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Isoform-Specific Effects of Transforming Growth Factor-β on Endothelial to Mesenchymal Transition

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Abstract

Endothelial to mesenchymal transition (EndMT) was first reported in the embryogenesis. Recent studies show that EndMT also occurs in the disease progression of atherosclerosis, cardiac and pulmonary fibrosis, pulmonary hypertension, diabetic nephropathy, and cancer. Although transforming growth factor-β (TGFβ) is crucial for EndMT, it is not clear which isoform elicits a predominant effect. The current study aims to directly compare the dose-dependent effects of TGFβ1, TGFβ2, and TGFβ3 on EndMT and characterize the underlying mechanisms. In our results, all the three TGFβ isoforms induced EndMT in human microvascular endothelial cells (HMECs) after 72 hours, as evidenced by the increased expression of mesenchymal markers N-cadherin and alpha-smooth muscle actin (αSMA) as well as the decreased expression of endothelial nitric oxide synthase (eNOS). Interestingly, the effect of TGFβ2 was the most pronounced. At 1 ng/ml, only TGFβ2 treatment resulted in significantly increased phosphorylation (activation) of Smad2/3 and p38-MAPK and increased expression of mesenchymal transcription factors Snail and FoxC2. Intriguingly, we observed that treatment with 1 ng/ml TGFβ1 and TGFβ3, but not TGFβ2 resulted in increased expression of TGFβ2 thus indicating that EndMT with TGFβ1 and TGFβ3 treatments was due to the secondary effects through TGFβ2 secretion. Furthermore, silencing TGFβ2 using siRNA blunted the expression of EndMT markers in TGFβ1 and TGFβ3 treated cells. Together, our results indicate that TGFβ2 is the most potent inducer of EndMT and that TGFβ1- and TGFβ3-induced EndMT necessitates a paracrine loop involving TGFβ2.

Keywords

TGFβ1; TGFβ2; TGFβ3; EndMT; Snail; FoxC2; N-cadherin

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

1. INTRODUCTION

Endothelial to mesenchymal transition (EndMT) is a phenomenon in which endothelial cells (ECs) lose their characteristic features and acquire mesenchymal properties (Azhar et al., 2009; Boyer et al., 1999). EndMT is not only an essential mechanism implicated in the embryonic cardiac development (Azhar et al., 2009) but also in the progression of diseases such as atherosclerosis, pulmonary hypertension, diabetic nephropathy, cardiac and pulmonary fibrosis, and many types of cancers (Arciniegas et al., 2007; Kizu et al., 2009; Lee and Kay, 2006; Long et al., 2009). Aberrant EndMT results in the uncontrolled conversion of ECs into mesenchymal cells (Medici et al., 2010), which further switch their phenotype to myofibroblasts (Zeisberg et al., 2008). Myofibroblast is a diverse mesenchymal cell type greatly implicated in wound healing (Gabbiani et al., 1971; Stone et al., 2016) and organ fibrosis (Gerarduzzi and Di Battista, 2016; Liu, 2006; Zeisberg et al., 2000). Upon activation by biochemical and mechanical signals, myofibroblasts secrete and organize extracellular matrix (ECM), develop specialized matrix adhesions (Hinz et al., 2003), and exhibit cytoskeletal organization characterized by contractile actin filaments (Gabbiani et al., 1971). This allows the re-establishment of mechanical integrity and stability to the damaged tissue thus assisting in both the wound closure and resolution, which can lead to pathological remodeling when aberrantly stimulated and goes unconstrained (Hinz and Gabbiani, 2010; Hinz et al., 2012).

EndMT is characterized by the loss of cell-cell adhesions and changes in cell polarity-inducing a spindle-shaped morphology (Manetti et al., 2011). These changes are accompanied by reduced expression of one or more of the endothelial markers such as VE-cadherin, eNOS, and CD31, and increased expression of mesenchymal markers like fibroblast-specific protein-1 (FSP-1), alpha-smooth muscle actin (αSMA), N-cadherin, and fibronectin (Potenta et al., 2008). Loss of cell-cell adhesion is mediated by transcription factors such as Snail, Slug, ZEB-1, Twist, and FoxC2 that suppress transcription of genes encoding proteins involved in the formation of adherens junctions and tight junctions (Liebner et al., 2004; Medici et al., 2008) that are integral to an intact endothelium. Transforming growth factor-β1 (TGFβ1) is a potent inducer of epithelial to mesenchymal transition (EMT) (Akhurst and Derynck, 2001), a phenomenon very similar in biology to that of EndMT. Whereas TGFβ2 is a more potent inducer of fibrosis than TGFβ1 in amphibians (Rosa et al., 1988), both TGFβ1 (Wermuth et al., 2016) and TGFβ2 (Kokudo et al., 2008; Liebner et al., 2004; Romano and Runyan, 2000) are implicated greatly in mediating myofibroblast activation, EMT, and EndMT in vertebrates leading to organ fibrosis. Although both TGFβ1 and TGFβ2 promote EndMT, only TGFβ2 gene ablation in mice prevented EndMT-mediated cardiac development, and while TGFβ1 or TGFβ3 knockout mice had normal heart development (Azhar et al., 2009). Interestingly, although TGFβ3 is implicated in EMT in cancer (Jalali et al., 2012), there are no reports on the effects of TGFβ3 on EndMT. Thus, it is not clear from the literature which isoform of TGFβ is the predominant inducer of EndMT.

In the current study, we directly compared the isoform-specific effects of TGFβ1, TGFβ2 and TGFβ3 in inducing EndMT in human microvascular ECs (HMECs) *in vitro* and their effect on the expression of EC markers, mesenchymal markers, transcription factors

regulating mesenchymal gene expression and the activity status of TGFβ-mediated canonical and non-canonical pathways. Our results demonstrated that TGFβ2 is the predominant mediator of EndMT in HMECs and that TGFβ1- and TGFβ3-induced EndMT needs EC-mediated paracrine loop through increased TGFβ2 secretion.

2. MATERIALS AND METHODS

2.1 Cell culture

Human dermal (Telomerase-immortalized) microvascular ECs (HMEC) (CRL-4025; ATCC, Manassas, VA) were maintained in EC Basal Medium-2 with a Growth Medium-2 Bullet Kit (Lonza; Walkersville, MD). All cultures were maintained in a humidified 5% CO2 incubator at 37 °C and routinely passaged when 80–90% confluent. TGFβ1, TGFβ2, and TGFβ3 were obtained from R&D Systems (Minneapolis, MN) and were reconstituted according to the manufacturer's protocol. HMECs monolayers were treated with 1, 2.5 and 5 ng/ml doses of TGFβ1, TGFβ2 and TGFβ3 in 5% serum-containing medium for 72 hours. The growth factors 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, and CA). Western blot analysis was performed as described previously (Abdalla et al., 2013; Al-Azayzih et al., 2015). Antibodies used include N-cadherin (4061), VE-cadherin (2158S), phosphorylated p-38 MAPK (9211S), total p38-MAPK (9212S), phosphorylated Smad2/3 (8828S), total Smad2/3 (8685S), FoxC2 (12974S), Snail (3879S), and GAPDH (2118L) from Cell Signaling Technology (Danvers, MA), αSMA (A2547) and β-actin (A5441) from Sigma (St. Louis, MO), eNOS (610297) from BD Pharmingen (San Diego, CA), and TGFβ2 (MAB612) from R&D (Minneapolis, MN). Band densitometry was done using NIH Image J software.

2.3 SiRNA-mediated TGFβ2 knockdown

HMECs were transfected with TGFβ2 SiRNA (50 nM) (Sigma, St. Louis, MO) using Lipofectamine 2000 (Thermo Scientific, Grand Island, NY) when 70–80% confluent according to the manufacturer's protocol. After transfection cells were incubated for 5 hours at 37° C in serum-free media (Thermo Scientific, Grand Island, NY). The medium was then discarded and cells were further cultured in the EBM-2 medium. After 12 hours of transfection, cells were treated with TGFβ1 or TGFβ3 (1 ng/ml) for 72 hours. Scrambled SiRNA was used as a control.

2.4 Cell scattering assay

HMECs were seeded at a low density and were allowed to grow to form small colonies. After the formation of small scattered colonies, the EBM-2 medium was replaced with fresh medium containing 5 % FBS and cells were treated with vehicle or 1 ng/ml TGFβ1, 2 and 3. This treatment was done daily for 3 days. Cell scattering images were taken using phase

contrast microscope and the images were qualitatively analyzed for consistency in the observations.

2.5 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 analysis, Student's two-tailed *t*-test or ANOVA test were used to determine significant differences between treatment and control values using the GraphPad Prism 4.03 and SPSS 17.0 software.

3. RESULTS

3.1 TGFβ1, 2 and 3- induced EndMT *in vitro* is a long-term process

To investigate the time response effects of TGFβ1, 2 and 3 on inducing EndMT, HMEC monolayers were treated with 1 ng/ml dose of the three TGFβ isoforms for 0, 12, 24, 48 and 72 hours and the cell lysates were subjected to Western blot analysis. We observed that although stimulation of HMECs with TGFβ isoforms results in a gradual increase in the expression of mesenchymal marker N-Cadherin (Figure 1A–C) and decrease in the expression of endothelial marker eNOS (Figure 1D–F) we observed that the earliest time point at which any of the isoforms significantly induce EndMT is 48 hours. By 72 hours all the three isoforms induced the expression of N-Cadherin (except for TGFβ3) and loss of eNOS promoting EndMT. These results indicate that TGFβ- induced EndMT *in vitro* is a long-term process.

3.2 TGFβ2 is the most potent inducer of mesenchymal markers *in vitro*

In order to determine which of the three TGFβ isoforms is more potent in inducing the expression of mesenchymal markers and hence EndMT, HMEC monolayers were treated with 1, 2.5, and 5 ng/ml doses of TGFβ1, TGFβ2, and TGFβ3 for 72 hours and the cell lysates were subjected to Western blot analysis. Our results showed that stimulation of HMEC by TGFβ2 at the lowest dose of 1 ng/ml results in a significantly higher expression of mesenchymal markers N-cadherin and αSMA as compared to control, TGFβ1 or TGFβ3 (Figure 2A–B, S1, and S2). The replicate blots are shown in Figures S3 and S4. These results indicate that TGFβ2 is more potent compared to that of TGFβ1 and TGFβ3 in inducing mesenchymal markers thus promoting EndMT.

3.3 TGFβ2 is the most potent suppressor of endothelial marker expression *in vitro*

Next, we determined the effects of 1, 2.5, and 5 ng/ml doses of TGFβ1, TGFβ2, and TGFβ3 on the expression of endothelial markers. Analysis of cell lysates after 72 hours of treatment revealed that TGFβ2 treatment resulted in a reduced expression of endothelial marker eNOS and the effect was much higher than similar doses of TGFβ1 and TGFβ3 (Figure 3A–B). However, there was no change in the expression of another endothelial marker VE-cadherin as known previously that epithelial adherens junction protein E-Cadherin expression is not affected by TGFβ (Morin et al., 2011). The replicate blots are shown in Figures S3 and S4.

These results indicate that 1 ng/ml dose of TGFβ2 is more potent in downregulating the endothelial marker than that of TGFβ1 or TGFβ3 at a similar dose (Figure 3A–B).

3.4 TGFβ2 is the most potent activator of canonical and non-canonical TGFβ-mediated pathways in HMECs

TGFβ superfamily ligands exert their downstream signaling effects via either canonical or non-canonical signaling pathways (Gauldie et al., 2007; Hanahan and Folkman, 1996; Kavsak et al., 2000; Santibanez et al., 2011; Suzuki et al., 2002). In order to investigate whether one or both these pathways are activated by TGFβ isoforms in EndMT, HMEC monolayers were treated with 1, 2.5, and 5 ng/ml doses of TGFβ1, 2, and 3 isoforms for 72 hours and the cell lysates were subjected to Western blot analysis. Our results showed that the lowest dose of 1 ng/ml of TGFβ2 promoted activation (phosphorylation) of receptor-regulated Smad2/3 (Figure 4A), the canonical effector of TGFβ signaling and p38-MAPK (Figure 4B, S1 and S2), the non-canonical effector of TGFβ signaling with higher efficiency as compared to the vehicle, TGFβ1 or TGFβ3 treated cells at the same dose suggesting the involvement of both canonical and non-canonical TGFβ signaling in promoting EndMT. These results indicate that TGFβ2 is the predominant inducer of EndMT in HMECs.

3.5 TGFβ2, but not TGFβ1 or TGFβ3, is the predominant regulator of expression of mesenchymal transcription factors Snail and FoxC2 in HMECs

We wanted to further examine the efficiency of different TGFβ isoforms in inducing EndMT promoting transcription factors Snail and FoxC2. Once again, HMEC monolayers were treated with 1, 2.5, and 5 ng/ml doses of TGFβ1, 2, and 3 isoforms for 72 hours and the cell lysates were subjected to Western blot analysis. We observed that 1 ng/ml dose of TGFβ2 is more potent in upregulating the mesenchymal transcription factors Snail and FoxC2 in HMECs than a similar dose of TGFβ1 or TGFβ3 (Figure 5A–B). The replicate blots are shown in Figures S3 and S4. These results indicate that TGFβ2 is the predominant inducer of mesenchymal transcription factors and hence EndMT.

3.6 TGFβ2 induces EndMT and cell scattering at lower doses compared to TGFβ1 or TGFβ3

Whereas both TGFβ1 and TGFβ3 showed dose-dependent effects at a range of 1, 2.5 and 5 ng/ml doses, TGFβ2 effects were almost the same between these doses suggesting that TGFβ2 may still induce EndMT on much lower levels compared to TGFβ1 and TGFβ3. To further investigate that 1 ng/ml dose of TGFβ2 is the right dose for the cell scattering assay, we determined the dose-dependent effect of TGFβ2 on a lower range of 50 pg/ml to 1 ng/ml. Our analysis indicated that the effect of TGFβ2 on mesenchymal marker N-cadherin expression peaks at 0.5 and 1.0 ng/ml doses (Figure 6A–D), thus confirming it as the most appropriate dose to compare the isoform specific-effects. Furthermore, cell scattering analysis, a method that is often used to qualitatively determine mesenchymal phenotype, indicated a predominant effect of 1 ng/ml TGFβ2 on cell scattering, compared to the similar doses of TGFβ1 or TGFβ2 (Figure 7).

3.7 TGFβ1- and TGFβ3-induced EndMT involve an endothelial TGFβ2-mediated paracrine loop

In order to investigate how TGFβ1 or TGFβ3 were able to induce EndMT in HMEC, we investigated if TGFβ1 or TGFβ3 could increase the production of TGFβ2 by HMEC. Our Western blot analysis indicated that treatment with both TGFβ1 and TGFβ3, but not TGFβ2 itself, promoted synthesis of TGFβ2 by the HMEC (Figure 8A–B). Next, we employed SiRNA-mediated TGFβ2 knockdown in HMECs to determine whether TGFβ2 deficient cells will be resistant to TGFβ1 and TGFβ3-induced EndMT. Our data indicated that HMECs transfected with TGFβ2 SiRNA resulted in >60% knockdown in TGFβ2 (Figure 8C–D) expression blunted the effects of TGFβ1 and TGFβ3 on N-cadherin (Figure 8E) and αSMA (Figure 8F) expression. These results further confirmed that TGFβ2 is the most potent inducer of EndMT and that TGFβ1 and TGFβ3 isoforms initiate a TGFβ2 paracrine loop to indirectly promote EndMT in HMECs (Figure 9).

4. DISCUSSION

Myofibroblasts or activated mesenchymal cells play a crucial role in tissue repair and contribute to the pathogenesis of various fibrotic and vascular diseases including but not limited to interstitial pulmonary fibrosis, systemic sclerosis, and liver or cardiac fibrosis (Hinz and Gabbiani, 2010; Hinz et al., 2012; Neilson, 2006). However, the source of these myofibroblasts remains fairly controversial and is gaining more attention recently due to the emergence of a new type of cellular transdifferentiation, a phenomenon known as EndMT (Abraham et al., 2007; Hinz et al., 2007). EndMT is a biological process in which ECs lose one or more of their specific markers such as VE-cadherin, eNOS, and CD31 and acquire mesenchymal markers such as N-cadherin, αSMA, FSP1 and collagen VI (Arciniegas et al., 2005; Arciniegas et al., 1992). EndMT attributes proliferative properties to the otherwise quiescent and adherent ECs, transforming them into myofibroblasts (Li and Bertram, 2010; Piera-Velazquez et al., 2011). In the recent past, quite a few studies reported the occurrence of EndMT in various fibrotic disorders like cardiac (Zeisberg et al., 2007), pulmonary (Hashimoto et al., 2010; Pinto et al., 2016), corneal (Li et al., 2013), and renal fibrosis (Li and Bertram, 2010). Both TGFβ1 and TGFβ3 have been implicated in the cardiac cushion formation in avian embryo (Nakajima et al., 1997a; Nakajima et al., 1997b; Nakajima et al., 2000; Yamagishi et al., 1999). Although EndMT is implicated in many diseases, the stimuli that trigger the initiation of this cellular differentiation and the mechanisms through which the transformation occurs remain elusive. Several signalling pathways are reported in EndMT, while the most important being TGFβ binding (Doerr et al., 2016; Montorfano et al., 2014; Nakajima et al., 2000; Zeisberg et al., 2008; Zeisberg et al., 2007). Given the popularity of TGFβ as a potent cell differentiation cytokine and the extensive involvement of its signaling in the pathogenesis of fibrotic diseases (Rosenbloom et al., 2010; Wynn, 2008), several groups have investigated its role in the generation of myofibroblasts via EndMT (Hinz et al., 2007; Hinz et al., 2012). Although it is widely accepted that TGFβ1 is a potent inducer of fibrosis via generation of myofibroblasts in various fibrotic models, several emerging reports advocate the involvement of TGFβ2 in promoting EndMT (Chrobak et al., 2013; Medici et al., 2011; Nie et al., 2014; Shi et al., 2016). Whereas TGFβ and its downstream effectors as EndMT inducers are being extensively studied by several groups in

various fibrotic and vascular diseases, appropriate knowledge on the contributions of different TGFβ isoforms, TGFβ1, 2, and 3 remains unknown.

In order to identify the most potent inducer of EndMT, we directly compared the dose-dependent effects of TGFβ1, TGFβ2, and TGFβ3 on EndMT *in vitro*. We examined changes in the expression of endothelial and mesenchymal markers, transcription factors that promote mesenchymal transition, and the activation of TGFβ-induced canonical and non-canonical pathways by treating HMECs with various doses of these TGFβ isoforms for 0–72 hours. Stimulation of HMECs with 1 ng/ml dose of the three isoforms TGFβ1, 2 and 3 for 72 hours revealed that TGFβ is involved in EndMT which was evident from the upregulation of mesenchymal markers N-cadherin and αSMA and a decrease in expression of endothelial marker eNOS. We noticed that the earliest time point at which the EndMT changes start to occur is 48 hours and that at 72 hours most if not all the isoforms induce EndMT. Together these results indicate that TGFβ induced EndMT *in vitro* is a long-term process.

Among the different receptor-regulated Smads involved in TGFβ signaling, Smad3 was reported as the pro-fibrotic member of the Smad family as its activation (phosphorylation) promotes the progression of fibrosis (Darland et al., 2003; Hirschi et al., 1998). On a similar note, we observed that TGFβ2 increases the phosphorylation (activation) of Smad2/3 and p38 MAPK greater than that of TGFβ1 and TGFβ3 suggesting the predominance of TGFβ2 in inducing mesenchymal transition of ECs and the involvement of both canonical and non-canonical TGFβ signaling pathways in promoting EndMT. We also observed that TGFβ2 increases the expression of EndMT promoting transcription factors Snail and FoxC2 with significantly higher efficiency than the other two TGFβ isoforms further confirming our observation and in agreement with the recent finding reporting a TGFβ2-mediated activation of the ALK5-Smad2/3-Snail pathway (Zeng et al., 2013) leading to EndMT. Interestingly, two most recent studies indicate the unique role of Smad1/5 pathway in EndMT (Ramachandran et al., 2018; Sniegon et al., 2017), in addition to the observed role of Smad2/3-Snail pathway.

Biochemical changes in the expression of EndMT markers must also be accompanied by changes in its morphology and/or functional behavior. Cancer cells that invade and metastasize to distant organs exhibit the ability of cell scattering, invasion and transendothelial migration *in vitro* (Al-Azayzih et al., 2015; Gao et al., 2015). Unlike cancer cells, motility and adhesion of endothelial cells is a measure of endothelial barrier function and angiogenesis (Chen et al., 2005; Somanath et al., 2007). Although a scattering of endothelial cells in a monolayer is not as robust as the colony forming cancer cells, for a 72-hour long effect of TGFβ isoforms on EndMT, cell scattering is still the best possible assay to be performed *in vitro*. Our qualitative analysis indicated that 72-hour treatment of endothelial cells with 1 ng/ml of TGFβ2 induced cell scattering more effectively than the comparable doses of TGFβ1 or TGFβ3. Although endothelial cells appeared to be scattered in TGFβ3 wells, this, however, was also contributed by increased cell death with TGFβ3 treatment compared to TGFβ1 or TGFβ2, thus demonstrating that TGFβ2 is a more robust inducer of EndMT that TGFβ1 or TGFβ3.

Another important question that we wanted to address was how TGFβ1 and TGFβ3 were able to induce EndMT, albeit at higher doses. Are the functions of these three isoforms redundant? To address this, we determined the expression changes in TGFβ2 post 72-hour treatment with TGFβ1, TGFβ2, and TGFβ3. The most intriguing and prominent finding of our study that came from this experiment was that both TGFβ1 and TGFβ3, but not TGFβ2, stimulated the expression of TGFβ2 by the HMECs. This indicated the existence of a positive feedback loop between different TGFβ isoforms via paracrine effects involving TGFβ2 synthesis in inducing EndMT. Together, our results demonstrate that although all the three isoforms of TGFβ moderately induce EndMT, TGFβ2 is the most potent inducer. This is in agreement with observations from several reports that suggested the predominance of TGFβ2 in inducing EndMT of mouse embryonic stem cell-derived ECs (Kokudo et al., 2008) and that TGFβ2 is a more potent fibrotic inducer than TGFβ1 in amphibians (Hsuan, 1989; Rosa et al., 1988). To our knowledge, this is the first study to examine and directly compare the dose-dependent effects of three different TGFβ isoforms- TGFβ1, TGFβ2, and TGFβ3 on EndMT demonstrating a paracrine TGFβ2-mediated EndMT loop in HMECs stimulated by TGFβ1 and TGFβ3. Here we indicate the predominance of TGFβ2 in inducing EndMT thus paving a way to direct future investigations on this pathway in EndMT to yield a better understanding of the mechanisms involved.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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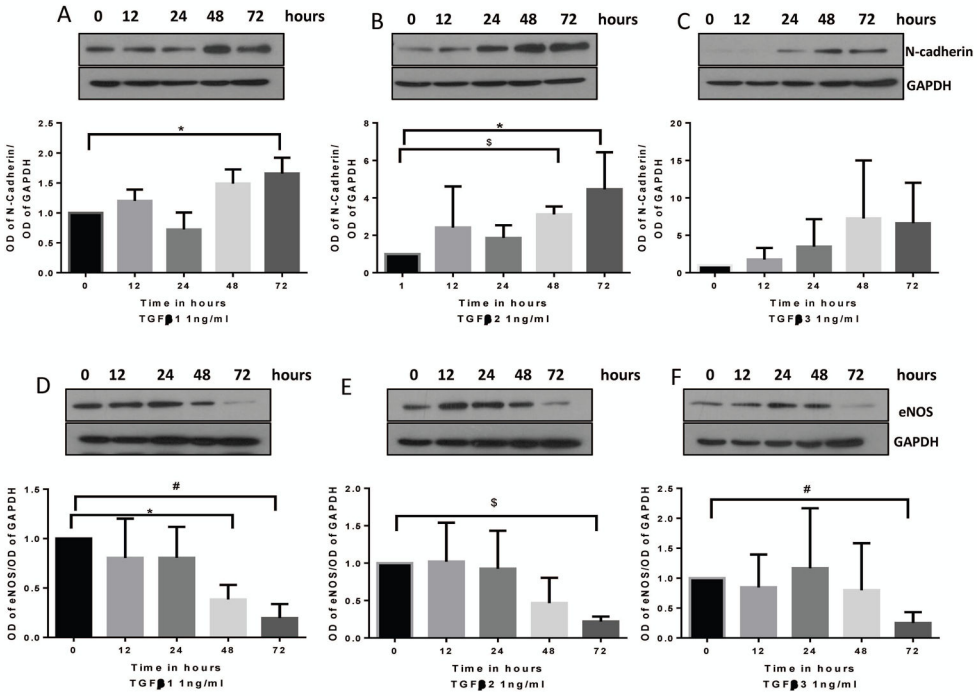

FIGURE 1. TGFβ- induced EndMT in HMECs is a long-term process

(A–C) Representative Western blot images and the corresponding bar graph of band densitometry showing a gradual increase in the expression of mesenchymal marker N-Cadherin in HMECs treated with 1 ng/ml of TGFβ1, 2 and 3 for 0, 12, 24, 48 and 72 hours**.** **(D–E)** Representative Western blot images and the corresponding bar graph of band densitometry showing a gradual decrease in the expression of endothelial marker eNOS in HMECs treated with 1 ng/ml of TGFβ1, 2 and 3 for 0, 12, 24, 48 and 72 hours. Data are represented as mean ± SD. (n=3–5), **p<0.05*; *#p<0.0;*, *$p<0.001.*

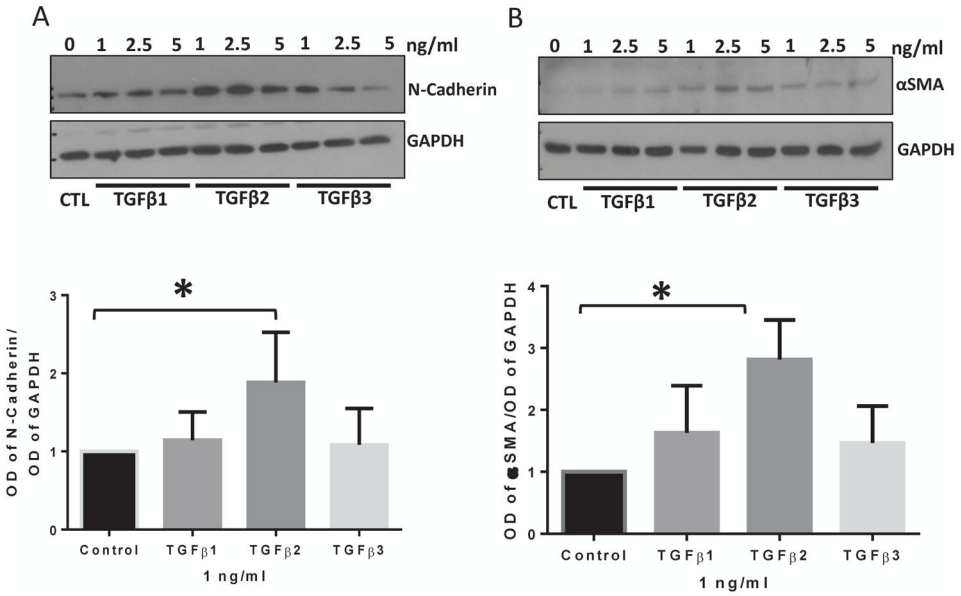

FIGURE 2. TGFβ2 is a more potent inducer of mesenchymal markers in HMECs compared to TGFβ1 and TGFβ3

(A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of mesenchymal marker N-cadherin in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of the mesenchymal marker αSMA in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean ± SD. (n=3–5), *$p<0.05$.

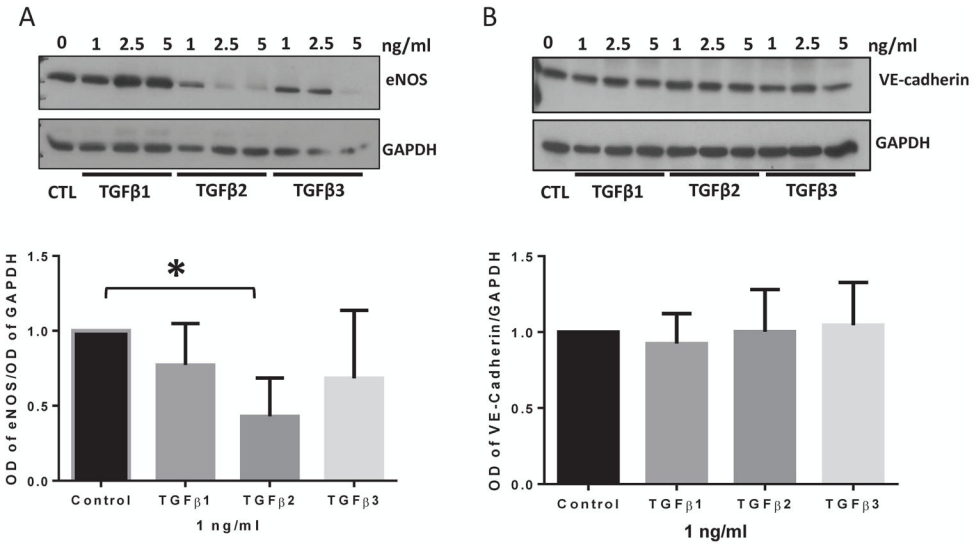

FIGURE 3. TGFβ2 is a more potent suppressor of the endothelial marker expression in HMECs compared to TGFβ1 and TGFβ3

(A) Representative Western blot images and the corresponding bar graph of band densitometry showing reduced expression of endothelial marker eNOS in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing no significant change in the expression of endothelial receptor VE-cadherin in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean ± SD. (n=3–5), *$*p<0.05$*.

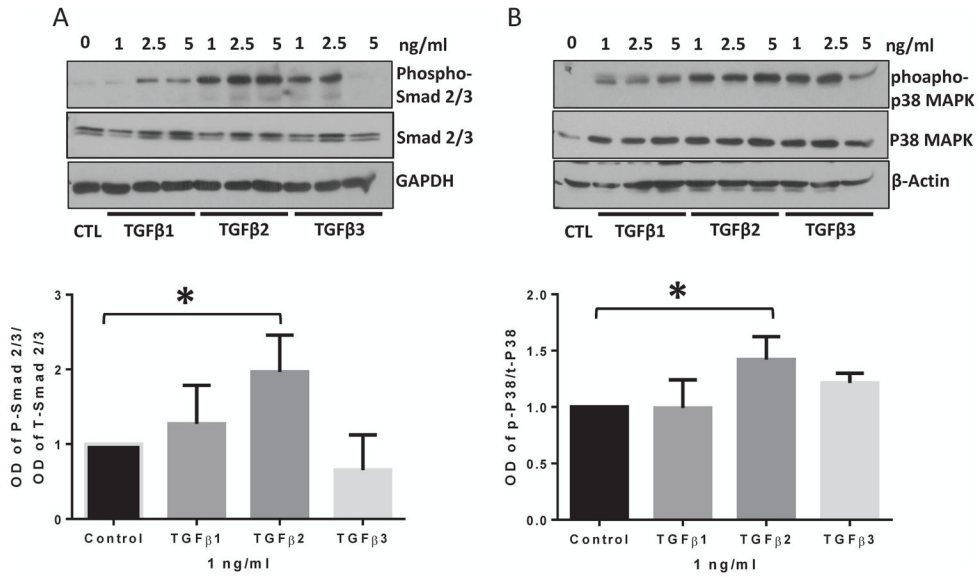

FIGURE 4. TGFβ2 exhibits higher potency in activating both canonical and non-canonical pathways in HMECs compared to TGFβ1 and TGFβ3

(A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased phosphorylation and total expression of canonical transcription factor Smad2/3 in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased phosphorylation of non-canonical, stress-induced p38 MAPK in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean ± SD. (n=3–5), **p<0.05*.

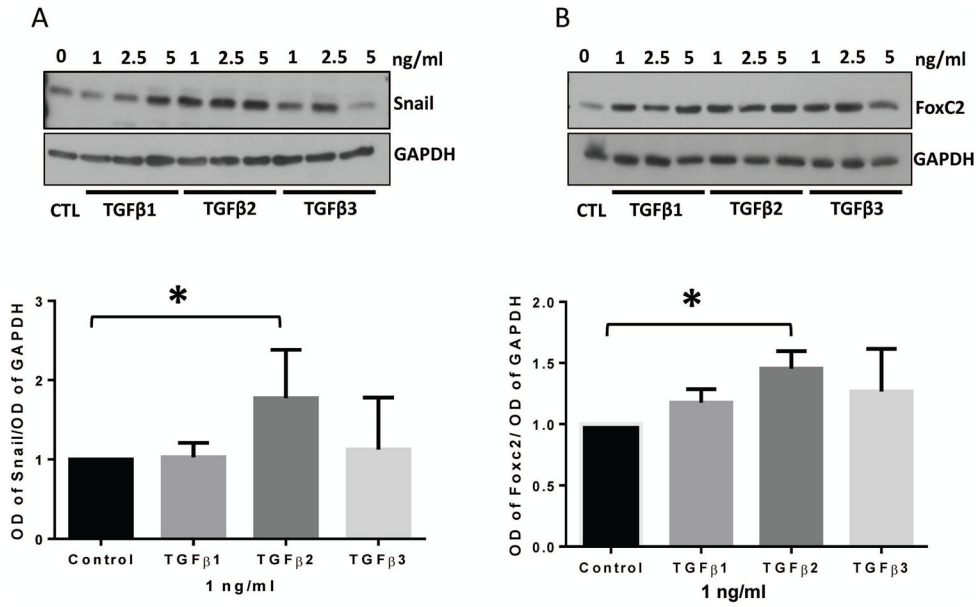

FIGURE 5. TGFβ2 is a more potent stimulator of mesenchymal transcription factor expression in HMECs compared to TGFβ1 and TGFβ3

(A) Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of mesenchymal transcription factor Snail in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Representative Western blot images and the corresponding bar graph of band densitometry showing increased expression of mesenchymal transcription factor FoxC2 in HMECs in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. Data are represented as mean ± SD. (n=3–5), *$p<0.05$.

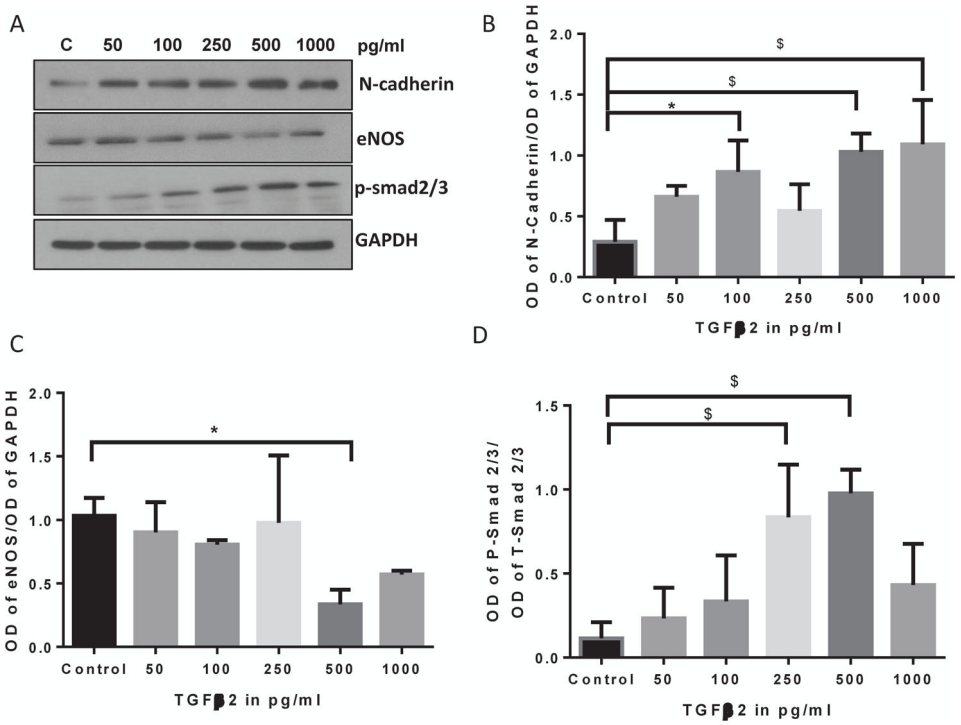

FIGURE 6. TGFβ2 induces EndMT at lower doses

(A) Representative Western blot images showing a gradual increase in the expression of mesenchymal marker N-Cadherin associated with increased p-smad2/3 and decreased eNOS levels in HMECs treated with TGFβ1 for 72 hours with 0, 50, 100, 250, 500 and 1000 pg/ml doses**. (B–D)** Corresponding bar graph of band densitometry showing a gradual decrease a gradual increase in the expression of mesenchymal marker N-Cadherin associated with increased p-smad2/3 and decreased eNOS levels in HMECs treated with TGFβ1 for 72 hours with 0, 50, 100, 250, 500 and 1000 pg/ml doses. Data are represented as mean ± SD. (n=3–5), **p<0.05*; $*p<0.001*.

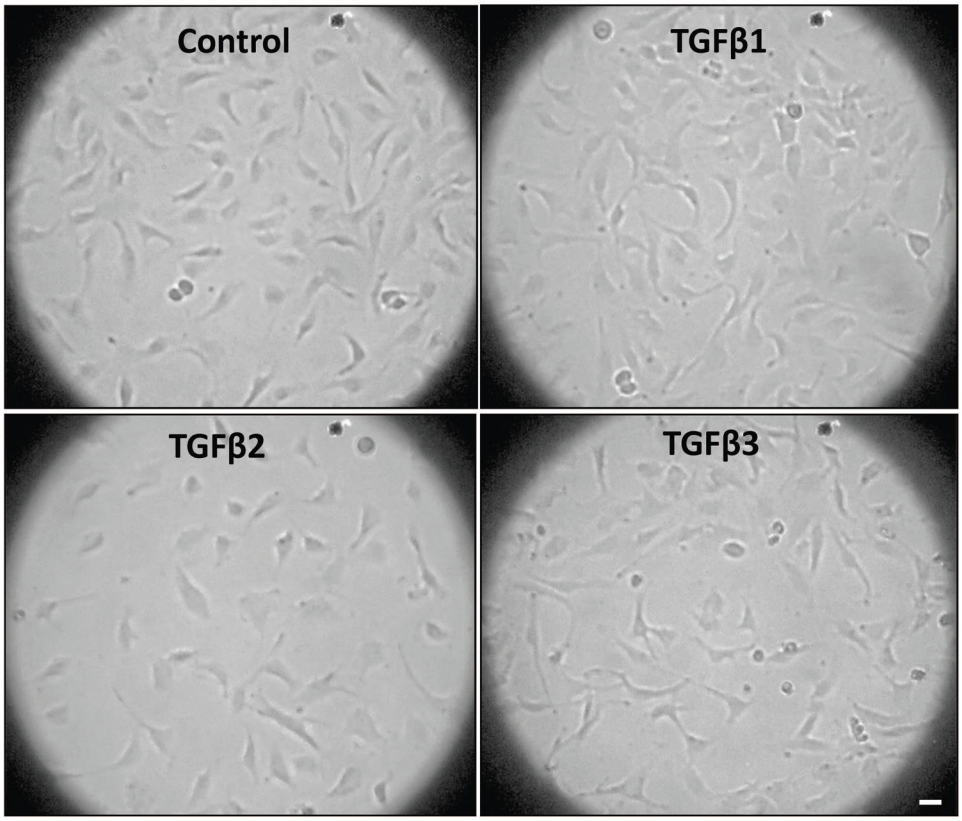

FIGURE 7. TGFβ2 predominantly induce endothelial cell scattering compared to other isoforms

Representative images showing the predominant effect of TGFβ2 on cell scattering, a feature of the invasive mesenchymal cells compared to control, TGFβ1 and TGFβ2 treated cells after 72 hours of treatment. *Scale bar: 20 μM.*

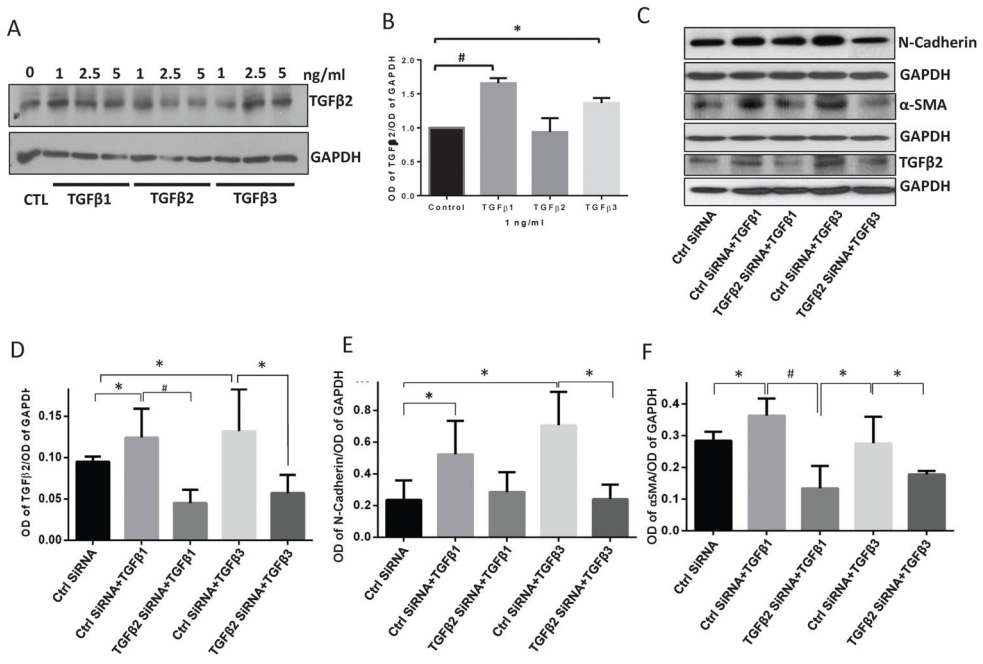

FIGURE 8. TGFβ1- and TGFβ3-induced EndMT needs activation of a paracrine loop in HMECs involving TGFβ2

(A) Representative Western blot images showing increased expression of the most potent EndMT stimulating TGFβ isoform, TGFβ2, in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(B)** Bar graph of Western blot band densitometry analysis showing increased expression of TGFβ2 in response to 1, 2.5 and 5 ng/ml of TGFβ1, TGFβ2, and TGFβ3 for 72 hours. **(C)** Representative Western blot images showing increased expression of N-Cadherin, αSMA, and TGFβ2 by both TGFβ1 and TGFβ3, both of which were blunted upon SiRNA-mediated knockdown of TGFβ2. **(D–E)** Bar graph of Western blot band densitometry analysis showing increased expression of N-Cadherin and αSMA by both TGFβ1 and TGFβ3, which were blunted upon SiRNA-mediated knockdown of TGFβ2. Data are represented as mean ± SD. (n=3–5), **p<0.05; #p<0.01.*

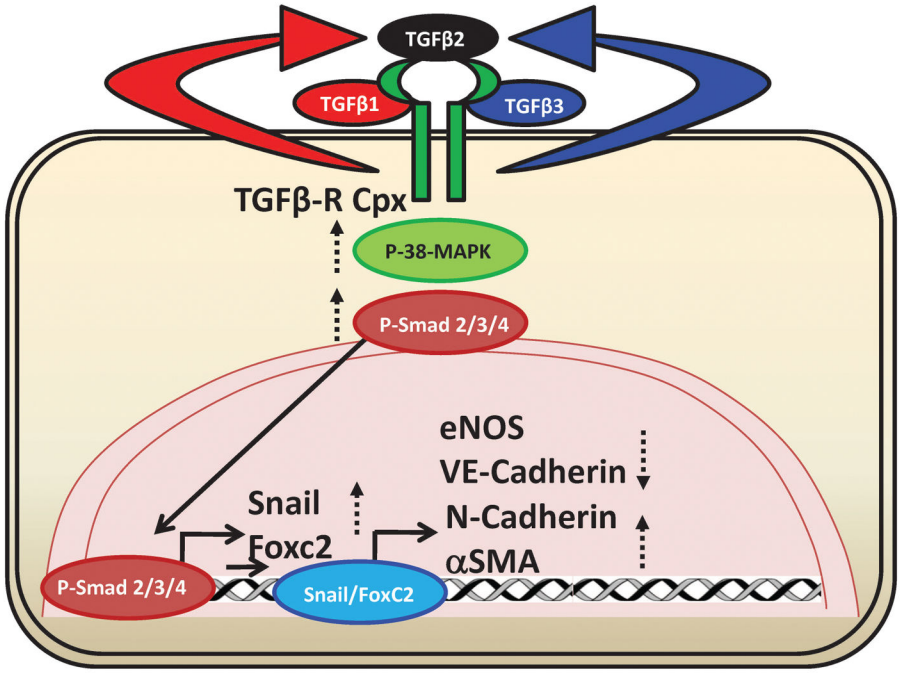

FIGURE 9. Diagrammatic sketch of the working hypothesis

Both TGFβ1 and TGFβ3, but not TGFβ2 itself, induces the generation of TGFβ2 in HMECs, which in turn, promote EndMT pathways leading to Snail and FoxC2-induced transcriptional activation of mesenchymal markers and repression of endothelial markers.