Valvular heart disease due to congenital abnormalities or pathology is a major cause of mortality and morbidity. Understanding the cellular processes and molecules that regulate valve formation and remodeling is required to develop effective therapies. In the developing heart, epithelial-mesenchymal transformation (EMT) in a subpopulation of endocardial cells in the atrioventricular cushion (AVC) is an important step in valve formation. Transforming Growth Factor β (TGFβ) has been shown to be an important regulator of AVC endocardial cell EMT in vitro and mesenchymal cell differentiation in vivo. Recently Par6c (Par6) has been shown to function downstream of TGFβ to recruit Smurf1, an E3 ubiquitin ligase, which targets RhoA for degradation to control apical-basal polarity and tight junction dissolution. We tested the hypothesis that Par6 functions in a pathway that regulates endocardial cell EMT. Here we show that the Type I TGFβ receptor ALK5 is required for endocardial cell EMT. Overexpression of dominant negative (dn) Par6 inhibits EMT in AVC endocardial cells while overexpression of wildtype Par6 in normally nontransforming ventricular endocardial cells results in EMT. Overexpression of Smurf1 in ventricular endocardial cells induces EMT. Decreasing RhoA activity using dnRhoA or siRNA in ventricular endocardial cells also increases EMT while overexpression of constitutively active (ca) RhoA in AVC endothelial cells blocks EMT. Manipulation of Rac1 or Cdc42 activity is without effect. These data demonstrate a functional role for Par6/Smurf1/RhoA in regulating EMT in endocardial cells.

Congenital heart defects occur at a rate of almost 1 in 100 live births (1,2) and are responsible for the largest proportion of deaths due to birth defects in the first year of life and remain a major cause of death during childhood (3). The majority of these defects include abnormalities of valve formation. Valvular injury in adults occurs as a result of infection (4), pharmacological therapy (5), or tumorogenesis (6). Together, these factors result in over 100,000 Americans a year undergoing surgery to repair or replace heart valves (7). Valve formation is initiated in the region between the common atria and ventricle and in the distal aspect of the common outflow tract (OFT) when the heart is a simple tubular structure comprised of two concentric cylinders of epithelia separated by a gel-like matrix termed the cardiac jelly. A critical step in valvulogenesis is the transformation of a subpopulation of endocardial cells from the inner cell layer into mesenchymal cells that invade the cardiac jelly and later contribute to the connective tissue of the valves and septa of the adult heart (8). Transformation occurs at the atrioventricular boundary to initiate formation of the mitral and tricuspid valves and somewhat later in the OFT to initiate aortic and pulmonary valve formation. Endocardial cell transformation is dependent on factors derived from the myocardium that diffuse through the cardiac jelly (9). Since only a subset of endothelial cells become mesenchyme it is important to reveal the mechanisms regulating transformation that balance the ratio of transformed to non-
transformed cells. Although, much of the later mechanisms of valve maturation are poorly understood it is clear that cushion mesenchyme is critical to the process of valve formation (8,10).

Transformation in the atrioventricular cushion (AVC) has been studied extensively in avian systems using an in vitro assay in which the AVC is excised and placed on a collagen gel (11). In this assay, transformation can be divided into three steps based on cellular morphology. Endocardial cells separate from the epithelial sheet and elongate in a step termed activation. Next, elongate mesenchymal cells enter the matrix, a step termed invasion. Finally, cells migrate through the gel in the migration step. These three steps – activation, invasion, and migration – constitute EMT. As in the developing heart, EMT is tightly restricted where endocardial cells in AVC explants undergo EMT whereas endocardial cells in the ventricle do not (9).

Transforming cells alter their pattern of gene expression downregulating molecules such as the endothelial marker PECAM-1 and upregulating smooth muscle alpha actin and procollagen type I (12,13). EMT can be quantified by counting the number of cells in the gel and molecules may be scored for the ability to induce loss-of-function in AVC explants or gain-of-function in ventricular explants.

TGFβ is a key regulator of endocardial cell EMT (reviewed in (11)). TGFβ signals through three high affinity cell surface receptors, the TGFβ Type I (TGFβR1), Type II (TGFβR2), and Type III (TGFβR3) receptors. In the canonical signaling pathway (14) ligand binding to TGFβR2 results in recruitment of the TGFβR1, activin receptor like kinase (ALK) 5, to the complex. The constitutively active kinase of TGFβR2 phosphorylates and activates the kinase domain of ALK5 which subsequently phosphorylates and activates downstream receptor associated (R-) Smads 2 and 3 (15). TGFβ can activate additional downstream effectors including RhoA (16-20), Ras (21), MAP kinases (17,19,22,23), and PI3K/Akt (24) although the mechanisms by which TGFβ regulates these effectors is less well described.

TGFβR3 or betaglycan has a short, highly conserved intracellular domain with no apparent signaling function (25-27). TGFβR3 is required for AVC EMT in vitro (28) and targeted inactivation of the gene encoding TGFβR3 results in embryonic death at E14.5 associated with abnormalities of epicardial cell EMT and a failure of coronary vessel development (29).

Recently a novel signaling pathway has been described in which Par6 acts downstream of TGFβ to control apical-basal polarity in mouse NMuMG cells (30). Par6 is co-localized to the tight junctions with ALK5 by occludin. TGFβR2 is recruited to the tight junctions by TGFβ addition and phosphorylates Par6 resulting in the recruitment of Smurf1 (31). Smurf1 ubiquitination of RhoA leads to degradation of RhoA promoting tight junction dissolution and EMT (32). Here we ask if the Par6 signaling pathway regulates EMT in endocardial cells. Using an in vitro model of endocardial cell transformation we demonstrate that ALK5 activity is required for EMT in AVC explants. Overexpression of wildtype Par6 induces EMT in ventricular explants while dominant negative (dn)Par6 blocks EMT in AVC explants. Similarly, overexpression of Smurf1 causes EMT in ventricular endocardial cells. Consistent with a role for Smurf1 in targeting RhoA for degradation, both dnRhoA and siRNA to RhoA caused gain-of-function in ventricular explants while constitutively active (ca)RhoA effectively blocked EMT in AVC explants. Similar manipulations of Rac1 and Cdc42 did not alter EMT, suggesting a selectivity for RhoA in mediating these effects. These data demonstrate that the Par6 pathway is an important mediator of endocardial cell EMT in vitro.

**Experimental Procedures**

**Construction of Adenoviral Constructs**

Adenoviruses were generated using the pAdEasy system (33). 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 Rho GTPase dominant negative and constitutively active recombinant adenoviruses were prepared and characterized as previously described (34). Titers of virus ranged between $10^9$ to $10^{14}$ pfu/ml. Injections were adjusted to achieve infection of 20-50% of endocardial cells.

_Viral Injections and Collagen Gel Assays-_ 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 39°C. After 24 h, ventricular or AVC explants were excised from the infected hearts and placed in culture as described previously (35), Figure S1A, B). After 48 h, the explants were fixed in 0.8% formaldehyde, 0.05% glutaraldehyde. GFP expression by virally infected cells was observed on an inverted fluorescent microscope. The phenotype of each GFP-expressing cell was determined and scored as epithelial, activated, or transformed as described (35), Figure S1C, D).

_Ligand and Inhibitor Addition-_ TGFβ2 was purchased from R & D Systems. Ligand addition occurred 12 h post placement of explants on collagen pads to allow for attachment of explants. SB431542 was purchased from Sigma and Calbiochem 616541 was purchased from Calbiochem. Inhibitors were added to conditioning media to preincubate 12 hours prior to placement of explants on collagen pads, following removal of media at the time of explant placement.

_siRNA Treatment of AVC and Ventricular Explants-_ AVC or ventricular explants 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 in a final volume of 100 μl M199 for 10 minutes (min) at RT. Next, the appropriate final concentration (for 300 μl total volume) of siRNA derived from 21 bp 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. Next, the 200 μl mixture was added to the 100 μl containing AVCs 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 hours. After 48 h explants were fixed in 0.8% formaldehyde, 0.05% glutaraldehyde for 5 min at RT and washed twice with PBS. Fixed explants were scored for number of mesenchymal cells invading the gel. The siRNA target sequence for RhoA was: 5’(AATTATGTAGCAGATTGAA)3’.

_CONTROL siRNA, a scrambled 21 oligonucleotide template containing the same number of the bases of the RhoA siRNA target that did not blast to any gene in the chicken genome, 5’(AGACTGTCCGCTGCTCTGTC)3’ was used as previously described (36). RhoA siRNA and control siRNA were a generous gift from R. Runyan (37). Two independent siRNA constructs against each chicken Par6c and Smurf1 (designated Par6c-A, Par6c-B, Smurf1-A, and Smurf1-B) were designed using Silencer Select custom siRNA (Ambion). The siRNA target sequences for Par6c-A were: sense 5’(GCCUCCAACUCAUUGCAtt)3’ and antisense: 5’(UCUGCAAUAGUUGGAGGCGa)3’; Par6c-B sense: 5’(GCAGUACCUCAGCAACUAAtt)3’ and antisense: 5’(UAGUUGCUCGAGGCUACG Gag)3’; Smurf1-A sense: 5’(GCACAAGGGUUUAAACGAAtt)3’ and antisense: 5’(UUCGUUAAAACCCUUCUGCag)3’; Smurf1-B sense: 5’(GGAACUUGAGCUAAUCAUAtt)3’ and antisense: 5’(UAUGAUUGACUCCAGGUUCCtt)3’. Control siRNA was used for these experiments as described above. All siRNA constructs were confirmed for their ability to decrease mRNA measured by real-time PCR in chick embryonic fibroblasts (Figure S2). Additional methods are found in the Supplemental Materials.

**RESULTS**

ALK5 kinase activity is required for EMT in endocardial cells. We have previously demonstrated that ALK2 is sufficient and required for endocardial cell EMT, whereas
ALK5 is not sufficient (35). Several recent studies have shown that even if not sufficient, ALK5 activity may be required for TGFβ signaling (38-40). To test whether ALK5 kinase activity is required for endocardial cell EMT, we took advantage of the availability of several specific, small molecule inhibitors of ALK5 kinase activity. Two ALK5 inhibitors with different potency and distinct structural characteristics were chosen, SB431542 and Calbiochem 616451. Each inhibitor or vehicle was added to the collagen gel 12 hours prior to the placement of AVC explants isolated from Stage 14 chick embryos. Explants were incubated for 48 hours, fixed, and the number of transformed cells quantitated in each group (Figure S1C,D). Transformed cells were defined as those that enter the collagen gel. Both compounds significantly inhibited the number of cells entering the gel. SB431542 at a final concentration of 2.5 μM inhibited the number of cells entering the collagen gel by 71±4% when compared to vehicle (Figure 1A). Calbiochem 616451 at a final concentration of 150 nM similarly reduced the number of cells that enter the gel by 77±3% when compared to control (Figure 1B). These data demonstrate that ALK5 kinase activity is required for AVC endocardial cell EMT.

We have previously determined that TGFβR3 is sufficient and required for endocardial cell EMT in vitro. Blockade of TGFβR3 in AVC explants inhibits EMT. Overexpression of TGFβR3 in nontransforming ventricular endocardial cells, which normally lack TGFβR3 expression, results in EMT following the addition of TGFβ ligand (28). To determine if TGFβR3-mediated EMT requires ALK5 kinase activity, we overexpressed TGFβR3 in ventricular endothelial cells and incubated explants in the presence or absence of 2.5μM SB431542 plus or minus 250 pM TGFβ2. Stage 10-12 chick embryos were harvested and injected with adenovirus expressing either GFP alone or TGFβR3 and GFP (see Figure S1). Injected embryos were cultured 18-24 hours and ventricular explants from embryos between Stages 13-15 were excised and placed on collagen gels preincubated with vehicle or SB431542 as above. Twelve hours after explant placement either vehicle or 250pM TGFβ2 was added and the incubation continued for an additional 36 hours. Explants were fixed and GFP positive cells scored as epithelial (round cells in a sheet on the surface of the gel), activated (elongated, individual cells on the surface of the gel), or transformed (elongated, individual cells in the collagen gel). Infection with GFP alone defined the basal distribution of cells as epithelial, activated, or transformed (Fig 1C). The addition of TGFβ did not alter this distribution. Cells infected with TGFβR3 and GFP incubated with vehicle had a distribution comparable to cells infected with GFP alone. In contrast, the addition 250 pM TGFβ2 resulted in a significant increase in the percent of transformed cells (557%) and a concomitant decrease in the percent of cells scored as epithelial (57%). Incubation with SB431542 prevented the ability of TGFβ2 to stimulate EMT in TGFβR3 expressing cells. Importantly, incubation with SB431542 did not alter the distribution of cells expressing TGFβR3 and incubated with vehicle. These data demonstrate that ALK5 kinase activity is required for TGFβR3-dependent EMT in endocardial cells.

Par6 is Sufficient and Required for EMT. Par6 has been demonstrated to play a role in TGFβ-stimulated dissolution of tight junctions during EMT (30). Par6 is expressed in several epithelial cell populations in the chick embryo as well as in endocardial cells during embryonic development (data not shown). To test the hypothesis that Par6 activity regulates endocardial cell EMT we took advantage of our assay where both gain- and loss-of-function experiments can be performed in ventricular and AVC explants, respectively. We chose to overexpress wildtype Par6 in ventricular explants to score for gain-of-function and overexpress dnPar6 in AVC explants to score for loss-of-function. Embryos were injected, ventricular or AVC explants harvested, and scored as described above. Representative cell phenotypes are depicted (Figure 2A-D). Injection of an adenoviral construct coexpressing wildtype Par6 and GFP into normally nontransforming ventricle induced a statistically significant
75% increase in transformed cells with a concomitant decrease in epithelial cells (Figure 2E). When compared to explants infected with GFP alone, infection by dnPar6-GFP adenovirus in normally transforming AVCs resulted in a larger percentage of GFP positive cells which are epithelial, with a concomitant 35% decrease in the percentage of GFP positive cells that are transformed (Figure 2F). These data demonstrate that Par6 is both sufficient and required for endocardial cell EMT in vitro.

As a second independent approach to decrease Par6 activity we delivered siRNA constructs against Par6 to AVC explants. Explants from Stage 14- embryos were harvested. Following incubation with siRNA as described in methods the explants were placed on a collagen gel. Explants were fixed and scored after 48 hours. As compared to control siRNA, two independent siRNA constructs to Par6 decreased the number of transformed cells by 70% (Figure 2G,H). These data are consistent with those obtained using dnPar6 and support a role for Par6 activity in mediating endocardial cell transformation.

**Smurf1 is Sufficient and Required for EMT.** Given that ALK5 and Par6 are required for EMT in endocardial cells, we sought to determine if Smurf1, which is activated downstream of Par6, is sufficient for transformation. Adenovirus coexpressing Smurf1 and GFP or GFP alone was injected into the heart of Stage 10-12 embryos followed by the harvest of both ventricles and AVCs. Following incubation of explants for 48 hours, GFP-positive cells were scored as described. Smurf1 overexpression in the ventricle led to a statistically significant gain-of-function (i.e. EMT) as measured by a 300% increase in the number of transformed cells by 220% increase in transformed cells with a concomitant decrease in epithelial cells (Figure 2A). Expression of Smurf1 in AVC had no effect (Figure 2A). When compared to explants infected with GFP alone, infection by caRhoA-GFP adenovirus in normally transforming AVCs resulted in a larger percentage of GFP positive cells which are epithelial, with a concomitant 77% decrease in the percentage of GFP positive cells that are transformed (Figure 4A). Expression of caRhoA in ventricular explants also decreased the number of transformed cells, in this case by 90% (Figure 4A). These data demonstrate that a decrease in RhoA activity is sufficient to induce EMT while increased RhoA activity blocks EMT.

As a second independent approach to decrease RhoA activity we delivered siRNA constructs against Smurf1 to AVC explants as described earlier. Each construct decreased the number of transformed cells by at least 70% when compared to control siRNA (Figure 3B,C). These data demonstrate that Smurf1 is both sufficient and required for endocardial cell EMT in vitro.
and support a role for a decrease in RhoA activity in mediating endocardial cell transformation.

These data are not consistent with several studies that target an indirect measure of RhoA activity by targeting the Rho kinase (ROCK1/2) downstream of RhoA using a small molecule inhibitor, Y27632 (41,42). This apparent discrepancy may be due to the use of high concentrations of ROCK inhibitor. The IC$_{50}$ for the target is 0.22 μM (43), while typical studies employ 5-150 μM. To address this issue directly we generated a dose response curve of AVC endothelial cell EMT to Y27632 (Figure 5A). At a concentration of 0.3 μM, just above the reported IC$_{50}$, we noted a statistically insignificant decrease in EMT. Concentrations of 3 μM and higher decreased EMT with an EC$_{50}$ around 50 μM. Since the EC$_{50}$ required to significantly inhibit EMT was over 200 fold higher than the reported IC$_{50}$ we choose to examine the effects of a low (0.5 μM) and high (30 μM) concentration on ventricular explants (Figure 5B). The lower concentration of Y27632 significantly enhanced EMT, consistent with a loss of RhoA being required for EMT. However, at 30 μM, Y27632 did not enhance EMT consistent with nonspecific effects that act to inhibit EMT.

**Neither Cdc42 nor Rac1 are Sufficient or Required for EMT.** To address the specificity of the effect on endocardial cell transformation seen by altering RhoA activity, we examined the related small GTPases, Rac1 and Cdc42. Although neither Rac1 nor Cdc42 are substrates for Smurf1 (44) these small GTPases have been shown to coordinately regulate signaling with RhoA (45-48). Adenovirus coexpressing GFP and ca and dn forms of Cdc42 and Rac1 were injected into the heart lumen and explants harvested and scored as described. In ventricular or AVC explants, neither caRac1 nor Cdc42 is without effect. Taken together, these data support a role for a decrease in RhoA activity in mediating endocardial cell transformation.

**DISCUSSION**

Endocardial cell EMT is a necessary step in valvulogenesis in the heart. Using small molecule inhibitors we demonstrate that ALK5 kinase activity is required for TGFβR3-stimulated endocardial cell transformation. We also show that the recently described role of Par6 in regulating EMT in NMuMG cells is operative in endocardial cells. Overexpression of dnPar6 inhibits EMT in AVC explants while wildtype Par6 induces EMT in ventricular endocardial cells. Consistent with Par6 regulating Smurf1 activity, overexpression of Smurf1 induces EMT in ventricular cells. In NMuMG cells, Smurf1 is proposed to target RhoA for degradation and thereby allow dissolution of tight junctions and cell migration (30). Targeting of RhoA activity either by dnRhoA or siRNA supports EMT of ventricular endocardial cells while caRhoA inhibits EMT. Manipulation of Rac1 or Cdc42 activity does not support a role for these molecules. Together, these data support the notion that the Par6/Smurf1/RhoA pathway regulates endocardial cell transformation.

In the canonical TGFβ signaling pathway, ligand binding activates ALK5 kinase activity followed by the phosphorylation and subsequent nuclear translocation of Smads (49). Our studies reveal a requirement for ALK5 activity. In a variety of systems both Smad-dependent and Smad-independent pathways have been described (50). The deletion of Smad4 clearly identifies TGFβ responses independent of the canonical Smad signaling pathway (51). TGFβ can signal via several pathways independent of Smads activation including RhoA (16-20), Ras (21), MAP kinases (17,19,22,23), and PI3K/AKt (24) although cross talk with Smad pathways occur (50). It has previously been shown that dissolution of tight junctions in response to TGFβ in NMuMG cells occurs via the Par6/Smurf1/RhoA pathway and is
disassociated from Smad activation (30). We tested whether this pathway regulates EMT in endocardial cells. NMuMG cells contain prototypic tight junctions whereas endothelial cells have a modified junctional complex (52). Most notably, desmosomes are absent in the endothelium while gap junctions are present (52). Our results reveal that, despite these differences, the modified complex found in endothelial cells is subject to the same regulatory pathways as those in cells containing prototypic tight junctions. This regulation of endothelial cell junctional complexes is important in cell movement not just in embryonic development but also in angiogenesis and leukocyte extravasation in adults (53,54). The ubiquitination of RhoA by Smurf1 plays a general role in regulating cell motility and the formation of cell protrusions promotes neurite outgrowth (55) and cell shape change, motility, and invasion in tumor cells (56).

Our data demonstrate that loss of RhoA activity leads to EMT in the ventricle. Some investigators have suggested that RhoA upregulation (37,41,42,57) or RhoA activation and not degradation enhances EMT in vitro (18,37,41,42,57). Several of these studies use an indirect measure of RhoA activity by targeting the Rho kinase (ROCK1/2) downstream of RhoA using a small molecule inhibitor, Y27632. These conflicting data may be due to the use of high concentrations of ROCK inhibitor as the IC_{50} for the target is 0.22 \mu M (43), while typical studies employ 5-150 \mu M. Indeed we independently generated a dose response curve of endothelial cell EMT to Y27632 (Figure 5A) and determined that at concentrations above 0.5 \mu M there is a linear decrease in cell motility suggestive of a nonspecific effect on cell viability. Further, at a concentration of 0.5 \mu M, Y27632 enhanced EMT in ventricular explants, consistent with inhibition of RhoA activity being required for EMT. However, a concentration of 30 \mu M did not enhance EMT, suggesting that off target effects at high concentrations of Y27632 act to inhibit EMT. These data highlight the importance of choosing appropriate concentrations of small molecule inhibitors.

Additionally, contradictory to published reports, it has been recently been reported that basal ROCK activity is present at endothelial cellular junctions both in vitro and in situ (58). Inhibition of basal ROCK activity or depletion of ROCK was shown to disrupt the integrity of endothelial barriers (58), suggesting that ROCK inhibition may be necessary for breakdown of junctions for protrusive behavior leading to EMT. Our data is consistent with this report, as loss of ROCK activity is a consequence of inhibition of RhoA activity.

Given the conflicting data concerning RhoA activity in mediating EMT, in addition to the use of ca and dn constructs, we used siRNA to target RhoA in endocardial cells. We obtained a chick-specific siRNA construct reported to reduce AVC transformation in vitro (37). We were unable to demonstrate loss-of-function in the AVC but noted a significant gain-of-function in the normally nontransforming ventricle consistent with our results with dnRhoA. Our data provide internally consistent results in gain- and loss-of-function in ventricle and AVC respectively concerning RhoA activity and EMT. Overall these data demonstrate that loss of RhoA activity is sufficient for endocardial cell EMT and suggest a role for the Par6/Smurf1/RhoA pathway in endothelial cells.

Members of the Rho family of GTPases share guanine nucleotide exchange factors and signaling is often coordinated among these proteins [Reviewed in (59,60)]. For example, RhoA can interact with activated Cdc42 or Rac GTPases via a Cdc42-Rac interaction binding (CRIB) motif (61). There is a large body of evidence suggesting that RhoA and Rac1 play antagonizing roles in cell signaling (45-48). In the regulation of endothelial cell barriers, RhoA has been shown to increase actomyosin contractility to facilitate the breakdown of cellular junctions, whereas Rac1 stabilizes these junctions (59). Neither the overexpression of ca nor dnRac1 had an effect on endocardial cell EMT in vitro, suggesting that Rac1 does not play a role in endocardial cell EMT. We also addressed the role of Cdc42. The addition of dnCdc42 to ventricular explants caused a small but statistically
significant increase in the number of transformed cells with no change seen in AVC transformation. However, we did not see any opposing effects, defined as a loss-of-function, with the addition of caCdc42 in the AVC. We hypothesize that the effect on transformation seen with dnCdc42 in the ventricle is due to the known role of RhoA binding Cdc42, thus effectively reducing the amount of available RhoA. Taken together these data suggest that Cdc42 does not play a role in endocardial cell EMT and are consistent with a report demonstrating Cdc42 does not regulate endothelial cell permeability (62). Further neither Rac1 nor Cdc42 are substrates for Smurf1 (44). Since EMT is altered only with the manipulation of RhoA activity, and not Rac1 or Cdc42 activity, these data argue that other Rho GTPases cannot compensate for RhoA in mediating endocardial cell EMT.

Our data confirm our previous findings that the Type III TGFβ receptor, TGFβR3, is sufficient and required for endocardial cell EMT in vitro (28). Little is known concerning the signaling pathways downstream of TGFβR3. We demonstrate that ALK5 kinase activity is required for TGFβR3-mediated EMT leading us to hypothesize that TGFβR3 may be upstream of the Par6/Smurf1/RhoA pathway in endocardial cells. The demonstration of a TGFβR3 linked Par6/Smurf1/RhoA pathway that regulates endocardial cell behavior provides potential novel therapeutic targets for modulating endothelial cell transformation or angiogenesis.

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**FIGURE LEGENDS**

**Fig 1.** ALK5 Inhibitors Block EMT in vitro. 
*A & B:* Effect of ALK5 Inhibitors (*A*) SB431542 and (*B*) Calbiochem 616451 EMT in AVC explants. Quantification of cells migrated into collagen gel. 
*A:* Means are derived from 4 separate experiments, normalized to vehicle. SB431542 statistically significant decreased number of cells migrating into gel versus vehicle (DMSO). SB431542: 29±4.1%; (mean±SEM). vehicle: 100±8.9%. Two-tailed Student’s t-test: P=9.8E-10 (* P<0.01). The number of AVC explants examined and cells in each category were as follows: SB431542 (n=45; total number of cells in gel, 801), n=number of explants. Vehicle (n=40; total number of cells in gel, 2454). 
*B:* Means are derived from 3 separate experiments, normalized to vehicle. Calbiochem 616451 statistically significant decreased number of cells migrating into gel versus vehicle (DMSO). Calbiochem 616451: 23±3.0%; (mean±SEM). vehicle: 100±9.1%. Two-tailed Student’s t-test: P=1.0E-10 (* P<0.01). The number of AVC explants examined and cells in each category were as follows: Calbiochem 616451 (n=50; total number of cells in gel, 246), n=number of explants. 
*C:* ALK5 Kinase Activity is Required for TGFβR3-Stimulated Transformation. Average percent of total GFP-expressing cells scored as epithelial, activated or transformed. Means are derived from 3 separate experiments. TGFβR3 plus 250 pM TGFβ2 induced statistically significant increases in transformed cells with a concomitant decrease in epithelial cells. This effect was abolished in the presence of 2.5 μM SB431542. For actual counts and statistical analysis refer to Supplemental Table 1.

**Fig 2.** Par6 is sufficient and required for EMT in vitro. 
*A-D.* Representative merged brightfield and fluorescent photomicrographs of GFP-positive cells to illustrate the phenotypes of infected cells (at 200X). 
*A & B:* ventricular explants. 
*A:* GFP-only with the plane of focus at the surface of the collagen pad. Rounded, adjacent cells scored as epithelial cells (asterisks) are seen adjacent to the cardiac muscle. 
*B:* Par6 and GFP with the plane of focus in the collagen pad. Cells scored as transformed have elongated, separated from adjacent cells, and entered the collagen pad (white arrowheads). 
*C & D:* AVC explants. 
*C:* GFP-only with the plane of focus at the surface of the collagen pad. Many activated or transformed cells are evident (white arrowheads). 
*D:* dnPar6 and GFP with the plane of focus at the surface of the collagen pad. GFP positive cells are more likely to be epithelial (asterisk) while uninfected cells transform (white arrowhead). 
*E & F:* Average percent of total GFP-expressing cells scored as epithelial, activated or transformed. Means are derived from 4 separate experiments. 
*E:* Par6 is sufficient for EMT in ventricular explants. Par6 induced statistically significant increases in transformed cells with a concomitant decrease in epithelial cells. 
*F:* dnPar6 blocks EMT in AVC explants. dnPar6 led to statistically significant decreases in transformed cells with a concomitant increase in epithelial cells. For actual counts and statistical analysis refer to Supplemental Table 1. 
*G & H:* siRNA knockdown of Par6 blocks AVC EMT. Addition of two independent siRNA constructs, (*G*) Par6c-A or (*H*) Par6c-B blocked AVC transformation to 32±6.7% and 26±3.8% (mean±SEM) of control siRNA respectively. The number of ventricular explants examined and cells in each category were as follows: Control (n=29; total number of cells in gel, 3442), n=number of explants. 
*G:* Par6c-A siRNA (n=30; total number of cells in gel, 1069). Two-tailed Student’s t-test: P=0.006. 
*H:* Par6c-B siRNA (n=30; total number of cells in gel, 862). Two-tailed Student’s t-test: P=0.002.

**Fig 3.** Smurf1 is Sufficient and Required for EMT. 
*A:* Smurf1 adenovirus caused statistically significant enhancement of EMT in the ventricle. Average percent of total GFP-expressing cells scored as epithelial, activated or transformed. Means are derived from 3 separate experiments.
GFP adenovirus alone served as a negative control to define basal levels of transformed cells. For actual counts and statistical analysis refer to Supplemental Table 1. B & C. siRNA knockdown of Smurf1 blocks AVC EMT. Addition of two independent siRNA constructs, (B) Smurf1-A or (C) Smurf1-B blocked AVC transformation to 29±3.3% and 23±6.4% (mean±SEM) of control siRNA respectively. The number of ventricular explants examined and cells in each category were as follows: Control (n=29; total number of cells in gel, 3442), n=number of explants. B: Smurf1-A siRNA (n=30; total number of cells in gel, 977). Two-tailed Student’s t-test: P=0.001. C: Smurf1-B siRNA (n=30; total number of cells in gel, 765). Two-tailed Student’s t-test: P=0.005.

Fig 4. Inhibition of RhoA activity is sufficient for EMT. A: Average percent of total GFP-expressing cells scored as epithelial, activated or transformed. Means are derived from 3 separate experiments. GFP control adenovirus is used as a control to define basal levels of transformation in both the ventricle and AVC. dnRhoA induced statistically significant increases in transformed cells with a concomitant decrease in epithelial cells in ventricular explants. caRhoA induced statistically significant decreases in transformed and activated cells with a concomitant increase in epithelial cells in both the ventricle and AVC. For actual counts and statistical analysis refer to Supplemental Table 1. B & C: RhoA siRNA leads to endocardial cell EMT. Quantification of cells migrated into collagen gel. Means are derived from 3 separate experiments, normalized to negative control siRNA. B: RhoA siRNA leads to EMT in ventricular explants. RhoA siRNA led to a statistically significant increase in number of cells migrating into gel verses negative control siRNA. Control: 100±19% (mean±SEM). RhoA siRNA: 690±160%. Two-tailed Student’s t-test: P=0.0006 (* P<0.01). The number of ventricular explants examined and cells in each category were as follows: Control (n=42; total number of cells in gel, 187), n=number of explants. RhoA siRNA (n=42; total number of cells in gel, 1289). C: RhoA siRNA has no effect on EMT in AVC explants. RhoA siRNA led to no change in number of cells migrating into gel verses negative control siRNA. Control: Normalized to 100%. RhoA siRNA: 101±6.9% (mean±SEM). Two-tailed Student’s t-test: P=0.921. The number of AVC explants examined and cells in each category were as follows: Control (n=42; total number of cells in gel, 6432), n=number of explants. RhoA siRNA (n=42; total number of cells in gel, 6441).

Fig 5. ROCK1/2 inhibition via Y27632 leads to ventricular EMT. A & B: Data points are mean number of cells invading the collagen gel, derived from three independent experiments normalized to vehicle. A: Y27632 does not significantly block AVC transformation when used near the published IC50 value of 0.22 μM, but does significantly reduce EMT at high concentrations. Vehicle (DMSO): normalized to 100%. 0.3 μM: 89±4.9%. 3.0 μM: 78±4.7%. 30 μM: 60±10.4%. 60 μM: 40±5.0%. The number of AVC explants examined were as follows: Vehicle (n=23; total number of invaded cells, 3186), n=number of explants, 0.3 μM (n=25; total number of invaded cells, 3087), 3.0 μM (n=26; total number of invaded cells, 2831), 30 μM (n=33; total number of invaded cells, 2751), 60 μM (n=24; total number of invaded cells, 1318). We observe a linear decrease in cell motility above 0.5 μM, suggestive of nonspecific effects on cell viability at higher concentrations. B: Y27632 leads to EMT in ventricular explants. Vehicle (DMSO): normalized to 100%. 0.5 μM: 368±23%. 30 μM: 92±11%. The number of ventricular explants examined were as follows: Vehicle (n=30; total number of invaded cells, 219), n=number of explants, 0.5 μM (n=30; total number of invaded cells, 800), 30 μM (n=30; total number of invaded cells, 199).

Fig 6. Cdc42 and Rac1 have no effect on endocardial cell EMT. Average percent of total GFP-expressing cells scored as epithelial, activated or transformed. Means are derived from 3 separate experiments. GFP control adenovirus is used as a control to define basal levels of transformation.
in both the ventricle and AVC. **A**: Effects of Constitutively Active and Dominant Negative Rac1 on EMT. Neither dn- or caRac1 induced statistically significant changes in transformed cells. **B**: Effects of Constitutively Active and Dominant Negative Cdc42 on EMT. In ventricular explants dnCdc42 induced a small but statistically significant increase in transformed cells. For actual counts and statistical analysis refer to Supplemental Table 1.
Figure 1

A  
Mean Number Of Cells In Gel
Vehicle  SB431542

B  
Mean Number Of Cells In Gel
Vehicle  Calbiochem 616451

C  
Percent of Total GFP Positive Cells
epithelial  activated  transformed
GFP + vehicle  GFP + TGFβ2  TGFβR3 + vehicle  TGFβR3 + TGFβ2  TGFβR3 + vehicle + SB431542  TGFβR3 + TGFβ2 + SB431542

***  **  ***  ****
Figure 2
Figure 3

A
Percent of Total GFP Positive Cells

epithelial
activated
transformed

GFP, Ventricles
Smurf1, Ventricles
GFP, AVC
Smurf1, AVC

B
Mean Number Of Cells In Gel

Control siRNA
Smurf1 siRNA-A

C
Mean Number Of Cells In Gel

Control siRNA
Smurf1 siRNA-B
Figure 4
Figure 5

A

Transformed Cells (Percent of Control)

Log Concentration (μM)

B

Mean Number Of Cells In Gel

Vehicle

0.5 μM Y27632

30 μM Y27632

**
Figure 6

A
Percent of Total GFP Positive Cells

GFP, Ventricle
GFP, AVC
caRac1, Ventricle
caRac1, AVC
dnRac1, Ventricle
dnRac1, AVC

epithelial   activated   transformed

B
Percent of Total GFP Positive Cells

GFP, Ventricle
GFP, AVC
cacdc42, Ventricle
cacdc42, AVC
dncdc42, Ventricle
dncdc42, AVC

epithelial   activated   transformed