Par6 in TGF β 2-induced invasion, we used two independent siRNA constructs to target Par6. Cells transfected with control siRNA and incubated with either TGF β 1- or TGF β 2-induced ~2 and 2.6 fold invasion, respectively. Transfection with either construct targeting Par6 completely abolished the ability of either TGF β 1 or TGF β 2 to induce invasion cells (Figure 2D). Neither of the siRNA's had any effect on cell number (Figure 2E). Collectively, these data indicate that Par6 is sufficient and required for the loss of epithelial character and invasion in epicardial cells.

3.3 Smurf1 is sufficient to drive epicardial EMT and invasion

Having established that Par6 is operant in epicardial cells and functions in epicardial EMT and invasion, we next investigated the role of Smurf1, which is activated by Par6 in this pathway. Overexpression of Smurf1 was sufficient to induce redistribution of ZO1 (Figure 3A) and invasion (Figure 3B). Smurf1 overexpression induced a 3-fold change in invasion over GFP expressing, vehicle incubated cells (Figure 3B), while having no effect on cell number (Figure 3C). Knockdown of Smurf1 using two independent siRNA constructs abolished TGF β 1- and TGF β 2-induced invasion (Figure 3D) and did not alter cell number (Figure 3E). These studies demonstrate that Smurf1 is sufficient for loss of epithelial character and required and sufficient for invasion, consistent with a role downstream of Par6.

3.4 RhoA activity regulates epicardial EMT and invasion

Once activated, Smurf1 targets RhoA for degradation leading to EMT in NMuMG [30] and endocardial cells [29]. To determine the role of RhoA activity in epicardial EMT we infected cells with adenovirus expressing either dominant negative (dn) or constitutively active (ca) RhoA. Cells expressing caRhoA retained epithelial character as determined by the presence of ZO1 at the tight junctions, whereas cells expressing dnRhoA showed redistribution of ZO1 (Figure 4A). Overexpression of dnRhoA induced invasion independent of ligand addition whereas caRhoA abolished TGF β 2-induced invasion (Figure 4B). Expression of either dnRhoA or caRhoA did not affect cell number (Figure 4C). Knockdown of RhoA using two independent siRNA constructs resulted in invasion. These data support our initial observation and indicate that RhoA stability is required to maintain epithelial character while degradation of RhoA promotes loss of epithelial character and drives invasion (Figure 4D). Targeting RhoA by siRNA did not affect cell number (Figure 4E). Given that multiple GTPases have been implicated in regulating cytoskeletal rearrangement [41-43], and that the small GTPases, Cdc42 and Rac1, have been reported to act in concert with RhoA to mediate intracellular signaling [44, 45], we asked whether these other two small GTPases could be playing a role in epicardial cell invasion. To discern the roles of these GTPases on epicardial invasion, we overexpressed ca- or dn- Cdc42 or Rac1. Neither isoforms of the GTPases had any effect on relative invasion levels (Figure 5A and B) or cell number (Figure 5C). Therefore, these GTPases do not participate in epicardial cell invasion. Taken together these data support the role for Par6/Smurf1/RhoA being operant in epicardial cells to regulate loss of epithelial character and cell invasion.

3.5 BMP-2 can access Par6/Smurf1/RhoA pathway in epicardial cells

Given that TGF β and BMP-2 both access the Par6/Smurf1/RhoA pathway in endocardial cells via TGF β R3 [28] we next asked whether BMP-2 acts through the Par6/Smurf1/RhoA pathway to regulate invasion. We targeted each member of the Par6 pathway with two independent siRNA constructs in *Tgfb3*^{+/+} epicardial cells incubated with 5nM BMP-2. In all cases, transfection with scrambled siRNA followed by incubation with 5nM BMP-2 induced ~ 2.25 fold invasion relative to vehicle incubated cells. Knockdown of ALK5, Par6, or Smurf1 with each siRNA abolished the ability of BMP-2 to induce invasion (Figure 6 A, B, C). Knockdown of RhoA resulted in levels of cell invasion that were not increased by the addition of BMP-2 (Figure 6D). These data indicate that in epicardial cells, as in endocardial cells [28, 29], the Par6/Smurf1/RhoA pathway can be accessed by both TGF β and BMP-2.

3.6 TGF β R3 regulates access to the Par6/Smurf1/RhoA pathway by TGF β and BMP-2 in epicardial cells

Given that TGF β and BMP-2 both require TGF β R3 to access the Par6/Smurf1/RhoA pathway in endocardial cells [28, 29] we sought to determine whether access to this pathway in epicardial cells required TGF β R3. We took advantage of our existing *Tgfb3*^{-/-} immortalized epicardial cells to determine if the Par6/Smurf1/RhoA pathway is downstream of TGF β R3. Overexpression of full length TGF β R3 (FL-TGF β R3) in *Tgfb3*^{-/-} cells was coupled with knock down of ALK5, Par6, or Smurf1 and scoring the ability of epicardial cells to invade in response to TGF β 2 or BMP-2. GFP-infected *Tgfb3*^{-/-} cells transfected with control siRNA and incubated with TGF β 2 or BMP-2 exhibited low levels of invasion (1.54 and 1.49-fold, respectively) relative to vehicle incubated cells (Figure 7A, B, C). In contrast, cells infected with FL-TGF β R3 and transfected with control siRNA showed higher levels of invasion (2.22 and 2.17-fold, respectively) when incubated with TGF β 2 or BMP-2 (Figure 7A, B, C). Cells infected with FL-TGF β R3 and transfected with siRNA targeting ALK5 (Figure 7A), Par6 (Figure 7B), or Smurf1 (Figure 7C) showed a reduced ability of FL-TGF β R3 to rescue responsiveness to TGF β 2 or BMP-2-induced invasion.

Overexpression of wtPar6, Smurf1 or dnRhoA was sufficient to induce invasion in *Tgfb3*^{-/-} cells, although to a lesser extent than in wildtype cells, demonstrating that the pathway components are present in the absence of *Tgfb3* in these cells (Figure 8A). There was no effect on cell number (Figure 8B). Expression of dnPar6 or caRhoA did not induce changes in invasion or cell number (Figure 8B). Together these data establish that TGF β R3 is required in epicardial cells to access the Par6/Smurf1/RhoA pathway in response to TGF β 2 or BMP-2.

4.0 Discussion

Here we demonstrate that in epicardial cells both TGF β 2 and BMP-2 require TGF β R3 to access the Par6/Smurf1/RhoA pathway via ALK5 to mediate cell invasion. This establishes a novel pathway downstream of TGF β R3 in epicardial cells that contributes to coronary vessel formation by regulating the delivery of epicardially-derived mesenchyme to the subepicardial space and myocardium. Loss of TGF β R3 results in failed coronary vessel formation [14] associated with decreased mesenchymal cell invasion [26], but the molecular mechanisms regulating cell invasion downstream of TGF β R3 have been unclear. Recently we reported that epicardial cell invasion *in vitro* requires both the specific cytoplasmic residues of TGF β R3 that bind the scaffolding protein, GAIP-interacting protein, C terminus (GIPC), as well as GIPC [26]. The data presented here suggests that integration of GIPC signaling and TGF β R3-dependent regulation of the Par6/Smurf1/RhoA together mediate epicardial cell invasion. In endocardial cells, TGF β R3 is required to activate the Par6/Smurf1/RhoA pathway by TGF β 2 and BMP-2 to induce EMT and invasion [28, 29]. This

activity in endocardial cells is similarly dependent on the cytoplasmic domain of TGF β R3 and GIPC [27]. While these findings in epicardial and endothelial cells are consistent and suggest a general mechanism for TGF β R3-mediated regulation of EMT, how these two pathways intersect is not currently known.

Although to our knowledge no prior report has addressed the role of Par6 or Smurf1 in epicardial cell EMT, invasion, or differentiation there is considerable data on the role of ALK5. We have previously established that ALK5 is required and sufficient to drive EMT and smooth muscle differentiation in epicardial cells [37, 46]. ALK5 is also required but not sufficient for cell transformation and invasion in endocardial cells [6, 47] downstream of TGF β 2 and BMP-2 [17, 29]. ALK5 signaling has also been shown to be particularly important for proper formation of the epicardium during cardiac development *in vivo*. Mice with conditional deletion of *Alk5* from the epicardium survive until birth but the coronary arteries display a defective smooth muscle layer and epicardial cells cultured from these mice fail to undergo TGF β -induced EMT *in vitro* [48]. These data confirm the role of ALK5 in mediating epicardial cell EMT and invasion but also suggest that additional mechanisms are available *in vivo* to at least partially rescue epicardial cell invasion. These additional mechanisms may include signaling initiated by HA [39, 49], PDGF [50], or FGF [51] which have all been demonstrated to regulate epicardial cell invasion (reviewed in [52]).

Par6 has been shown regulate cell polarity through interaction with the small GTPases, Cdc42 and Rac1 [53, 54] and TGF β R3 has been reported to regulate directional persistence through its interaction with Cdc42 [25]. However, neither constitutively active nor dominant negative forms of these small GTPases interfered with the ability of epicardial cells to respond to TGF β 2- or BMP-2- induced invasion. In contrast, both dnRhoA and siRNA targeting RhoA induced epicardial cell invasion while caRhoA inhibited TGF β 2-stimulated invasion. These results in epicardial cells are consistent with those seen in endocardial cells where TGF β R3 is required for cell transformation and invasion in response to TGF β 2 or BMP-2 [17, 28]. The introduction of dnRhoA or siRNA to ventricular endocardial cells induced cell transformation and invasion while caRhoA did not [29]. Constitutively active or dominant negative forms of Cdc42 and Rac1 were without effect on transformation or invasion. These findings, along with the fact that neither Cdc42 nor Rac1 are substrates for Smurf1 [55], support the hypothesis that TGF β 2- and BMP-2- induced invasion is mediated through RhoA degradation in response to Par6 activation of Smurf1 as has been described in NMuMG cells [30].

The integration of TGF β 2 and BMP-2 signaling through TGF β R3 is especially relevant to the interpretation of the phenotype of the *Tgfbr3*^{-/-} mouse. The consideration of the mechanisms that underlie the failed coronary vessel development seen in *Tgfbr3*^{-/-} mice must now include the contribution of BMP-2 signaling through TGF β R3 as well as the previously recognized role of the TGF β 's in signaling via TGF β R3. Similarly, a potential role for TGF β R3, as well as the canonical BMP signaling receptors, may be considered in understanding the reported roles of BMP-2 in development. For example, *Bmp2*^{-/-} mice have deficits in endocardial EMT [56, 57] and in proepicardial explants, BMP-2 in cooperation with FGF2, regulates differentiation into myocardial or epicardial lineage [58]. TGF β R3 is expressed in both of these tissues which would allow for a contribution by TGF β R3 to BMP-2 signaling ([23], unpublished).

Par6 has been shown to play a significant role in cancer metastasis. Blockade of Par6 activity maintains the epithelial character of nontransformed mammary cells and decreases protrusions from transformed mammary cells [32]. In an orthotopic model of breast cancer metastasis the inhibition of Par6 activity enhanced the epithelial character of tumor cells and decreased metastasis [32]. These data suggests a central role for Par6 signaling in tumor

progression and metastasis. Data in epicardial cells presented here suggest that TGF β R3 must be considered as a possible regulator of Par6 in tumor metastasis, especially given abundant data demonstrating that TGF β R3 regulates tumor cell migration [59-61]. Similarly BMP-2, in addition to TGF β , may access Par6 in tumor cells via TGF β R3. In summary, we demonstrate that TGF β 2 and BMP-2 in epicardial cells can assess the Par6/Smurf1/RhoA pathway via TGF β R3 to mediate epicardial cell invasion during coronary vessel formation. These data force us reconsider the roles and contributions of these ligands with respect to activation of canonical signaling pathways versus signaling through TGF β R3 in both development and disease.

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Abbreviations

ALK	Activin Receptor-Like Kinases
AVC	Atrioventricular Endocardial Cushion
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
Rac1	Ras-related C3 botulinum toxin substrate 1
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 TGF β receptor
TGFβR3	Type III TGF β receptor
TGFβR3-FL	Type III TGF β receptor-full length
TGFβR3-CYTO	Type III TGF β receptor-lacking the entire cytoplasmic domain
TGFβR3	Type III TGF β receptor-lacking the 3 C-terminal amino acids

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Highlights

TGF β 2 and BMP-2 signal epicardial cell transformation and invasion

Activation of the Par6/Smurf1/RhoA pathway is required for both ligands

TGF β 2R3 is required to access the Par6/Smurf1/RhoA pathway

Fig 1. ALK5 is required for epicardial cell invasion

(A) *Tgfbr3*^{+/+} cells infected with adenovirus expressing GFP alone (left) or GFP and caALK5 (right), and immunostained for the tight junction protein, ZO-1. (B and C) Quantitation of TGFβ-induced invasion using a modified Boyden chamber assay in the presence of either: (B) ALK5 inhibitors, SB431452 (2.5μM) or Cal616451 (150 nM) or (C) siRNA targeting ALK5 (n=3; *p<0.05).

Fig 2. Par6 is required and sufficient for epicardial cell invasion

Tgfbr3^{+/+} cells infected with adenovirus expressing GFP + wtPar6 or GFP + dnPar6. (A) Immunohistochemistry to visualize redistribution of the tight junction protein, ZO-1. Quantitation of invasion using a modified Boyden chamber assay in *Tgfbr3*^{+/+} cells (B) infected with adenovirus co-expressing GFP + wtPar6 or GFP + dnPar6, or (D) transfected with siRNA targeting Par6. (C) Quantitation of cell number using MTS assay in *Tgfbr3*^{+/+} cells infected with adenovirus co-expressing GFP + wtPar6 or GFP + dnPar6. (E) Relative expression levels following transfection with siRNA targeting Par6 (n=3; *p<0.05).

Fig 3. Smurf1 is required and sufficient for epicardial cell invasion

Tgfb β 3^{+/+} cells infected with adenovirus co-expressing GFP alone or GFP + Smurf1. (A) Immunohistochemistry to visualize redistribution of the tight junction protein, ZO-1. Quantitation of invasion using a modified Boyden chamber assay in *Tgfb β 3^{+/+}* cells (B) infected with adenovirus co-expressing GFP alone or GFP + Smurf1, or (D) transfected with siRNA targeting Smurf1. (C) Quantitation of cell number using MTS assay in *Tgfb β 3^{+/+}* cells infected with adenovirus co-expressing GFP alone or GFP + Smurf1. (E) Relative expression levels following transfection with siRNA targeting Smurf1 (n=3; *p<0.05).

Fig 4. Loss of RhoA is required and sufficient for epicardial cell invasion

Tgfb3^{+/+} cells infected with adenovirus co-expressing GFP + caRhoA or GFP + dnRhoA. (A) Immunohistochemistry to visualize redistribution of the tight junction protein, ZO-1. Quantitation of invasion using a modified Boyden chamber assay in *Tgfb3*^{+/+} cells (B) infected with adenovirus co-expressing GFP + caRhoA or GFP + dnRhoA, or (D) transfected with siRNA targeting RhoA. (C) Quantitation of cell number using MTS assay in *Tgfb3*^{+/+} cells infected with adenovirus co-expressing GFP + caRhoA or GFP + dnRhoA. (E) Relative expression levels following transfection with siRNA targeting RhoA (n=3; *p < 0.05).

Fig 5. cdc42 and Rac1 are not required for epicardial cell invasion

Tgfb3^{+/+} cells infected with adenovirus co-expressing GFP + caCdc42, GFP + dnCdc42, GFP + caRac1 or GFP + dnRac1. (A, B) Quantitation of invasion using a modified Boyden chamber assay. (C) Quantitation of cell number using MTS assay (n=3; *p<0.05).

Fig 6. BMP-2 can induce invasion through the Par6/Smurf1/RhoA

Cells were transfected with siRNA targeting (A) ALK5, (B) Par6, (C) Smurf1, or (D) RhoA, and the ability to invade a collagen gel in response to 5nM BMP-2 was assessed using a modified Boyden chamber assay (n=3; *p<0.05).

Fig 7. TGFβR3 regulates access to the Par6/Smurf1/RhoA pathway by TGFβ2 and BMP-2 in epicardial cells

Quantitation of invasion using a modified Boyden chamber assay. *Tgfr3*^{-/-} cells infected with adenovirus expressing GFP or GFP + FL-TGFβR3 followed by transfection with siRNA directed at (A) ALK5, (B) Par6 or (C) Smurf1 and incubation with TGFβ2 or BMP-2 to access the ability of epicardial cells to invade collagen gel (n=3; *p<0.05).

Fig 8. *Tgfr3*^{-/-} cells have a reduced ability to access the Par6/Smurf1/RhoA pathway
Tgfr3^{-/-} cells infected with adenovirus co-expressing GFP and caALK5, WTPar6, dnPar6, Smurf1, caRhoA, or dnRhoA (A) Quantitation of invasion using a modified Boyden chamber assay (B) Quantitation of cell number using MTS assay. (n=3; *p<0.05)