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ALK-1 to ALK-5 ratio dictated by the Akt1-β-catenin pathway regulates TGFβ-induced endothelial-to-mesenchymal transition

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Abstract

Endothelial-to-mesenchymal transition (EndMT) indispensable in embryogenesis also occurs in several human pathologies. Although transforming growth factor-β (TGFβ) has been demonstrated to induce EndMT, the type-I receptors (ALK-1 and ALK-5) responsible for TGFβ-induced EndMT is unclear. In the current study, we investigated the role of the Akt1 pathway in ALK1 and ALK5 expression regulation in response to TGFβ1 and TGFβ2 in human microvascular endothelial cells (HMECs). Whereas treatment with TGFβ1 and TGFβ2 or Akt1 gene silencing promoted EndMT accompanied by increased ALK5 expression and reduced ALK1 expression accompanied by increased expression of N-cadherin and reduced expression of eNOS in HMECs, treatment with ALK-5 inhibitor (SB431542) blunted these effects. Importantly, the inhibitor of β-catenin (ICG-001) suppressed TGFβ1- and TGFβ2-induced ALK5 expression in both normal and Akt1 deficient HMECs indicating the integral role of Akt1-β-catenin pathway in the regulation of ALK5 expression promoting EndMT.

Keywords

ALK1; ALK5; TGFβ; EndMT; Akt1; β-catenin

1. Introduction

The occurrence of Endothelial-to-mesenchymal transition (EndMT) has been confirmed in several cancers (Akatsu et al. 2019; Choi et al. 2018; Zeisberg et al. 2007a), atherosclerosis (Jiang et al. 2019; Ma et al. 2013; Souilhol et al. 2018), cardio-pulmonary pathologies (Geng and Guan 2017; Isobe et al. 2019; Lv et al. 2018; Ma et al. 2013; Sabbineni et al. 2019;

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Zeisberg et al. 2007b) and diabetic retinopathy (Thomas et al. 2019), etc. EndMT causes endothelial cells to shun endothelial markers (E.g. eNOS) and acquire mesenchymal markers (N-cadherin, and FoxC2, etc.) (Li et al. 2018; Sabbineni et al. 2018). Although EndMT can be triggered by all three transforming growth factor β (TGFβ) isoforms (Yun et al. 2019), TGFβ2 serves as the primary inducer (Sabbineni et al. 2018). We recently reported that reduced activity of serine/threonine kinase, Akt1, in endothelial cells results in the loss of tight junction claudins (Artham et al. 2019; Gao et al. 2016), barrier disruption (Chen et al. 2005) and EndMT-related pulmonary vascular remodeling (Sabbineni et al. 2019), thus demonstrating the integral role of Akt1 in endothelial-barrier protection and prevention of EndMT.

TGFβ utilizes two receptors with intrinsic serine-threonine kinase activity namely type I and type II TGFβ receptors (Derynck and Zhang 2003; Miettinen et al. 1994). Even though there are seven human type-I receptors and five type-II receptors in the entire TGFβ superfamily, the three TGFβ isoforms bind only to a single type-II receptor (TβRII) and two type-I receptors, namely, ALK-1 and ALK-5 (TβRI/activin receptor-like kinase 1 and 5) (Heldin and Moustakas 2016). In the endothelium, it has been previously reported that ALK-1 and ALK-5 elicit opposing effects (Goumans et al. 2003). While ALK-1 promotes endothelial cell proliferation and migration through the Smad1/5 signaling pathway, ALK-5 activates Smad2/3, and opposes ALK-1 functions (Goumans et al. 2003). Whereas ALK-5 promotes TGFβ-induced endothelial pathology, ALK-1 has been reported to have paradoxical effects (Goumans et al. 2003). However, molecular mechanisms that regulate differential expression of ALK-5 and ALK-1 in ECs in the modulation of TGFβ-induced EC dysfunction, such as EndMT, is poorly studied. Whereas TGFβ2 is the predominant isoform that induces EndMT in endothelial cells, TGFβ2 is also the only isoform that, in contrast, has rather a low affinity to TβRII and requires co-receptors like beta glycan to assist TGFβ2 into assembling stable receptor complex (Heldin and Moustakas 2016; Sabbineni et al. 2018).

Apart from the canonical SMAD pathway, TGFβ signaling through phosphorylation or direct interaction with signaling intermediates can also initiate several pathways including PI3K/AKT signaling (Hamidi et al. 2017; Zhang et al. 2013). However, the regulation of TGFβ type I receptors and TGFβ signaling by endothelial Akt1, loss of which promotes EndMT, needs further exploration. Here we show that loss of endothelial Akt1 results in differential expression of ALK-1 and ALK-5 in promoting TGFβ-induced EndMT *via* β-catenin, a downstream molecule in the Akt1 pathway.

2. Materials and methods

2.1. Cell culture and preparation of ShAkt1 stable cell lines

Human dermal microvascular endothelial cells (HMEC) (CRL-4025; ATCC, Manassas, VA) were maintained in Endothelial Cell Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD) in a humidified 5% CO2 incubator at 37°C and routinely passaged when 80–90% confluent. Stable ShControl and ShAkt1 (ACGCTTAACCTTTCCGCTG) knockdown HMEC cells were generated using SMART vector 2.0 lentivirus particles (109 pfu) (Thermo Scientific, Waltham, MA) mixed in 1 ml Hyclone SFM4Transfx-293 (Fisher, Hanover Park, IL) and added along with 1 μl Polybrene

(10 mg/ml, American Bioanalytical, Natick, MA). Three days later, transfection efficiency was tested through Turbo- GFP expression and subjected to 4 μg/ml puromycin (Life Technologies, Grand Island, NY) selection until all cells expressed GFP. Human TGFβ1 and TGFβ2 were obtained from R&D Systems (Minneapolis, MN). SB431542 (ALK-5 inhibitor) and ICG-001 (β-catenin inhibitor) were purchased from Selleckchem (Houston, TX) and reconstituted according to the manufacturer's Protocol. HMEC monolayers were treated with TGFβ1 (5ng/ml), TGFβ2 (1ng/ml), SB431542 (ALK-5 inhibitor) and ICG-001 (β-catenin inhibitor) in 5% serum-containing medium for 72 hours as previously standardized in the laboratory (Abdalla et al. 2015; Sabbineni et al. 2018; Sabbineni et al. 2019). The growth factors and inhibitors were replenished every 24 hours.

2.2. Western blot analysis

Cell lysates were prepared using complete lysis buffer (EMD Millipore, San Diego, CA), with protease and phosphatase inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Protein quantification was performed using DC protein assay from Bio-Rad (Hercules, CA). Western blot analysis was performed as described previously (Al-Azayzih et al. 2015; Sabbineni et al. 2019). Antibodies used include Akt1 (2938), N-cadherin (4061), phosphorylated p38-MAPK (9211), total p38-MAPK (9212), phosphorylated Smad2/3 (8828), total Smad2/3 (8685), FoxC2 (12974), phosphorylated β-catenin (9561), total β-catenin (8480) and GAPDH (2118) from Cell Signaling Technology (Danvers, MA). Anti-β-actin (A5441) was from Sigma (St. Louis, MO), anti-eNOS (610297) was from BD Pharmingen (San Diego, CA), anti-ALK-5 (ab31013) was purchased from Abcam (Cambridge, UK) and anti-ALK-1 (NBP1-90254) was obtained from Novus Biologicals. Anti-phosphorylated-Smad1/5/8 (AB3848-I) from Millipore Sigma and anti-Smad1/Smad5/Smad8 (SAB2702532) from Sigma-Aldrich. Band densitometry was performed using NIH Image J software (Bethesda, MD).

2.3. Statistical analysis

All the data are presented as Mean ± SD and were calculated from multiple independent experiments performed in quadruplicates. For normalized data analysis, data was confirmed that normality assumption was satisfied and analyzed using paired sample *t-test* (dependent *t-test*) and/or further confirmed with non-parametric test Wilcoxon signed-rank test. For all other analyses, Student's two-tailed t-test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 6.0 software.

3. Results

3.1. Akt1 loss sensitizes endothelial cells for TGFβ-induced EndMT via differentially regulating ALK-1 and ALK-5 expression

We first determined the direct effect of Akt1 loss in HMECs (Fig. 1A-B) on EndMT and, in turn, on TGFβ-induced ALK-1 and ALK-5 receptor expression. Western blot analysis of ShAkt1 HMEC lysates revealed increased ALK-5 and reduced ALK-1 expression compared to ShControl HMECs, which was exacerbated by treatment with TGFβ1 or TGFβ2 for 72 hours thus promoting EndMT (Fig. 1A-D). A net reduction in the ALK-1/ALK-5 ratio, a measure of pathological effects of TGFβ signaling, was observed in ShAkt1 compared to

ShControl HMECs (Fig. 1E), accompanied by a decreased endothelial marker eNOS and increased mesenchymal marker N-cadherin expression (Fig. 1F-G). Together these results indicated that Akt1 is directly involved in the differential expression of ALK-1 and ALK-5 in HMECs preventing EndMT.

Next, we determined the expression of phosphorylated SMADs that represent canonical TGFβ signaling as well as phosphorylated P38MAPK that are associated with non-canonical TGFβ signaling in HMECs in response to Akt1 loss in the presence and absence of TGFβ1 and TGFβ2. We found that lack of Akt1 or treatment with either TGFβ1 or TGFβ2 significantly increased phosphorylation of SMAD2 (Fig. 2A-C), p38MAPK (Fig. 2D-E) and phosphorylation of SMAD1/5 (Fig. 2F-G) indicating the involvement of both canonical and non-canonical pathways in EndMT induced by the TGFβ-Akt1 pathway. Overall, our results indicate that the Akt1 loss primes the endothelial cells for pathological TGFβ-induced EndMT through increased ALK-5 expression and reduced ALK-1 expression.

3.2. ALK-5 inhibitor (SB431542) partially abrogates EndMT in control and Akt1-deficient HMECs via inducing changes in βcatenin expression.

To investigate the role of ALK-5 in EndMT due to Akt1 loss, we determined the effect of ALK-5 inhibitor on TGFβ-induced EndMT in control and Akt1-deficient HMECs. Treatment with SB431542, although it did not have any effect on reduced eNOS expression in ShAkt1 HMECs (Fig. 3A-B), it significantly decreased the expression of mesenchymal marker FoxC2 specifically in ShAkt1 HMECs (Fig. 3A and C), and not in ShControl HMECs. Interestingly, we found that ALK-5 inhibition in control HMECs further decreased phosphorylated β-catenin expression as compared to their already decreased levels in ShAkt1 compared to ShControl HMECs (Fig. 3D). We also found that ALK-5 inhibition did not decrease total β-catenin expression in control HMECs but modestly increased phosphorylated β-catenin expression in ShAkt1 HMECs at higher doses (Fig. 3D-E). These results indicate that ALK-5 inhibition in ShAkt1 HMECs partially inhibits EndMT, which is accompanied by changes in β-catenin expression.

In order to determine the role of β-catenin in the expression of ALK-1 and ALK-5 in Akt1 silenced HMECs, we next determined the effect of the β-catenin inhibitor on the ALK-1/ALK-5 ratio in ShControl and ShAkt1 HMECs. Surprisingly, treatment with β-catenin inhibitor ICG001 significantly decreased the expression of both ALK-1 and ALK-5 in normal as well as Akt1-deficient HMECs (Fig. 4A-C). Intriguingly, the ALK-1/ALK-5 ratio in ShControl and ShAkt1 HMECs were significantly increased (Fig. 4D). Whereas the loss of Akt1 in HMECs was associated with reduced ALK-1/ALK-5 ratio and worsened TGFβ-induced EndMT, the inhibition of β-catenin in the HMECs reversed the ALK-1/ALK-5 ratio (Fig 4D) suggesting the potential involvement of β-catenin in the regulation of ALK1/ALK-5 ratio.

4. Discussion

Endothelial Akt1 signaling plays a vital role in the maintenance of vascular homeostasis and loss of Akt1 in ECs results in aberrant vascular changes as observed in acute lung injury (Artham et al. 2019), vascular remodeling (Sabbineni et al. 2019), fibrosis (Abdalla et al.

2015) and vascular permeability (Chen et al. 2005; Gao et al. 2016). TGFβ suppresses Akt activity in ECs in a non-canonical pathway resulting in endothelial-barrier breakdown and EndMT (Gao et al. 2017; Sabbineni et al. 2018; Sabbineni et al. 2019). TGFβ isoforms initially bind to TβRII and subsequently transmits the signals downstream through a type I TGFβ receptor but TGFβ signaling in endothelial cells is unique as it involves two type I TGFβ receptors, namely ALK-1 and ALK-5 (Goumans et al. 2003). Literature suggests that while ALK-5 is associated with endothelial and vascular dysfunction induced by TGFβ, ALK-1 may be more or less opposing the ALK-5 effects (Goumans et al. 2003). For example, extracellular matrix remodeling in aorta involves TGFβ-induced ALK-5 signaling in ECs that is responsible for podosome rosette assembly, which is mitigated by ALK-1 signaling induced by TGFβ or BMP9 (Curado et al. 2014). The mechanisms regulating the ALK-5 to ALK-1 ratio and their paradoxical effects in response to TGFβ stimulation remains elusive.

In the current study, we tested the hypothesis that EndMT, because of Akt1 loss and/or TGFβ-stimulation, is associated with a change in the ALK-1 to ALK-5 ratio. Our results indicated that loss of Akt1 promoted EndMT as evidenced by the reduced endothelial marker eNOS and increased mesenchymal marker N-cadherin was associated with an increase in ALK-5 and a corresponding decrease in ALK-1 expression, which was exacerbated by treatments with TGFβ1 or TGFβ2. Interestingly, there was no significant difference between untreated control ECs compared to either TGFβ1 or TGFβ2 treatments, although there was a trend towards decreasing eNOS expression with TGFβ2 treatment for 72 hours. Although we do not have an answer for this intriguing information, the potential reason could be that the TGFβ treatment does not inhibit Akt1 similar to the level of Akt1 gene silencing by ShRNAs (Gao et al. 2017; Sabbineni et al. 2019) and the available active Akt1 in TGFβ-stimulated ECs may be enough to keep eNOS expression at basal levels.

The overall ALK-1 to ALK-5 ratio was decreased in ShAkt1 compared to ShControl HMECs indicating its relevance to EndMT. This decrease in the ALK-1/ALK-5 ratio could be also a result of proteasomal degradation of ALK-1. The loss of phosphorylated β-catenin in ShAkt1 HMECs was modestly restored by treatment with ALK-5 inhibitor treatment. The reduced phosphorylated β-catenin expression in ShAkt1 HMECs indicates their subsequent degradation as evidenced by the reduced levels of total β-catenin in the same cell lysates. This decreases phosphorylated β-catenin to total β-catenin ratio indicates the predominant availability of non-phosphorylated (transcriptionally active) β-catenin in Akt1 deficient or TGFβ-treated HMECs compared to respective controls.

The expression of both ALK-1 and ALK-5 might not be exclusively regulated by the Akt1-β-catenin pathway but may be reliant on alternate signaling pathways or secondary effects. It is possible that Akt1 pathway reconciles its effect with another pathway in the regulation of the ALK1/ALK5 ratio. This was confirmed by our data indicating pharmacological suppression of β-catenin significantly inhibits ALK-5 expression in ShAkt1 HMECs. Thus, our results indicated the prominent role of β-catenin in ALK-5 expression and the promotion of EndMT due to the loss of Akt1. The observed pathway is expected to further influence the EndMT *in vivo* and associated vascular complications in vascular injury.

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Abbrevations list:

HMECs	Human microvascular endothelial cells
EndMT	Endothelial-to-mesenchymal transition
TGFβ	Transforming growth factor-β
ALK1	Activin Linked Kinase-1
ALK5	Activin Linked Kinase-5
TβRI	Transforming growth factor-β type-I receptor
TβRII	Transforming growth factor-β type-II receptor

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HIGHLIGHTS

- Akt1 loss in endothelial cells promote mesenchymal transition
- Akt1 loss promotes ALK-5 and reduces ALK-1 expression in endothelial cells
- ALK-5 suppression blunts mesenchymal transition in Akt1 null endothelial cells
- β-catenin inhibition blunts mesenchymal transition in Akt1 null endothelial cells

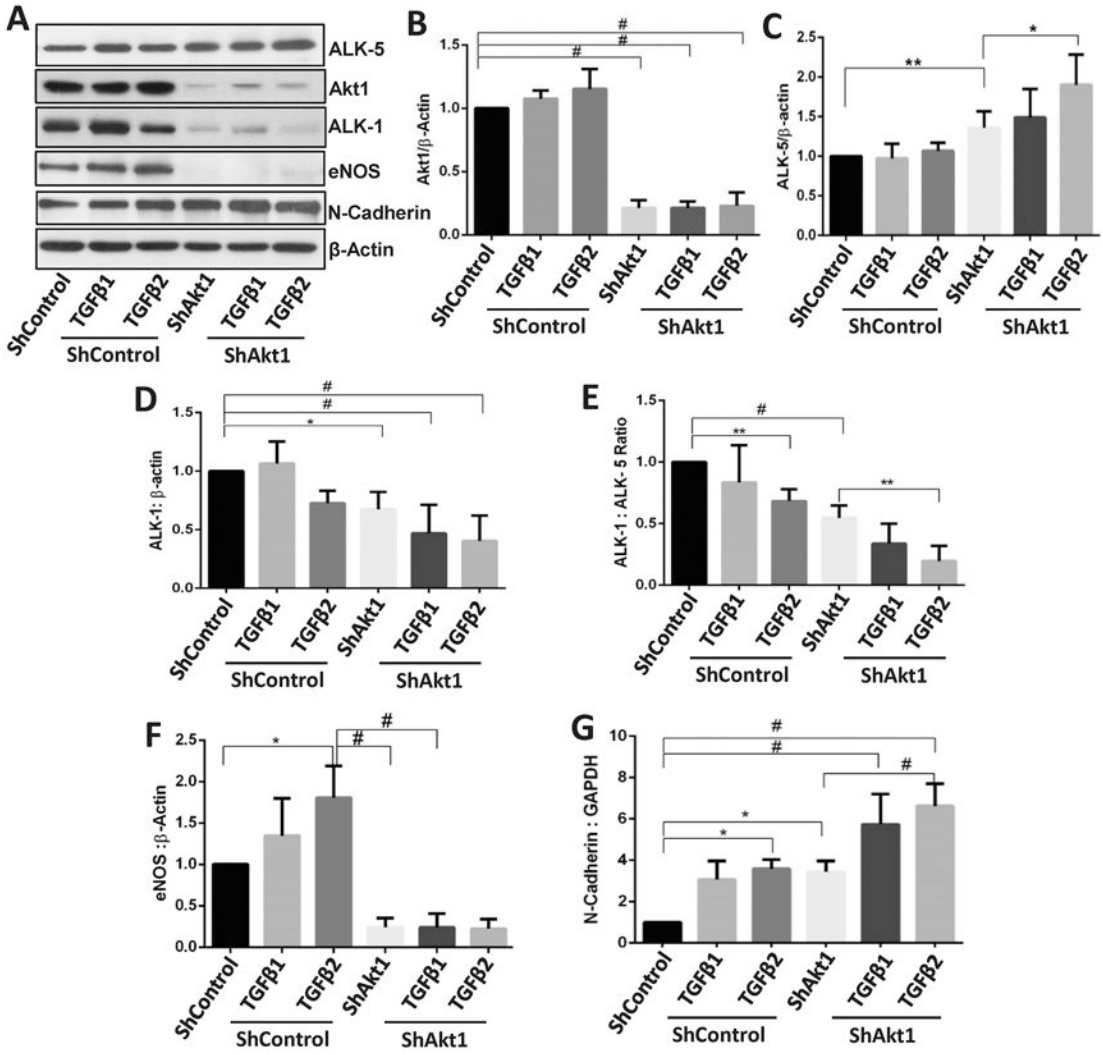

Figure 1: Endothelial Akt1 loss differentially regulates ALK-1 and ALK-5 expression in endothelial cells promoting EndMT.

A) Representative Western blot images of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing changes in Akt1, ALK-5, ALK-1, Akt1, eNOS and N-cadherin expression in ShAkt1 HMECs compared to ShControl HMECs. **B)** Band densitometry analysis of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing reduced Akt1 expression in Akt1 silenced HMECs. **C-D)** Band densitometry analysis of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing increased ALK-5 and decreased ALK-1 expression in Akt1 silenced HMECs, which was exacerbated with TGFβ2 treatment. **E)** Bar graph showing significantly decreased ALK-1/ALK-5 expression ratio in ShAkt1 HMECs with TGFβ treatment compared to ShControl HMECs. **F-G)** Band densitometry analysis of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing decreased endothelial marker eNOS and increased mesenchymal marker N-cadherin expression in ShAkt1 HMECs compared to ShControl HMECs. Data represented as Mean ± SD. (n=6), *p<0.05, **p<0.01, #p<0.001.

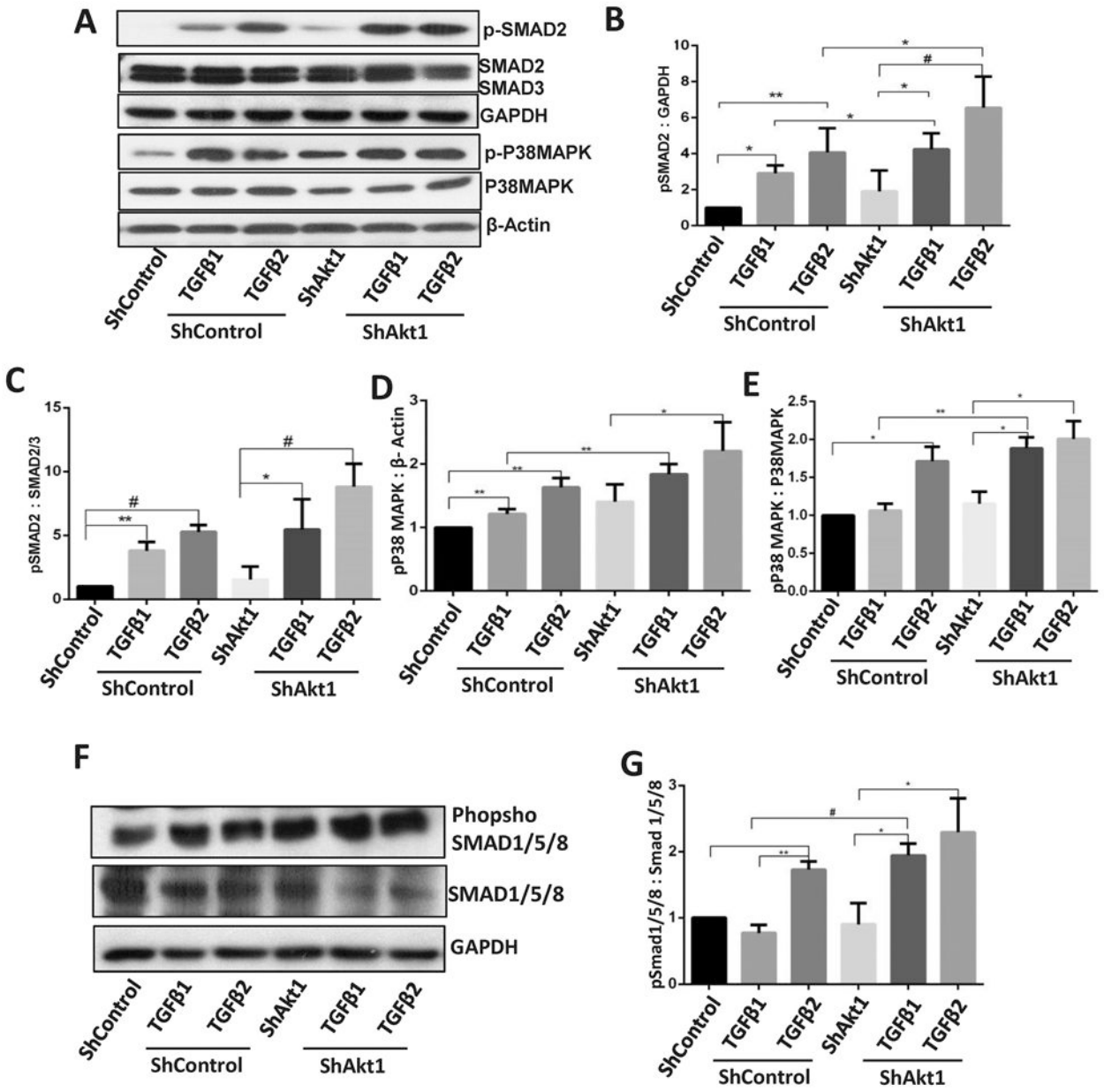

Figure 2: TGFβ-induced EndMT and differential expression of the ALK-1/ALK-5 ratio is associated with changes in SMAD and P38-MAPK activities.

A) Representative Western blot images of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing changes in phosphorylated SMAD2/3 and Phospho-P38MAPK expression in ShAkt1 HMECs compared to ShControl HMECs. **B-C)** Band densitometry analysis of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing increased phosphorylated SMAD2/3 expression in Akt1 silenced HMECs, which was exacerbated with TGFβ2 treatment. **D-E)** Band densitometry analysis of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing increased Phospho-P38MAPK expression in Akt1 silenced HMECs, which was exacerbated with TGFβ2 treatment. **F-G)** Band densitometry analysis of TGFβ-induced ShControl or ShAkt1 HMECs for 72 hours showing changes in

phosphorylated SMAD1/5/8 expression in Akt1 silenced HMECs, which was exacerbated with TGFβ2 treatment. Data represented as Mean ± SD. (n=6), *p<0.05, **p<0.01, #p<0.001

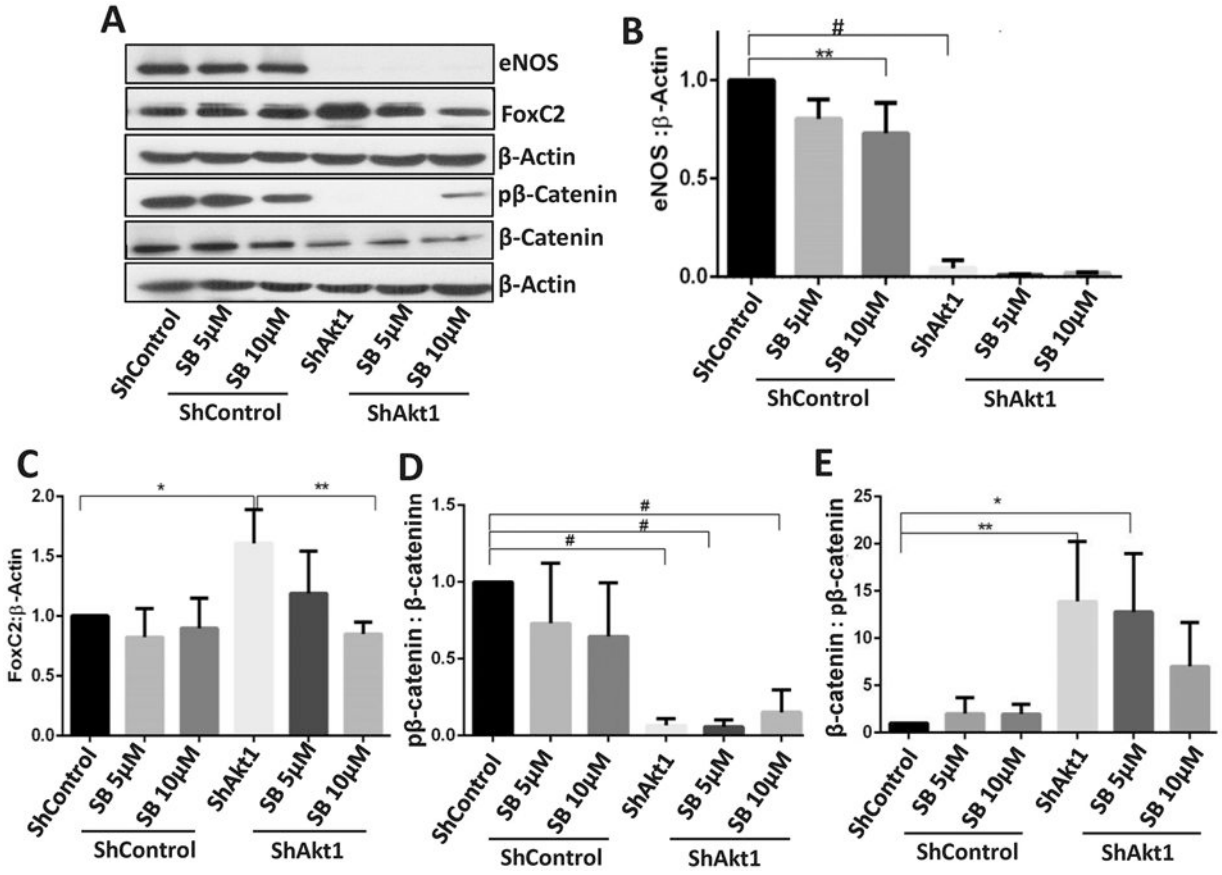

Figure 3: ALK-5 inhibitor (SB431542) partially inhibited EndMT due to Akt1 loss.

A) Representative Western blot images of ShControl or ShAkt1 HMEC lysates treated with or without ALK-5 inhibitor (SB505124) for 72 hours showing changes in eNOS, FoxC2, phosphorylated β-catenin and β-catenin expression in ShAkt1 HMECs compared to ShControl HMECs. **B-C)** Band densitometry analysis of ALK-5 inhibitor-treated ShControl or ShAkt1 HMECs for 72 hours showing decreased eNOS and increased FoxC2 in untreated ShAkt1 cells, which was decreased with ALK-5 inhibitor treatment. **D-E)** Band densitometry analysis of ALK-5 inhibitor-treated ShControl or ShAkt1 HMECs for 72 hours showing changes in phosphorylated β-catenin levels and β-catenin to phosphorylated β-catenin ratio. Data represented as Mean ± SD. (n=4), *p<0.05, **p<0.01, #p<0.001.

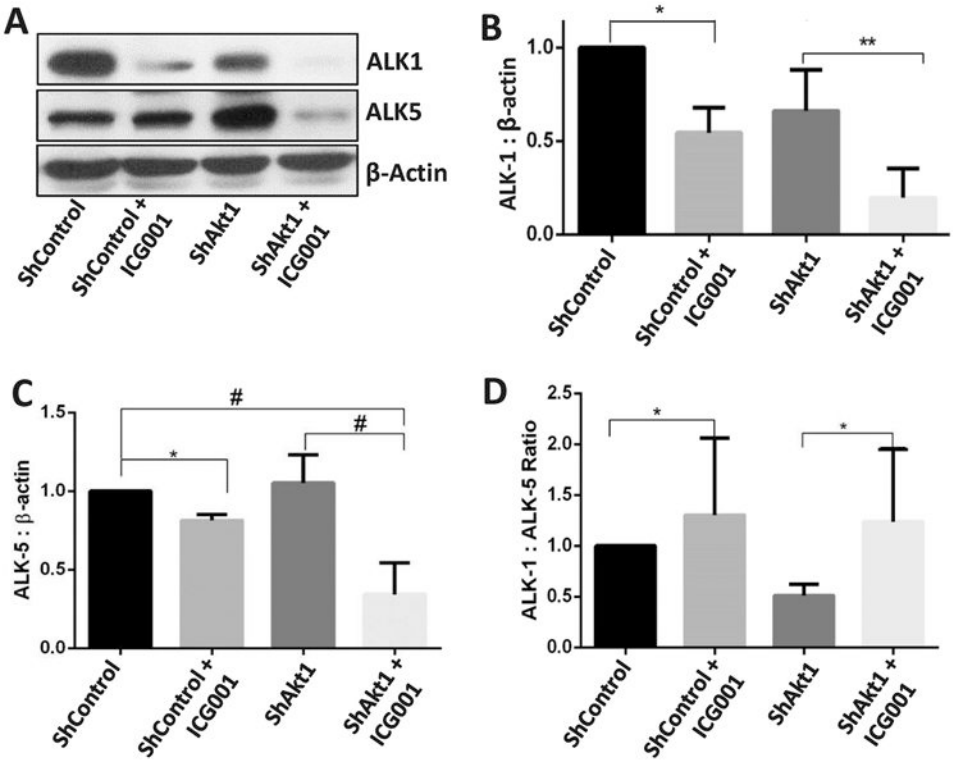

Figure 4: β-catenin inhibition is associated with reduced Alk-5 expression in endothelial cells. **A)** Representative Western blot images of ShControl and ShAkt1 HMEC lysates showing expression changes in ALK-1 and ALK-5 by treatment with β-catenin inhibitor (ICG001) for 72 hours. **B-C)** Band densitometry analysis of ShControl and ShAkt1 HMEC lysates showing a significant decrease in ALK-1 and ALK-5 expressions with β-catenin inhibitor (ICG001) treatment for 72 hours compared to untreated controls. **D)** Bar graph showing increased ALK-1/ALK-5 ratio with β-catenin inhibitor treatment in ShControl and ShAkt1 HMECs. Data represented as Mean ± SD. (n=6), *p<0.05, **p<0.01, #p<0.001.