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Common pathways regulate Type III TGF β receptor–dependent cell invasion in epicardial and endocardial cells

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Abstract

Epithelial-Mesenchymal Transformation (EMT) and the subsequent invasion of epicardial and endocardial cells during cardiac development is critical to the development of the coronary vessels and heart valves. The transformed cells give rise to cardiac fibroblasts and vascular smooth muscle cells or valvular interstitial cells, respectively. The Type III Transforming Growth Factor β (TGF β R3) receptor regulates EMT and cell invasion in both cell types, but the signaling mechanisms downstream of TGF β R3 are not well understood. Here we use epicardial and endocardial cells in *in vitro* cell invasion assays to identify common mechanisms downstream of TGF β R3 that regulate cell invasion. Inhibition of NF- κ B activity blocked cell invasion in epicardial and endocardial cells. NF- κ B signaling was found to be dysregulated in *Tgfbr3*^{-/-} epicardial cells which also show impaired cell invasion in response to ligand. TGF β R3-dependent cell invasion is also dependent upon Activin Receptor-Like Kinase (ALK) 2, ALK3, and ALK5 activity. A TGF β R3 mutant that contains a threonine to alanine substitution at residue 841 (TGF β R3-T841A) induces ligand-independent cell invasion in both epicardial and endocardial cells *in vitro*. These findings reveal a role for NF- κ B signaling in the regulation of epicardial and endocardial cell invasion and identify a mutation in TGF β R3 which stimulates ligand-independent signaling.

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Keywords

transforming growth factor beta; bone morphogenic protein; endocardial cell; epicardial cell; invasion; nuclear factor-kappa B

1.0 Introduction

Transforming Growth Factor Beta (TGF β) signaling is altered in multiple human diseases including cancer, cardiovascular disease, fibrotic disease, and tissue injury (review [1]). These findings have driven efforts to explore the components of the TGF β signaling pathway as therapeutic targets. However, experience with current TGF β -based therapies under development for cardiovascular diseases (review [1–4]) and cancer ([5] & review [6–8]) have revealed that our current knowledge does not allow for efficiently meeting the task of drug development. The lack of detailed understanding of how TGF β signaling is regulated and integrated impairs our ability to predict safety and efficacy; thus presenting a significant obstacle to developing therapeutic agents.

In particular, our understanding of the role of Type III TGF β receptor (TGF β R3) in TGF β signaling is not well understood. Targeting TGF β R3 in mice [9] and cardiac cushion explants [10] revealed a unique & non-redundant role for TGF β R3 in TGF β signaling. Deletion of *Tgfbr3* in mice is embryonic lethal due to failed coronary vessel development [9] associated with decreased proliferation and invasion of the epicardial cells required for coronary vessel development [11]. Similarly, targeting TGF β R3 in cardiac cushion explants revealed a requirement of the receptor for the endocardial cell invasion that is essential for the formation of the heart valves [10]. TGF β R3 contains a glycosylated extracellular domain and a 43 amino acid intracellular domain devoid of catalytic activity [12, 13]. TGF β R3 binds TGF β 1 & TGF β 3, is required for high affinity binding of TGF β 2 [14], and also binds and signals in response to BMP2 [15] and inhibin [16].

TGF β R3 has been reported to act as a co-receptor to augment signaling via the canonical TGF β signaling pathway through Smads activation after presenting ligand to the Type I (TGF β R1) or Activin Receptor Like Kinase (ALK) 5 & Type II (TGF β R2) TGF β receptors [17]. Although the cytoplasmic domain of TGF β R3 is not required for ligand presentation to TGF β R1 & TGF β R2, the regulation of migration and invasion of several cell types have been shown to require the cytoplasmic domain of TGF β R3. These include several cancer cell lines [18, 19] as well as both endocardial [20] and epicardial cells [11]. Therefore, efforts to understand TGF β R3 signaling have focused on the identification of proteins that interact with the cytoplasmic domain. The 3 C-terminal amino acids of TGF β R3, STA, serve as a Class I PDZ binding motif and bind the scaffolding protein, GIPC (GAIP-interacting protein, C terminus). GIPC stabilizes TGF β R3 at the cell surface which has been proposed to enhance TGF β signaling [21]. The interaction between TGF β R3 and GIPC has been reported to mediate the inhibition of breast cancer cell migration *in vitro* and cancer progression *in vivo* [22]. However, in both epicardial [11] and endocardial [20] cells, ligand-stimulated cell invasion has been found to be dependent on the cytoplasmic domain of TGF β R3, specifically the 3 C-terminal amino acids that interact with GIPC. In a second,

distinct region of the cytoplasmic domain, phosphorylation of Thr841 by TGF β R2 is required for β arrestin2 (β Arr2) binding which leads to TGF β R3 internalization [23]. TGF β R2 is trafficked with TGF β R3 leading to the down-regulation of TGF β signaling. Mutation of Thr841 to alanine (TGF β R3-T841A) prevents phosphorylation by TGF β R2 and renders TGF β R3 unable to interact with β Arr2. The loss of β Arr2 interaction with TGF β R3 resulted in enhanced TGF β signaling as measured by TGF β -mediated growth inhibition in keratinocytes. The interaction between TGF β R3 and β Arr2 has also been suggested to regulate cell migration in cancer cell lines through β Arr2-mediated activation of Cdc42 [19] and through negatively regulating NF- κ B signaling [24]. Taken together, these data show a critical role for the cytoplasmic domain of TGF β R3 in the regulation of TGF β R3-dependent cell migration and invasion. Here we exploit both cultured epicardial and endocardial cells to investigate common signaling mechanisms that regulate cell invasion downstream of TGF β R3.

2.0 Material and Methods

2.1 Immortalized Epicardial Explant Culture

Multiple immortalized epicardial cell lines from *Tgfbr3*^{+/+} and *Tgfbr3*^{-/-} E11.5 littermate pair mouse embryos were generated as described previously [25]. To sustain the cell's immortalized state, they were grown at 33 °C in immorto media: 10 % fetal bovine serum (FBS), 100 U/ml Penicillin/Streptomycin (P/S), 1 X Insulin-Transferrin-Selenium (ITS; 1 μ g/ml insulin, 5.5 $\times$ 10⁻⁴ μ g/ml transferrin, 0.677 μ g/ml selenium), and 10 U/ml interferon γ (INF γ). Once the cells were ready to be used in an experiment, they were transferred to standard DMEM medium (10 % FBS and 100 U/ml P/S) and cultured at 37 °C.

2.2 Growth Factors and Inhibitors

Reagents were obtained from the following sources: TGF β 1, TGF β 2, BMP2, and FGF2 were purchased from R&D Systems; SB431542 from Sigma-Aldrich; SN-50 from Enzo; BMS-345541 from Calbiochem. DMH1 was a generous gift from Dr. Charles Hong (VUMC).

2.3 Adenovirus

Adenoviruses were generated using the pAdEasy system [26]. 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 TGF β R3 (FL), TGF β R3 lacking the cytoplasmic domain (CYTO), TGF β R3 lacking the last 3 amino acids (3), and TGF β R3 with T841A mutation (T841A).

Epicardial 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 experiments. Equivalent levels of expression and appropriate cellular localization of receptor constructs has been previously shown under these same conditions [11].

2.4 Plasmids

Epicardial cells were plated at a density of 50,000 per well of a 6-well plate in immorto media without P/S and allowed to adhere overnight at 33 °C. The cells were transfected the following day with 2 µg of either pAdTrackCMV vector alone or expressing constitutively active (ca) ALK2, caALK3, or caALK5 or NF-κB-luciferase and cmv-β-galactosidase and 6 µl of X-treme gene DNA transfection reagent (Roche) in immorto media. The media was changed 24 h after transfection to 10 % FBS/DMEM without P/S and cells were incubated at 37 °C for the duration of the experiments. Similar percent transfection efficiency (~50%) was seen for each plasmid after 48 h (data not shown).

2.5 Invasion Assay

Invasion assays were performed using a modified Boyden chamber assay as previously described [11]. 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 [27] in the top chamber. Epicardial 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).

2.6 Cell viability Assay

Epicardial cell viability was scored using a MTS assay as previously described [11, 28], 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/96-well plate in a total of 100 µl of 10 % FBS/DMEM +/- inhibitor at 37 °C. After 24 h, 20 µl 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.

2.7 Luciferase Assay

NF-κB activity was analyzed by transfecting epicardial cells with a NF-κB-luciferase reporter construct. 24 h after transfection, the cells were serum starved for 4 h and then stimulated with TGFβ1, TGFβ2, BMP2, or FGF2 for 24 h at 37 °C. Luciferase activity was normalized for transfection efficiency using a vector coding for β-galactosidase. After transfection and subsequent incubation with ligand, luciferase and β-galactosidase levels were detected using the Luciferase Assay System (Promega) and the Luminescent β-gal Detection Kit (Clontech), respectively, according to manufacturer's protocol.

2.8 Immunohistochemistry

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 were fixed with 2 %

paraformaldehyde (PFA) for 30 min at room temperature and permeabilized with ice cold 1:1 methanol/acetone solution for 30 min. The cells were incubated for 1 h in 10 % FBS/PBST solution to block non-specific binding and incubated with primary antibody (Phospho c-Rel; Cell Signaling; 1:100) overnight at 4 °C. Primary antibody detection with biotinylated goat anti-rabbit IgG and Dylight 549 streptavidin was performed at RT for 1 h. Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI; VectaShield mounting media). Photomicrographs were captured with Nikon Eclipse E800 microscope and SPOT imaging software. The total number of phospho c-Rel positive cells were counted and expressed as a percentage of a given population of DAPI positive cells

2.9 Collagen Gel Assay

Atrioventricular cushions (AVC) or ventricles were excised from HH stage 16 chicken embryos. Explants were placed endocardial side down on a collagen gel previously pre-incubated with media as described by Bernanke and Markwald [29]. Explants were then incubated at 37 °C, 5 % CO₂ for 48 h prior to 4 % PFA fixation.

2.10 Gene Targeting by siRNA

AVC or ventricular explants from stage 16–18 were collected in 100 µl room temperature (RT) M199 medium for each treatment condition. For transfection, first 4 µl of siPORT NeoFX (Ambion) was incubated with 96 µl M199 medium to a final volume of 100 µl for 10 minutes (min) at RT. Next, the appropriate final concentration (for 300 µl total volume) of siRNA derived from 21bp RNA (target sequence) was added to a final volume of 100 µl. These two tubes were mixed and incubated for 15 min at RT to allow siRNA complexes to form. This was followed by the 200 µl mixture being added to the 100 µl containing AVC or ventricular explants, and this solution was incubated at 37 °C, 5 % CO₂ for 45 min. Explants were then placed on conditioned collagen gels and incubated under the same conditions for 48 h. After 48 h, explants were fixed in 0.8 % formaldehyde, 0.05 % glutaraldehyde or 4 % PFA for 5 min at RT and washed twice with PBS. Fixed explants were scored for number of mesenchymal cells invading the gel. For control siRNA, a scrambled 21-oligonucleotide template containing the same number of the bases of the siRNA target gene that did not BLAST to any gene in the chicken genome, 5'(AGACTGT CGCGTGTCTGTCC)3' was used as previously described [30] and served to identify any toxicity due to the concentration of oligo or transfection reagent. siRNA targeted to TGFβR3 was used as a positive control for inhibition of EMT, 5' (GGAAGUAAAUCUACUUGAATT) 3'. Two independent siRNA constructs against each chicken ALK (2, 3 and 5) were designed using Silencer Select custom siRNA (Ambion). For each construct targeted against a specific ALK, that sequence is lacking in other genes of interest. At the concentrations and conditions used for the siRNA's, we have published the percent knockdown for each [11, 20, 28, 31]. All constructs show a greater than 70% knockdown in both chick and mouse. The siRNA target sequences are listed in Table 1.

2.11 Cell Death Assay in Explants

Explants harvested from Stage 16 chick embryos were placed on collagen gels. After 48 h, explants were incubated with 2 µM lysotracker (Invitrogen) for 15 min at 37 °C while rocking, washed for 15 min with PBS, incubated with DAPI (1:1000) for 5 min, and washed

a final time. Explants were fixed in 4 % PFA and followed by mounting on a slide. Lysotracker and DAPI positive cells were photographed under appropriate filter settings and corresponding photomicrographs were overlaid. The total number of lysotracker positive cells were counted and expressed as a percentage of a given population of DAPI positive cells.

2.12 Cell Proliferation in Explants

BrdU—AVC explants were excised from HH Stage 16 chick embryos and incubated on collagen gels for 48 h, incubated with 1 mM BrdU (Roche) for 1 h, and fixed with 4 % PFA. Explants were washed 3 times with PBS and permeabilized with 0.5 % tritonX-100. Antigen retrieval was accomplished with 2 M HCL. Explants were blocked with 5 % normal donkey serum and 0.05 % PBST for 1 h and incubated with monoclonal Alexafluor 594 conjugated BrdU antibody (1:50 dilution, Invitrogen) overnight. Explants were rinsed 3 times with PBS and stained with DAPI (dilution 1:1000) for 5 min. Stained explants were imaged with a fluorescent microscope. For each explant, approximately a hundred random cells were counted and scored for the presence of BrdU staining in the nucleus.

Histone H3 Phosphorylation—AVC explants were excised as previously described and fixed with 4 % PFA after 48 h incubation. Explants were washed 3 times with PBS and permeabilized with 0.5 % tritonX-100. Next, explants were blocked with 5 % normal donkey serum and 0.05 % PBST for 1 h at 4 °C. Explants were then incubated in primary antibody, mouse phospho histone-H3 (monoclonal, 1:300, Sigma), overnight at 4 °C. Explants were washed 3 times with PBST and incubated with secondary antibody, Goat anti-mouse IgG conjugated with Cy3 (1:50, Jackson Laboratories) overnight at 4°C. This was followed with explants being rinsed 3 times with PBS and stained with DAPI (1:1000) for 5 min. Stained explants were imaged with a Fluorescent scope (Nikon) and cells were digitally counted with Image J software.

2.13 Viral Introduction into Explants

Stage 10–12 chick embryos were harvested onto Whatman paper rings and immediately injected with adenovirus in the heart lumen. Injections were performed using 20–50 pulses, approximately 50 ms each, delivered by a picospritzer. Fast Green Dye (Sigma) was used to monitor injection of the virus. Immediately after injection, the embryos were placed on egg agar and incubated at 37 °C. After 24 h, ventricular or AVC explants were excised from the infected hearts and placed in culture as described previously [32]. After 48 h, the explants were fixed in either 0.8 % formaldehyde, 0.05 % glutaraldehyde or 4 % PFA. GFP expression by virally infected cells was observed on an inverted fluorescent microscope. The phenotype of each GFPexpressing cell was determined and scored as epithelial, activated, or transformed as described [32].

2.14 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 TGF β R3-T841A promotes ligand-independent invasion in epicardial cells

Tgfr3^{-/-} epicardial cells have decreased invasion into a collagen matrix in response to ligand when compared to *Tgfr3*^{+/+} cells [11]. The rescue of invasion in *Tgfr3*^{-/-} epicardial cells by TGF β R3 requires specific amino acid residues in the cytoplasmic domain that interact with GIPC [11, 28]. Here we wished to explore the role of threonine residue 841 found in the cytoplasmic domain of TGF β R3. Phosphorylation of this residue by TGF β R2 leads to a phosphorylation-dependent interaction with β Arr2 that results in the internalization of TGF β R3 and downregulation of signaling [5, 7]. A point mutation introduced at position 841 substituting alanine for threonine, TGF β R3-T841A, is sufficient to prevent these events [7]. We asked if TGF β R3-T841A overexpression in epicardial cells alters known TGF β -stimulated, TGF β R3-mediated responses. *Tgfr3*^{-/-} epicardial cells were infected with adenovirus expressing GFP alone or GFP in combination with full length TGF β R3 (TGF β R3-FL), TGF β R3 lacking the entire cytoplasmic domain (TGF β R3-CYTO), TGF β R3 lacking the C-terminal 3 amino acids (TGF β R3- Δ 3), or TGF β R3-T841A and scored for invasion. Consistent with prior results [11] the addition of TGF β R3-FL, but not TGF β R3-CYTO or TGF β R3- Δ 3, rescued invasion with the addition of ligand (Fig. 1A). Cells overexpressing TGF β R3-FL in the presence of 250 pM TGF β 2 have increased levels of invasion (150%) when compared to vehicle-incubated cells overexpressing TGF β R3-FL. *Tgfr3*^{-/-} cells expressing TGF β R3-T841A showed significantly increased levels of invasion when compared to cells expressing full length receptor (TGF β R3-FL) in the absence of TGF β 2 (Fig. 1A). The addition of ligand to *Tgfr3*^{-/-} cells expressing TGF β R3-T841A did not further increase invasion when compared to cells expressing TGF β R3-T841A in the absence of ligand (Fig. 1A). These data demonstrate that the introduction of TGF β R3-T841A into *Tgfr3*^{-/-} cells results in ligand-independent cell invasion.

3.2 Ligand-independent invasion stimulated by TGF β R3-T841A requires ALK activity

We next used small molecule inhibitors to specifically block effectors known to be downstream of TGF β R3 to determine if these same effectors were acting downstream of TGF β R3-T841A. Previous work by our laboratory demonstrated a requirement for ALK2, ALK3, and ALK5 for ligand-stimulated invasion in *Tgfr3*^{+/+} epicardial cells [28]. *Tgfr3*^{-/-} epicardial cells were infected with adenovirus overexpressing GFP, GFP and TGF β R3-FL, or GFP and TGF β R3-T841A (Fig. 1B). Cells overexpressing TGF β R3-FL increased cell invasion in response to TGF β 2 whereas cells overexpressing TGF β R3-T841A increased invasion independent of ligand addition. Incubation with either 2.5 μ M of the ALK5 kinase inhibitor SB431542 or 2 μ M of the ALK2/ALK3 inhibitor DMH1 [33] significantly decreased both TGF β 2-dependent invasion in cells overexpressing TGF β R3-FL and TGF β 2-independent invasion in cells overexpressing TGF β R3-T841A. Neither inhibitor altered cell viability (Fig. 1E). As expected, overexpression of TGF β R3-FL rescued a modest, but significant, deficit in cell proliferation seen in *Tgfr3*^{-/-} cells when compared to *Tgfr3*^{+/+} cells (Fig. 1F). Of note, TGF β R3-T841A, but not TGF β R3-CYTO or TGF β R3- Δ 3, also rescued this deficit. These data demonstrate that TGF β R3-T841A mediated ligand-independent cell invasion requires the activation of ALK2, ALK3, and ALK5 as does TGF β R3-FL.

3.3 NF- κ B activity in epicardial cells is required for ligand-dependent and ligand-independent invasion

Loss of even a single allele of *Tgfr3* results in decreased NF- κ B activity in mouse embryonic fibroblasts [34] and TGF β R3 phosphorylation by TGF β R2 at position 841 has been reported to modulate NF- κ B activity in breast epithelial and breast cancer cell lines [35]. Therefore, we next asked if the ligand-independent invasion seen in epicardial cells overexpressing TGF β R3-T841A was dependent on NF- κ B activity. *Tgfr3*^{-/-} epicardial cells overexpressing GFP, GFP and TGF β R3-FL, or GFP and TGF β R3-T841A were incubated with 10 μ M of the NF- κ B inhibitor BMS345541 which selectively targets I kappa B kinase (I κ B) (Fig. 1B). These data show significant inhibition of both TGF β 2-dependent invasion in cells overexpressing TGF β R3-FL and ligand-independent invasion in cells overexpressing TGF β R3-T841A. These findings suggest that NF- κ B activity is required downstream of both TGF β R3-FL and TGF β R3-T841A. To further address the role of NF- κ B in epicardial cell invasion, we pre-incubated *Tgfr3*^{+/+} epicardial cells with either 10 μ M BMS345541, an I κ B kinase inhibitor, or 2 μ M SN50, a cell permeable peptide inhibitor of NF- κ B translocation to the nucleus, prior to the addition of ligands known to require TGF β R3 for stimulating cell invasion [11]. Each inhibitor blocked invasion induced by TGF β 1, TGF β 2, BMP2, or FGF2 (Fig. 1C), all ligands known to stimulate TGF β R3-dependent cell invasion [11]. Neither inhibitor nor viral vectors containing receptor constructs altered cell viability (Fig. 1D, E).

3.4 NF- κ B activity is not required for ALK-mediated cell invasion in epicardial cells

We have previously reported that constitutive active (ca) constructs of ALK2, ALK3, or ALK5 induces invasion independent of ligand in *Tgfr3*^{+/+} or *Tgfr3*^{-/-} epicardial cells [28]. Since NF- κ B activity is required for invasion, we next asked if NF- κ B activity is downstream of ALK activation. *Tgfr3*^{-/-} were transfected with constructs expressing GFP alone, GFP and caALK2, GFP and caALK3, or GFP and caALK5, incubated with either vehicle or 10 μ M BMS345541, and scored for invasion. Overexpression of GFP or caALKs had no measurable effect on cell viability (Fig. 1F). BMS345541 inhibited invasion of *Tgfr3*^{-/-} epicardial cells overexpressing caALKs by 10% (Fig. 1D). The modest inhibition seen suggests that caALKs do not require NF- κ B activity to stimulate cell invasion.

3.5 NF- κ B activity is altered by the loss of TGF β R3

Given that *Tgfr3*^{-/-} epicardial cells have decreased invasion when compared to *Tgfr3*^{+/+} epicardial cells and that invasion stimulated by TGF β R3-T841A overexpression was blocked by the NF- κ B inhibitor BMS345541, we hypothesized that NF- κ B is activated downstream of TGF β R3 during epicardial cell invasion *in vitro*. Therefore, we asked if *Tgfr3*^{+/+} and *Tgfr3*^{-/-} epicardial cells activate NF- κ B in response to ligand. We incubated *Tgfr3*^{+/+} or *Tgfr3*^{-/-} epicardial cells with ligands known to induce epicardial cell invasion and scored for nuclear phospho c-Rel by immunostaining (Fig. 2A, B). Cells of both genotypes incubated with ligands known to stimulate cell invasion (either 250 pM TGF β 1 or TGF β 2, 5nM BMP2, or 10 ng/ml FGF2) demonstrated an increase in the number of phospho c-Rel-positive nuclei when compared to vehicle-incubated cells. Analysis of the percent of total nuclei positive for c-Rel immunostaining revealed that *Tgfr3*^{+/+} epicardial cells have a

larger percentage of positive nuclei after growth factor addition than *Tgfb β 3*^{-/-} epicardial cells (Fig. 2B). The addition of LPS, an inducer of I kappa B alpha phosphorylation and NF-kappa B activation, causes c-rel phosphorylation and nuclear translocation (Fig 2A, last row of panels) as indicated by immunostaining with the antibody. This important control demonstrates that there is sufficient expression of all the necessary components of the NF-kappa B pathway to support phospho c-rel nuclear translocation in *Tgfb β 3*^{-/-} cells. This apparent difference in NF-kB activation in *Tgfb β 3*^{+/+} cells when compared to *Tgfb β 3*^{-/-} cells was confirmed by measuring the activity downstream of an NF-kB reporter (Fig. 2B). Cells were co-transfected with a NF-kB-luciferase reporter construct and CMV- β -galactosidase. After 24 h, cells were incubated for 24 h with TGF β 1, TGF β 2, BMP2, or FGF2, ligands known to require TGF β R3 to stimulate invasion. The presence of luciferase in the media was analyzed and quantified. Our results demonstrate that *Tgfb β 3*^{+/+} epicardial cells activate NF-kB activity approximately 2-fold greater in response to TGF β 1, TGF β 2, BMP2, or FGF2 than do *Tgfb β 3*^{-/-} cells. Taken together, these data show that loss of TGF β R3 is associated with the loss of ligand-stimulated NF-kB activity coincident with the loss of ligand-dependent invasion in *Tgfb β 3*^{-/-} epicardial cells.

3.6 Invasion of endocardial cells requires ALK activity

Given that endocardial cell EMT represents a second system where TGF β R3 is required for ligand-stimulated cell invasion *in vitro* [36], we next examined TGF β R3 signaling mechanisms in this cell type in order to determine if the results seen in epicardial cells were shared between these two cell types. Experiments examining TGF β R3-dependent endocardial cell invasion in the chick atrioventricular cushion explant assay have revealed several similarities with this process in epicardial cells. Targeting TGF β R3 in cardiac cushion explants revealed a requirement of the receptor for endocardial cell invasion *in vitro* [10] which is dependent on the 3 C-terminal amino acids of TGF β R3 and interaction with GIPC [20]. Experiments in both endocardial cells and epicardial cells have also revealed a role for the Par6/Smurf/Rho pathway and multiple ALKs in cell invasion [20, 28, 31, 37, 38]. Here, incubation of atrioventricular cushion explants, which include endocardial cells that express TGF β R3 that undergo EMT and cell invasion in response to TGF β released from associated cardiac myocytes, with ALK-specific small molecule inhibitors (2 μ M DMH1; ALK2 & ALK3 or 2.5 μ M SB431542; ALK5) resulted in a 60% decrease in endocardial cell invasion when compared to vehicle incubated explants (Fig. 3A). To exclude toxicity of small molecule inhibitors, we measured cell death and proliferation after the explants were incubated in the presence of vehicle, SB431542 or DMH1. There was no significant change in cell death or proliferation between groups (Fig. 3B, C). We next asked directly if ALK2 and ALK3 were required downstream of the TGF β R3 by overexpressing GFP or GFP and TGF β R3-FL in ventricular explants, which include endocardial cells that lack TGF β R3 expression, and EMT was measured by scoring the percentage of total GFP-positive cells as epithelial, activated or transformed (Fig. 3D) as described previously [15, 20, 37, 38]. Ventricular endocardial cells overexpressing GFP alone show a small percentage of cells that undergo EMT as measured by the percentage of epithelial cells, activated cells (elongated cells on surface of gel), and transformed cells (cells that have entered the gel). However, the overexpression of TGF β R3-FL in the presence of TGF β 2 results in a significant increase in EMT and the number of cells that invade the gel. This increase in

invasion was decreased by 18% in the presence of the ALK2/ALK3 inhibitor DMH1. These data are consistent with prior studies using siRNA that demonstrated a requirement for ALK2 and ALK3 in endocardial cell invasion [20] and they further support the hypothesis that ALK2 and ALK3 are required for TGF β R3-dependent EMT and cell invasion.

3.7 NF- κ B activation is required for endocardial cell invasion

We previously identified NF- κ B activity as a regulator of endocardial cell invasion [39]. Here we asked if NF- κ B signaling was downstream of TGF β R3. Incubation of atrioventricular cushion explants with either 10 μ M BMS345541 or 2 μ M SN50 decreased cell invasion by 89% and 51% respectively when compared to vehicle-incubated explants (Fig. 4A). Incubation with 2.5 μ M of the ALK5 kinase inhibitor SB431542 was used as a positive control and decreased the number of invaded cells by over 60%. Since NF- κ B has a well-defined role in regulating cell proliferation [40–43] and apoptosis [43, 44] we examined cushion explants incubated with BMS or SN50 and measured changes in proliferation and cell death. There was a small, but significant change in cell death between vehicle and inhibitor incubated groups (Fig. 4B), but the modest increase noted (1% and 3%, respectively) cannot account for the magnitude of the decrease in cell invasion. No significant change in cell proliferation was identified (Fig. 4C). To determine if NF κ B activity acts downstream of TGF β R3, TGF β R3-FL was co-expressed with GFP in ventricular endocardial cells and scored as described. Overexpression of TGF β R3-FL in ventricular endocardial cells in the presence of 250pM TGF β 2 resulted in increased invasion to 47% when compared to ventricular endocardial cells overexpressing GFP alone (Fig. 4D). Ventricular explants expressing TGF β R3-FL with the addition of TGF β 2 and either 10 μ M BMS345541 or 2 μ M SN50 resulted in a significant decrease in the number of invaded cells when compared to the addition of ligand alone, 32% and 35% respectively. These data show that NF- κ B activity is required for TGF β R3-mediated cell invasion.

3.8 TGF β R3-T841A stimulates ligand-independent invasion of endocardial cells

To determine if TGF β R3-T841A-stimulated ligand-independent cell invasion in endocardial cells, TGF β R3-T841A was co-overexpressed with GFP in ventricular endocardial cells and EMT was measured as described above. As seen in epicardial cells, expression of TGF β R3-T841A results in ligand-independent cell invasion of endocardial cells contained in ventricular explants. To determine if the ligand-independent, TGF β R3-T841A-stimulated cell invasion requires ALKs, we targeted ALK2, ALK3, or ALK5 with either small molecule kinase inhibitors or specific siRNA. The number of transformed or invaded endocardial cells expressing TGF β R3-T841A from explants incubated with the ALK5 kinase inhibitor, SB431542 (2.5 μ M), decreased by 50% compared to explants incubated with vehicle alone (Fig. 5A). Ventricular endocardial cells that overexpress TGF β R3-T841A incubated with 2 μ M DMH1 showed a 41% decrease in the number of transformed or invaded cells when compared to explants incubated with vehicle alone. As a second, independent approach to explore a role for ALK activity in ligand-independent cell transformation we used siRNA to target ALK2, ALK3, or ALK5 [20]. Ventricular explants were infected with adenovirus that overexpressed TGF β R3-T841A and scored as described [32]. The targeting of ALK5 resulted in an average decrease of 29% in transformed or invaded cells compared to endocardial cells overexpressing TGF β R3-T841A (Fig. 5B). Targeting of ALK2 or ALK3

with 2 independent siRNA constructs resulted in an average decrease of 21% and 34%, respectively when compared to control siRNA. These results support our findings above with DMH1 and our prior report that both ALK2 and ALK3 are required downstream of TGF β R3 [20]. Furthermore, since siRNA is specific for ALK2 or ALK3 whereas DMH1 inhibits both, these results demonstrate that both ALK2 and ALK3 are required for endocardial cell invasion.

4.0 Discussion

Studies in cancer cell lines as well as epicardial and endocardial cells have identified TGF β R3 as a critical regulator of cell migration and invasion [10, 11, 45]. The cytoplasmic domain of TGF β R3 is particularly important in regulating these cell behaviors. The 3 C-terminal amino acids of TGF β R3 interact with GIPC; a scaffolding protein that stabilizes TGF β R3 at the cell surface to enhance TGF β signaling [21]. Our previous studies have demonstrated that both the 3 C-terminal amino acids of TGF β R3 and GIPC are required for the invasion of epicardial and endocardial cells *in vitro* [11, 20]. A second protein-protein interaction on the cytoplasmic domain of TGF β R3 includes Thr841 which is phosphorylated by TGF β R2 to allow for β Arr2 binding and the subsequent internalization of TGF β R3 [23]. The interaction of TGF β R3 and β Arr2 has also been associated with two distinct activities: the activation of Cdc42 to regulate cell migration [19] and the inhibition of NF- κ B activity [24]. Prior studies that interrogated the role of Cdc42 in epicardial [31] and endocardial [38] cell invasion failed to identify an effect of altered Cdc42 levels or activity on cell invasion. However, an examination of *Tgfb3*^{-/-} and *Tgfb3*^{+/-} mouse embryonic fibroblasts showed decreased NF- κ B activity associated with *Tgfb3* loss when compared to *Tgfb3*^{+/+} cells [34]. Since *Tgfb3*^{-/-} epicardial cells are impaired in the ability to invade a collagen matrix *in vitro*, we hypothesized that the loss of NF- κ B activity underlies the deficit in cell invasive activity. Here we used both epicardial and endocardial cell invasion as tool to identify shared pathways downstream of TGF β R3.

Our findings in epicardial cells reveal that TGF β R3-dependent cell invasion is associated with activation of the NF- κ B signaling pathway. Two distinct inhibitors of NF- κ B signaling suppress cell invasion in either *Tgfb3*^{-/-} epicardial cells that overexpress TGF β R3-FL to rescue ligand-dependent invasion or *Tgfb3*^{+/-} epicardial cells. Inhibition by NF- κ B inhibitors was seen in response to not only TGF β 1 and TGF β 2 but also BMP2 and FGF2, ligands known to interact with TGF β R3 and signal TGF β R3-dependent invasion of epicardial cells [11, 28]. Each of these ligands was also found to activate NF- κ B signaling as assessed by both phospho c-Rel translocation and NF- κ B-luciferase activity. To further probe the mechanisms by which TGF β R3 signals cell invasion, we examined the effect of a single amino acid substitution in the cytoplasmic domain of TGF β R3, TGF β R3-T841A, which prevents β Arr2 binding. TGF β R3-dependent regulation of NF- κ B signaling has been shown to require β Arr2 binding [34]. We hypothesized that since TGF β R3-dependent cell invasion requires NF- κ B signaling, TGF β R3-T841A might stimulate ligand-independent epicardial cell invasion associated with NF- κ B activation. Our results show that TGF β R3-T841A stimulated ligand-independent cell invasion is sensitive to NF- κ B inhibitors. The finding that NF- κ B activity regulates epicardial cell invasive behavior prompted us to ask if NF- κ B activity is downstream of the ALKs known to regulate TGF β R3-dependent invasion

in epicardial cells. Although we saw a modest effect from the NF- κ B inhibitor, BMS345541, on invasion induced by caALK2, caALK3, or caALK5 activity, our data are most consistent with the conclusion that NF- κ B activity is not required downstream of caALKs to induce invasion. To further probe the role of the interaction between ALK and NF- κ B signaling in the regulation of cell invasion, we took advantage of our finding that TGF β R3-T841A confers ligand-independent invasion. We used small molecule inhibitors that target and confirm that ALK2, ALK3, and ALK5, known mediators downstream of TGF β R3, are required for the ligand-independent invasion associated with TGF β R3-T841A in epicardial cells.

We next used endocardial cell invasion as a second system in order to determine if the observations made in epicardial cells may be generalized to other cell types. We had previously used gene regulatory network analysis to identify and validate NF- κ B signaling as a regulator of endocardial cell invasion [39]. Here we confirmed that ligand-dependent endocardial cell invasion is impaired in the presence of NF- κ B inhibitors. These data indicate that this mechanism is operative in at least two cell types which provides broad support for a role for NF- κ B signaling downstream of TGF β R3 in stimulating cell invasion. In endocardial cells, as in epicardial cells, TGF β R3-T841A stimulates ligand-independent cell invasion. We used a combination of small molecule inhibitors and siRNA to confirm that ALK2, ALK3, and ALK5, known mediators downstream of TGF β R3 [37], are required for ligand-independent invasion in endocardial cells. These results suggest that TGF β R3 activation results in a coordinated and concomitant stimulation of multiple ALKs to regulate cell invasion in both epicardial and endocardial cells. There is precedence for such a model of ALK activation. Endoglin, a TGF β co-receptor with structure similar to TGF β R3, has been demonstrated to result in the coordinate activation of ALK5 and ALK1 to regulate endothelial cell proliferation and migration [46].

The mechanisms by which TGF β R3 might activate the NF- κ B signaling pathway are not well understood. Our data using NF- κ B inhibitors indicates that increased NF- κ B activity is required for TGF β R3-dependent invasion in both epicardial and endocardial cells. Further, the introduction of TGF β R3-T841A into *Tgfbr3*^{-/-} epicardial cells results in ligand-independent invasion that is dependent on NF- κ B activity. Therefore, these results are consistent with a model where TGF β R3-T841A activates or allows NF- κ B activity to increase which results in invasion. Recent studies have shown that TGF β R3-dependent epicardial cell invasion stimulated by HMW-HA or the overexpression of TGF β R3-T841A is dependent on Src activation [47] which provides a well-described mechanism for the alteration of NF- κ B activity [reviewed in [48]] that may support cell invasion.

In summary, our studies sought to identify the common mechanisms of TGF β R3-dependent cell invasion in epicardial and endocardial cells. The introduction of TGF β R3-T841A into either cell type results in ligand independent cell invasion. Consistent with prior reports for ligand-stimulated, TGF β R3-dependent cell invasion, ligand-independent cell invasion associated with TGF β R3-T841A is also requires the activity of several ALKs. TGF β R3-dependent cell invasion is similarly dependent on NF- κ B activity in both cell types. Overall, these data demonstrate that TGF β R3 in epicardial and endocardial cells activates ALKs and NF- κ B signaling to initiate cell invasion. The identification of a mutant form of TGF β R3

that supports ligand independent cell invasion provides a useful tool for probing receptor function.

5.0 Conclusions

TGF β R3-mediated invasion in epicardial and endocardial cells shares a common signaling mechanism. Inhibition of NF κ B or ALKs impairs ligand-dependent invasion in both epicardial and endocardial cells. A threonine to alanine point mutation at residue 841 in TGF β R3 resulted in ligand independent cell invasion in both cell types.

TGF β R3-T841A mediated, ligand-independent invasion of epicardial and endocardial cells required activation of ALK 2, 3, and 5.

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ABBREVIATIONS

βArr2	Beta-arrestin2
ALK	Activin Receptor-Like Kinases
AV	Atrioventricular
BMP	Bone Morphogenetic Protein
ca	constitutively active
EMT	Epithelial-Mesenchymal Transformation
FGF	Fibroblast Growth Factor
GFP	Green Fluorescent Protein
GIPC	GAIP-interacting protein, C terminus
IκB	Inhibitor of kappa B protein
IKK	I kappa B kinase
NF-κB	Nuclear Factor- kappa B
SMαA	Smooth Muscle alpha Actin
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 TGFβ receptor-full length
TGFβR3-CYTO	Type III TGFβ receptor-lacking the entire cytoplasmic domain
TGFβR3- 3	Type III TGFβ receptor-lacking the 3 C-terminal amino acids
TGFβR3-T841A	Type III TGFβ receptor, threonine to alanine point mutation at residue 841

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Highlights

- A TGF β R3 mutant (TGF β R3-T841A) initiates ligand-independent invasion
- Epicardial and endocardial cell invasion requires activation of the NF- κ B pathway
- ALKs mediate both ligand-dependent and ligand-independent invasion
- NF κ B and ALK pathways may interact but both are independently required for invasion

Fig 1. TGFβR3-T841A caused ligand-independent epicardial cell invasion and required ALK2/3, ALK5, and NFκB

Quantification of invasion using a modified boyden chamber assay with *Tgfbr3*^{+/+} and *Tgfbr3*^{-/-} immortalized epicardial cells (E11.5). **A.** *Tgfbr3*^{-/-} epicardial cells were infected with GFP, TGFβR3-FL, TGFβR3-CYTO, TGFβR3-Δ3, or TGFβR3-T841A and scored for ligand-independent and ligand-dependent invasion. TGFβR3-T841A stimulated ligand-independent invasion that was not increased by the addition of ligand. TGFβR3-FL stimulated invasion in the presence of TGFβ2. TGFβR3-CYTO or TGFβR3-Δ3 did not

promote invasion. **B.** *Tgfb β 3*^{-/-} epicardial cells were infected with GFP, TGF β 3-FL, or TGF β 3-T841A and incubated with DMSO, TGF β 2, SB431542, DMH1, or BMS345541 during invasion. TGF β 3-T841A + DMSO, TGF β 3-FL + TGF β 2, and TGF β 3-T841A + TGF β 2 stimulated invasion which was inhibited by the presence of the small molecule inhibitors. **C.** *Tgfb β 3*^{+/+} epicardial cells were scored for invasion in the presence of indicated ligands with DMSO, SN-50, or BMS345541. Ligands + DMSO stimulated invasion while the addition of SN-50 or BMS345541 was inhibitory. **D.** *Tgfb β 3*^{-/-} epicardial cells were transfected with GFP, caALK2, caALK3, or caALK5. Prior to scoring for invasion they were treated with either DMSO or BMS345541. Cells invaded in response to caALKs + DMSO. Invasion of cells was modestly inhibited by BMS345541. **E, F.** Cell viability was analyzed in *Tgfb β 3*^{-/-} cells incubated in the presence of small molecule inhibitors or infected with adenovirus expressing the different receptor constructs. n=3, * = p < 0.05 compared to GFP control, \$ = p < 0.05 compared to DMSO.

Fig. 2. Loss of TGFβR3 in epicardial cells resulted in decreased NFκB activation

Tgfr3^{+/+} and *Tgfr3*^{-/-} immortalized epicardial cells (E11.5) were incubated with vehicle, TGFβ1, TGFβ2, BMP2, or FGF2 and scored for NFκB activity. **A.** Cells were stained with phospho c-Rel (cell signaling) following 24 h incubation with ligands. **B.** Cells were counted and the percent of phospho c-Rel⁺ cells / total Dapi⁺ cells was quantified. **C.** Cells were transfected with NFκB-luciferase reporter plasmid and incubated with ligands for 24 h. Luciferase luminescence was measured and graphed relative to cells incubated in vehicle. *Tgfr3*^{+/+} epicardial cells demonstrated up to a 2-fold increase in activation of NFκB signaling over *Tgfr3*^{-/-} cells. Data plotted as the mean percent ± SE of three independent experiments (*=p<0.05)

Fig 3. ALK2/3 inhibition blocked endocardial EMT

A. DMH1, a small molecule inhibitor specific to ALK2 and ALK3, decreased endocardial EMT in AVC by 50%. Specific ALK5 inhibitor, SB431542, is used as a positive control for small molecule inhibition of EMT. Data are derived from 3 independent experiments normalized explants incubated with vehicle. Quantification of the total number of explant and total number of transformed cells and normalized median $\pm$ SEM are as follows: Vehicle (n=31, total transformed cells = 9913; normalized median to 100% $\pm$ 22.1), SB431542 (n=31, total transformed cells = 4528; normalized median = 40.2 $\pm$ 14.1), DMH1 (n=31, total

transformed cells = 4472; normalized median = 47.2 ± 9.9). **B.** Cell death is not significantly changed between explants incubated with small molecule inhibitors targeted to ALK 2, 3 or 5 compared to those incubated with vehicle alone. In vehicle incubated explants and average of 2.2% of total cells were lysotracker-positive. Incubation with SB431542 and DMH1 resulted in no significant change in endocardial EMT. The total number of AVC explants examined (n), total number of DAPI and lysotracker positive cells, and average percent positive lysotracker cells $\pm$ SEM are as follows: Vehicle (n = 56; total DAPI positive cells 11525; lysotracker positive cells = 256; average percent positive lysotracker = 2.2 ± 1.9), SB431542 (n = 45; total DAPI positive cells 6882; lysotracker positive cells = 210; average percent positive lysotracker = 2.9 ± 2.1), and DMH1 (n=35; total DAPI positive cells = 4544; total lysotracker positive cells = 95; average percent positive lysotracker = $2.2 \pm .5$). **C.** The percentage of cells that stain positive for phosphorylated Histone 3 (P-His3) is unchanged in explants incubated with either vehicle or ALK 2, 3 or 5 small molecule inhibitors. The number of AVC explants examined (n), total number of DAPI and P-His positive cells, and average percent positive P-His cells $\pm$ SEM are listed. Vehicle (n = 38; total number of DAPI positive cells = 5130; P-His positive cells = 70; Average percent positive P-His = 1.1 ± 0.3); SB431542 (n = 48; total number of DAPI positive cells = 9636; P-His positive cells = 87; Average percent positive P-His = 0.9 ± 0.1) DMH1 (n = 33; total number of DAPI positive cells = 9548; P-His positive cells = 161; Average percent positive P-His = 1.7 ± 0.5). **D.** The effect of DMH1 on ventricular endocardial cell EMT. The average percent of total GFP-expressing cells are scored as epithelial, activated or transformed. Median percent $\pm$ SEM are depicted (n=3). Overexpression of full length TGF β R3 in ventricular endocardial cells along with the addition of 250pM TGF β 2 was used as a positive control for endocardial cell EMT. Expression of TGF β R3 in ventricular cells induced a statistically significant increase (50%) in transformed cells and a concomitant decrease in epithelial cells (44%) when compared to ventricular endocardial cells expressing GFP alone. The addition if the ALK2/3 inhibitor, DMH1, resulted in a significant decrease of 18% in the number of transformed cells. * = $p < 0.05$. The total number of GFP positive cells and SEM are listed in table 2.

Fig. 4. NF-κB is required for TGFβR3-mediated endocardial EMT

A: Incubation of AV cushion explants with two structurally different small molecule NFκB inhibitors, BMS-345541 (BMS) and SN50, decreased endocardial EMT in AVC by 89% and 51% respectively compared to vehicle. Specific ALK5 inhibitor, SB431542, is used as a positive control for small molecule inhibition of EMT. Data are derived from three independent experiments normalized explants incubated with vehicle. The number of AVC explants examined (n), total number of cells, and median number of transformed cells ± SEM (compared to cells incubated with vehicle) are listed. Vehicle (n=32, total transformed

cells = 7538; normalized median to $100 \pm 26\%$), SB431542 (n=37, total transformed cells = 3163; normalized median = $31.4 \pm 13\%$), BMS (n=34, total transformed cells = 748; normalized median = $10.8 \pm 3.4\%$) SN50 (n=36, total transformed cells = 3978; normalized median = $48.5 \pm 11.4\%$). **B:** Cell death is not significantly changed between explants incubated with small molecule inhibitors targeted to NF- κ B pathway compared to those incubated with vehicle alone. In vehicle incubated explants and average of 4% of total cells were lysotracker-positive. Incubation with SB431542 resulted in no significant change in endocardial EMT. However, incubation with BMS or SN50 resulted in a 1.4% and 3% increase. The total number of AVC explants examined (n), total number of DAPI and lysotracker positive cells, and median percent positive lysotracker cells $\pm$ SEM are as follows: Vehicle (n = 33; total DAPI positive cells 6701; lysotracker positive cells = 250; median percent positive lysotracker = 3.4 ± 0.6), SB431542 (n = 35; total DAPI positive cells 5098; lysotracker positive cells = 225; median percent positive lysotracker = 3.8 ± 1.2), and BMS (n=28; total DAPI positive cells = 3258; total lysotracker positive cells = 132; median percent positive lysotracker = 4.8 ± 0.9) SN50 (n=34; total DAPI positive cells = 4568; total lysotracker positive cells = 317; average percent positive lysotracker = 6.5 ± 1.4). **C:** The percentage of cells that stain positive for phosphorylated Histone 3 (P-Histone 3) is unchanged between explants incubated with either vehicle or small molecule inhibitors targeted to ALK5 or the NF κ B pathway. The number of AVC explants examined (n), total number of DAPI and P-His positive cells, and average percent positive P-His cells $\pm$ SEM are listed. Vehicle (n = 25; total number of DAPI positive cells = 7845; P-His positive cells = 152; Average percent positive P-His = $2.4.1 \pm 0.5$); SB431542 (n = 32; total number of DAPI positive cells = 8372; P-His positive cells = 216, Average percent positive P-His = 2.5 ± 0.6), BMS (n = 31; total number of DAPI positive cells = 5991; P-His positive cells = 177; Average percent positive P-His = 2.8 ± 0.5), SN50 (n=31; total DAPI positive cells = 8599; total lysotracker positive cells = 195; average percent positive lysotracker = 2.3 ± 1). **D:** The effect of NF- κ B inhibitors on ventricular endocardial cell EMT. The average percent of total GFP-expressing cells are scored as epithelial, activated or transformed. Median percent $\pm$ SEM are depicted (n=3). Overexpression of full length TGF β 3 in ventricular endocardial cells along with the addition of 250pM TGF β 2 was used as a positive control for endocardial cell EMT. Expression of TGF β 3 in ventricular cells induced a statistically significant increase $47 \pm 6\%$ in transformed cells and a concomitant decrease in epithelial cells (44%) when compared to ventricular endocardial cells expressing GFP alone. The addition if the NF κ B inhibitors, BMS and SN50, resulted in a significant decrease of $32 \pm 7\%$ and $35 \pm 6\%$ respectively of transformed cells. * = $p < 0.05$.

Fig. 5. Ligand-independent receptor action requires ALK2 and ALK3 in endocardial cells
The average percent of total GFP-expressing cells scored as epithelial, activated or transformed. Medians percent $\pm$ SEM are depicted ($n=3$). **A.** The effect of DMH1 on in ventricular endocardial cell EMT. Overexpression of TGFβR3-T841A in ventricular endocardial cells without the addition of TGFβ2 is used as a positive control for endocardial cell EMT. Expression of TGFβR3-T841A in ventricular cells induced a statistically significant increase ($52 \pm 3.5\%$) in transformed cells and a concomitant decrease in epithelial cells ($50 \pm 1.2\%$) when compared to ventricular endocardial cells expressing GFP

alone. The addition of the ALK2/3 inhibitor, DMH1, resulted in a significant decrease of 41% of transformed cells. * = $p < 0.05$. The total number of GFP positive cells and SEM are listed in table 1. **B.** ALK2 and ALK3 are required downstream of TGF β R3-T841A for endocardial EMT. Targeting of ALK5 by two independent constructs of siRNA was used as a positive control for siRNA-mediated inhibition of EMT Two independent siRNA's separately targeting ALK2 or ALK3 resulted in of 21% and 34% decrease, respectively, in transformed cells when compared to ventricular explants incubated with GC content-matched control siRNA. The total number of GFP positive cells and SEM are listed in table 2. (* = $p < .05$ to GFP, ‡ = $p < .05$ to GFP and TGF β R3-T841 + control siRNA)

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Fig. 6. A model for the common mechanisms downstream of TGFβR3-mediated epicardial and endocardial cell invasion

Table 1

siRNA Target Sequences

Smad1a	5'(GGAGUUCGCUCAGCUCUUATT)3'
Smad1b	5'(GCAGGGAGAUGAAGAAGAATT)3'
Smad2a	5'(GGAUUGAACUUCaucUGAATT)3'
Smad2b	5'(CCACCUCAGGAUAUAUCATT)3'
Smad3a	5'(GGAUGUAAUUGAAUUAUUTT)3'
Smad3b	5'(GGAUGUGCAGAUUCGGAUTT)3'
Smad4a	5'(CAAUGUCCAUCGCACCGAAtt)3'
Smad4b	5'(GCUAUUACCUUGGAUCGGGAtt)3'
Smad5a	5'(GCAAUACAAUGAUCCUCATT)3'
Smad5b	5'(GGUUUGCUCUCAAUGUUATT)3'
ALK2a	5'(GCAGAUUUUUGGACCAUUt)3'
ALK2b	5'(GGUUAGCAAUGGUAUAGUAtt)3'
ALK3a	5'(GAUUAACAGUGAACAAUGAtt)3'
ALK3b	5'(GGAGGAAGCUUGAAGUACAtt)3' ˆ
ALK5a	5'(GCUACGACAUGAAAACAUUt)3'
ALK5b	5'(GGAUAUUGCUGCCUUUAAtt)3'