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TGFβ and BMP-2 regulate epicardial cell invasion via TGFβR3 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. *Tgfbr3*$^{-/-}$ mice die due to failed coronary vessel formation associated with decreased epicardial cell invasion but the mediators downstream of TGFβR3 are not well described. TGFβR3-dependent endocardial EMT stimulated by either TGFβ2 or BMP-2 requires activation of the Par6/Smurf1/RhoA 1pathway where Activin Receptor Like Kinase (ALK5) signals Par6 to act downstream of TGFβ 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 TGFβR3 was required to access this pathway. Targeting of ALK5 in *Tgfbr3*$^{+/+}$ 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 *Tgfbr3*$^{-/-}$ epicardial cells which have a decreased ability to invade collagen gels in response to TGFβ2, overexpression of wtPar6, Smurf1, or dnRhoA had a diminished ability to induce invasion. Overexpression of TGFβR3 in *Tgfbr3*$^{-/-}$ cells, followed by siRNA targeting of Par6 or Smurf1, diminished the ability of TGFβR3 to rescue invasion demonstrating that the Par6/Smurf1/RhoA pathway is activated downstream of TGFβR3 in epicardial cells.

1. Introduction

Epithelial to mesenchymal transformation (EMT) is an important process during embryonic development and disease progression [1-4]. Transforming Growth Factor β (TGFβ) 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 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 *Tgfbr3* 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 (TGFβR1 or ALK5) and Type II (TGFβR2) receptors in TGFβ signaling are well understood [15] there remain significant gaps in our understanding of how the wide array of TGFβ-induced responses are signaled and regulated, particularly with respect to the contributions of TGFβR3. TGFβR3 binds TGFβ1 and TGFβ3 and is required for the high affinity binding of TGFβ2 [16]. TGFβR3 also binds and signals in response to BMP-2 [17] and functions as an inhibin receptor [18]. After TGFβ binding to TGFβR3, TGFβR3 presents ligand to the Type I (TGFβR1) and Type II (TGFβR2) TGFβ receptors to augment signaling through the canonical signaling pathway that requires the phosphorylation and nuclear translocation of the

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

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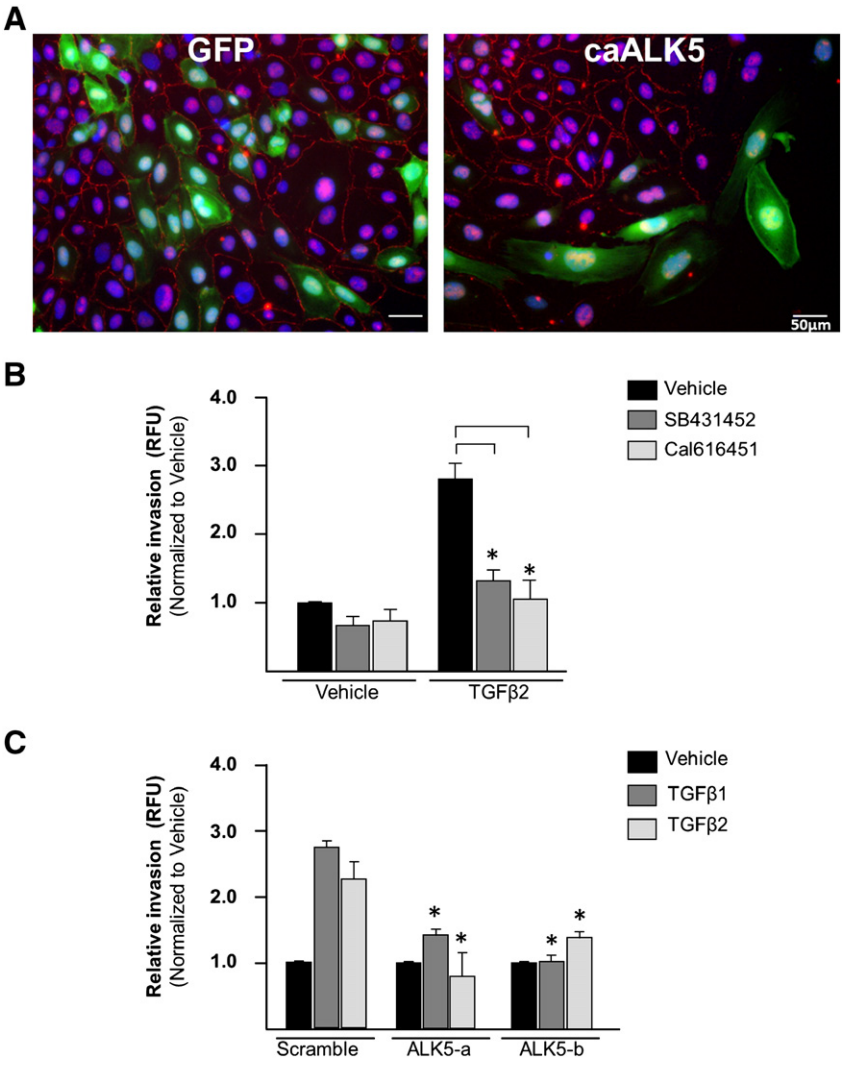

Fig. 1. ALK5 is required for epicardial cell invasion (A) *Tgfbr3*$^{+/+}$ cells infected with adenovirus expressing GFP alone (left) or GFP and caALK5 (right), and immunostained for the tight junction protein, ZO-1. (B and C) Quantitation of TGFβ-induced invasion using a modified Boyden chamber assay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

Smads [19]. TGFβR3 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 TGFβR3 to present ligand to TGFβR1 and TGFβR2 and augment canonical signaling [22]. TGFβR3 is postulated to play a unique and non-redundant role in TGFβ signaling in addition to ligand presentation based on the results of targeting TGFβR3 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 TGFβR3 suggesting the presence of a non-canonical signaling pathway activated by TGFβR3.

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 TGFβR1 or ALK5 at the tight junctions by occludin [34]. Upon TGFβ stimulation, TGFβR2 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 TGFβR3 in endocardial cells [17] and that BMP-2 activates the Par6/Smurf1/RhoA pathway in a TGFβR3-dependent manner [28].

TGFβR3 is also expressed in epicardial cells which are major contributors to the coronary vessels, and *Tgfbr3*$^{-/-}$ 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, Tgfbr3*$^{-/-}$ epicardial cells lose epithelial character in response to TGFβ but have a diminished ability to undergo TGFβ-induced invasion into a collagen gel *in vitro* [26]. Given the role of the Par6/Smurf1/RhoA pathway in mediating TGFβ and BMP-2-induced EMT in endocardial cushions and the requirement of TGFβR3 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 TGFβ and BMP-2 is regulated by TGFβR3.

2. 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),