rhBMP-7 suppresses TGF-β1-induced endothelial to mesenchymal transition in circulating endothelial cells by regulating Smad5

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Abstract. Endothelial to mesenchymal transition (EndMT) has been confirmed to participate in several cardiovascular diseases. In addition, EndMT of circulating endothelial cells (CECs) contributes to the pathology of musculoskeletal injury. However, little is known about the molecular mechanism of CECs undergoing EndMT. In the present study, human CECs were isolated and identified using anti-CD146-coupled magnetic beads. CECs were exposed to transforming growth factor (TGF)-β1 or TGF-β1 + recombinant human bone morphogenetic protein 7 (rhBMP-7) or TGF-β1 + rhBMP-7 + Smad5 antagonist Jun activation domain-binding protein 1. Vascular endothelial (VE)-cadherin and vimentin expression were detected by immunofluorescence staining in TGF-β1-treated CECs. The expression levels of von Willebrand factor (vWF), E-selectin, VE-cadherin, vimentin, fibronectin, α smooth muscle actin (α-SMA) and Smad2/3 were detected by reverse transcription-quantitative PCR or western blot analysis. It was identified that rhBMP-7 attenuated TGF-β1-induced endothelial cell injury. TGF-β1 could induce the EndMT process in CECs, as confirmed by the co-expression of VE-cadherin and vimentin. TGF-β1 significantly reduced the expression of VE-cadherin, and induced the expression of vimentin, fibronectin and α-SMA. rhBMP-7 reversed the effects of TGF-β1 on the expression of these genes. Additionally, Smad5 antagonist reversed the effects of rhBMP-7 on TGF-β1-induced EndMT, and upregulated rhBMP-7-inhibited Smad2/3 expression. In conclusion, TGF-β1 could induce EndMT in CECs and rhBMP-7 may suppress this process by regulating Smad5.

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Introduction

Endothelial-mesenchymal transition (EndMT) is a similar process to epithelial-mesenchymal transition, which involves a phenotypic conversion from endothelial cells to mesenchymal cells. During this process, endothelial cells lose their specific endothelial markers, including CD31, vascular endothelial (VE)-cadherin and von Willebrand Factor (vWF), and begin to express specific mesenchymal markers, including vimentin, fibronectin, collagens and α smooth muscle actin (α-SMA) (1). EndMT has been confirmed to participate in several cardiovascular diseases, including cardiac fibrosis, atherosclerosis, atrial fibrillation and pulmonary hypertension (2,3). For example, Kato *et al* (4) reported the occurrence of EndMT in the atria of patients with atrial fibrillation.

Circulating endothelial cells (CECs) represent desquamated mature cells sloughed off from vessel walls in response to endothelial injury. Previous studies have demonstrated that the number of CECs may be a biomarker for several vascular disorders, such as inflammatory vasculitis, cardiovascular diseases and metabolic pathologies (5-7). CECs also have important roles in tumor progression, and are involved in endothelial homeostasis and angiogenesis (8). It has been reported that increased counts of viable CECs are a marker of progressive disease in patients with cancer (9). Freestone *et al* (10) suggested that the numbers of CECs were increased in patients with atrial fibrillation and acute vascular complications. Alongside vascular endothelial cells, CECs may also undergo EndMT in response to injury (11). However, little is known about the molecular mechanism of CECs undergoing EndMT.

Several signaling molecules contribute to the process of EndMT, including transforming growth factor (TGF)-β, epidermal growth factor, Wnt, Notch and bone morphogenetic proteins (BMPs) (12). Among these, TGF-β1 has been identified as a potent inducer of EndMT in several diseases (13,14). TGF-β indirectly phosphorylates Smad2 and Smad3 through binding to type II TGF-β receptor, which phosphorylates the type I receptor. The phosphorylated Smad2 and Smad3 then interact with Smad4 and the complex containing Smad2, Smad3 and Smad4 was translocate into the nucleus, regulating the transcription of target genes. It has been demonstrated that TGF-β1-induced EndMT can be inhibited by BMP-7, which belongs to the TGF-β superfamily (15). BMP7 can inhibit Smad2/3 phosphorylation through phosphorylating Smad1, 5 and 8 (16). In the present study, the effects of recombinant

human BMP-7 (rhBMP-7) on TGF-β1-induced EndMT in CECs were assessed. Additionally, the role of Smad5 in the EndMT process of CECs regulated by rhBMP-7 was further investigated.

Materials and methods

Cell isolation, culture and treatments. Peripheral blood samples (100 ml) were collected from 10 healthy volunteers (6 male and 4 female, age range 20-40 years) at Taizhou Hospital (Taizhou, China) between October 2017 and March 2018, once informed consent was obtained. The present study was approved by the ethics committee of Taizhou Hospital. CEC isolation was performed according to previous studies (9,17). Briefly, after discarding the first 3-5 ml peripheral blood drawn through venipuncture, the remaining blood was incubated with magnetic beads conjugated to a monoclonal antibody against CD146 (also known as Sendo-I, cat. no. 361036, BioLegend, Inc.). PBS-BSA (0.1%, Shanghai Fanke Biotechnology Co., Ltd.) was used to rinse the bead-bound cell fraction. The viable endothelial cells within the isolate were quantified using a fluorescence microscope after staining with CalceinAM (Sigma-Aldrich; Merck KGaA). The selected bead-bound CECs were isolated using cytospin (5 min at 100 x g) on glass slides at 37˚C. The primary antibodies against CD31 (1:20; cat. no. ab28364; Abcam), von Willebrand factor (vWF) (1:400; cat. no. ab6994; Abcam) and vascular endothelial growth factor (VEGF)-receptor 2 (VEGF-R2) (1:50; cat. no. ab2349; Abcam) were used for the phenotypic analysis of CECs by immunofluorescence.

CECs were co-cultured with an endothelial feeder layer as described in a previous study (9). Human umbilical vein endothelial cells (HUVECs; Shanghai Hongshun Biological Technology Co., Ltd.; $7x10^4$/well) were used as the feeder layer. Carboxyfluorescein diacetate succinimidyl ester-labeled CECs (500 cells; Bio-Rad Laboratories, Inc.), obtained by incubating Carboxyfluorescein diacetate succinimidyl ester with CECs for 30 min at 37˚C, were co-cultured with HUVECs ($7x10^4$/well) in EGM-2 endothelial growth medium (Beijing Fubo Biotechnology Co., Ltd.) in a 6-well plate, which was pre-coated with fibronectin. For the treatments, CECs were divided into various groups: Control, TGF-β1 (10 ng/ml rhTGF-β1; 24 h; 37˚C; cat. no. GF346; Sigma-Aldrich; Merck KGaA) treatment group, TGF-β1 (10 ng/ml) + rhBMP (1 or 10 or 100 ng/ml; 24 h; 37˚C; cat. no. 354-BP-010; R&D Systems, Inc.) treatment group, and TGF-β1 (10 ng/ml) + rhBMP (100 ng/ml) + Jun activation domain-binding protein 1 (JAB1; 20 ng/ml; 24 h; 37˚C; cat. no. H00010987-P01; Abnova) treatment group.

Reverse transcription quantitative (RT-q)PCR. Total RNA was extracted from CECs using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Following quantification by NanoDrop (Thermo Fisher Scientific, Inc.), RNA was used as a template to synthesize cDNA using qRT SuperMix (Vazyme). The temperature protocol was as follows: 70˚C for 3 min, 42˚C for 60 min and 70˚C for 15 min. Subsequently, RT-qPCR was carried out using SYBR Green kits (Takara Biotechnology Co., Ltd., cat. no. RR820Q) and an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction steps were: 95˚C for 30 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 40 sec. Primer sequences used in RT-qPCR are presented in Table I. Each experiment was performed three times. The $2^{-\Delta\Delta Cq}$ method was used to calculate relative gene expression (18).

Western blot analysis. Total proteins in CECs were extracted using ice-cold cell extraction buffer (Invitrogen; Thermo Fisher Scientific, Inc.). Protein concentration was quantified using the BCA Protein Assay kit (Takara Biotechnology Co., Ltd.). Subsequently, equal amounts of protein (20 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked in 5% skimmed milk for 2 h at 37˚C and then incubated with primary antibodies, including anti-vWF (1:2,000; cat. no. ab218333; Abcam), anti-E-selectin (1:1,000; cat. no. ab18981; Abcam), anti-VE-cadherin (1:2,000; cat. no. ab33168; Abcam), anti-vimentin (1:1,000; cat. no. ab45939; Abcam), anti-fibronectin (1:2,000; cat. no. ab2413 Abcam), anti-α-SMA (1:2,000; cat. no. ab5694; Abcam), anti-Smad2 (1:2,000; cat. no. ab40855; Abcam), anti-Smad3 (1:1,000; cat. no. ab40854; Abcam) and anti-β-actin (1:2,000; cat. no. ab8227; Abcam) overnight at 4˚C. The secondary antibody used was a species appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; cat. no. ab7090) Abcam). Immunoreactive bands were visualized with the ECL detection system (EMD Millipore) and analyzed by ImageJ v1.8 (National Institutes of Health).

Immunofluorescence staining. Following exposure to 4% paraformaldehyde for 30 min at 37˚C, the CECs were co-incubated with antibodies against VE-cadherin (1:1,000; cat. no. ab33168; Abcam) or vimentin (1:1,000; cat. no. ab45939; Abcam), or CD31 (1:20; cat. no. ab28364; Abcam), von Willebrand factor (vWF) (1:400; cat. no. ab6994; Abcam) and vascular endothelial growth factor (VEGF)-receptor 2 (VEGF-R2) (1:50; cat. no. ab2349; Abcam) at 4˚C overnight. The samples were washed with PBS three times and incubated with the secondary HRP-conjugated immunoglobulin G antibody (1:1,000; cat. no. ab7090; Abcam) at room temperature for 2 h, then incubated with 100 ng/ml DAPI (Sigma-Aldrich; Merck KGaA) for 10 min at 37˚C to stain nuclei. For VE-cadherin and vimentin expression, fluorescence microscopy (Nikon Corporation,) and Image-Pro Plus v6.0 (Media Cybernetics, Inc.) were used.

ELISA of type I collagen content. CECs were cultured in six-well plates for 24 h and were then subjected to different treatments. The supernatants were collected to measure type I collagen content using a COL-I ELISA kit (Shanghai Walan Biotech Co., Ltd., cat. no. ABE10204) according to the manufacturer's protocol. Absorbance at 450 nm was determined using a microplate reader. Type I collagen concentration was calculated according to a standard curve.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed using SPSS v16.0 (SPSS Inc.). The differences between two groups were evaluated by Student's t-test or one-way ANOVA followed by Tukey's

Table I. Primer sequences used for reverse transcription-quantitative PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
vWF	TGCAACACTTGTGTCTGTCG	CGAAAGGTCCCAGGGTTACT
E-selectin	AAAGAGAGTGGAGCCTGGTC	CCTACCCAGACCCACACATT
VE-cadherin	TACCAGGACGCTTTCACCAT	AAAGGCTGCTGGAAAATGGG
Vimentin	GAGTCCACTGAGTACCGGAG	ACGAGCCATTTCCTCCTTCA
Fibronectin	GTATACGAGGGCCAGCTCAT	CCCAGGAGACCACAAAGCTA
α-SMA	ACCCAGCACCATGAAGATCA	TTTGCGGTGGACAATGGAAG
GAPDH	CCATCTTCCAGGAGCGAGAT	TGCTGATGATCTTGAGGCTG

vWF, von Willebrand Factor; VE, vascular endothelial; α-SMA, α-smooth muscle actin.

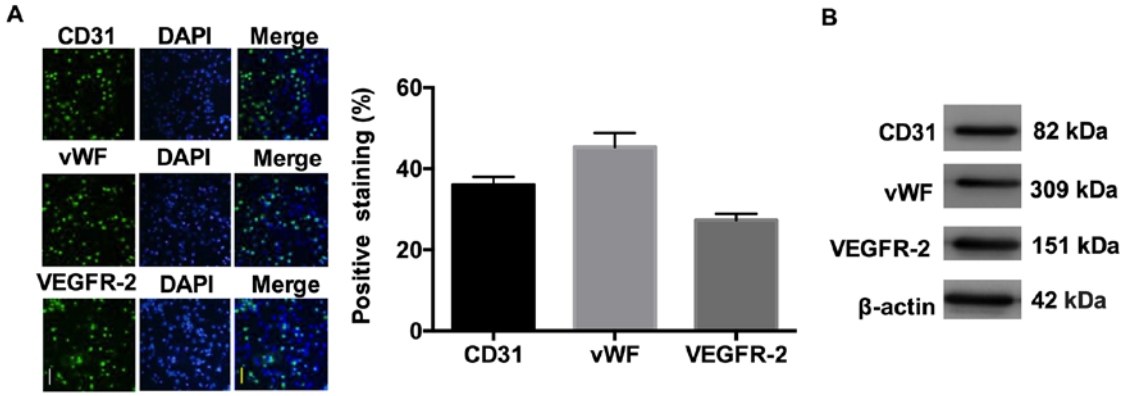

Figure 1. Isolation and identification of CECs. (A) CECs were stained for CD31, vWF and VEGFR-2. Scale bar=10 μm. The percentage of positively stained cells is presented. (B) Western blot analysis of CD31, vWF and VEGF-R2. n=3. CECs, circulating endothelial cells; vWF, von Willebrand factor; VEGF-R2, vascular endothelial growth factor receptor 2.

post hoc test was used for analyzing the differences of multiple groups. P<0.05 was considered statistically significant.

Results

Isolation and identification of CECs. CECs were isolated by anti-CD146-coupled magnetic beads and were identified by immunofluorescence using antibodies against CD31, vWF and VEGF-R2. As demonstrated in Fig. 1, the isolated CECs were positive for expression of CD31, vWF and VEGF-R2.

rhBMP-7 attenuates TGF-β1-induced endothelial cell injury. rhBMP-7 shares a similar signal transduction mechanism with TGF-β1, and can inhibit EndMT and collagen synthesis (19). Endothelial cells were treated with TGF-β1 or TGF-β1 + rhBMP-7 to elucidate the effect of rhBMP-7 on TGF-β1-induced endothelial cell injury. vWF and E-selectin are known biomarkers of endothelial cell injury. As demonstrated in Fig. 2A and B, TGF-β1 increased the mRNA expression levels of vWF and E-selectin in endothelial cells, which was decreased by rhBMP-7 in a dose-dependent manner. Western blotting further demonstrated the effects of TGF-β1 and rhBMP-7 on the protein levels of vWF and E-selectin (Fig. 2D). In addition, the expression of type I collagen was upregulated by TGF-β1 treatment, whereas rhBMP-7 reduced its expression (Fig. 2C).

rhBMP-7 inhibits TGF-β1-induced EndMT. To detect TGF-β1-induced EndMT, an immunofluorescence assay for endothelial marker VE-cadherin and mesenchymal marker vimentin expression was performed. As demonstrated in Fig. 3A, VE-cadherin and vimentin were co-expressed in TGF-β1-treated endothelial cells, indicating the occurrence of EndMT induced by TGF-β1. RT-qPCR results demonstrated that TGF-β1 greatly reduced the mRNA expression levels of VE-cadherin and induced the mRNA expression levels of vimentin, fibronectin and α-SMA (Fig. 3B-E). rhBMP-7 reversed the effects of TGF-β1 on the expression of these genes, indicating that rhBMP-7 inhibited TGF-β1-induced EndMT. These results were further validated by western blot analysis (Fig. 3F).

Smad5 antagonist reverses the effect of rhBMP-7 on TGF-β1-induced EndMT. To elucidate the mechanism underlying the effects of Smad5 on rhBMP-7 inhibiting TGF-β1-induced EndMT, the Smad5 antagonist Jab-1 was used to treat endothelial cells in the TGF-β1 + rhBMP-7 group. VE-cadherin expression was reduced by Jab-1, which conversely increased the expression of vimentin, fibronectin and α-SMA (Fig. 4). These results indicated that the Smad5 antagonist reversed the effect of rhBMP-7 on TGF-β1-induced EndMT.

Smad5 antagonist upregulates rhBMP-7-inhibited Smad2/3 expression. The present study further analyzed whether

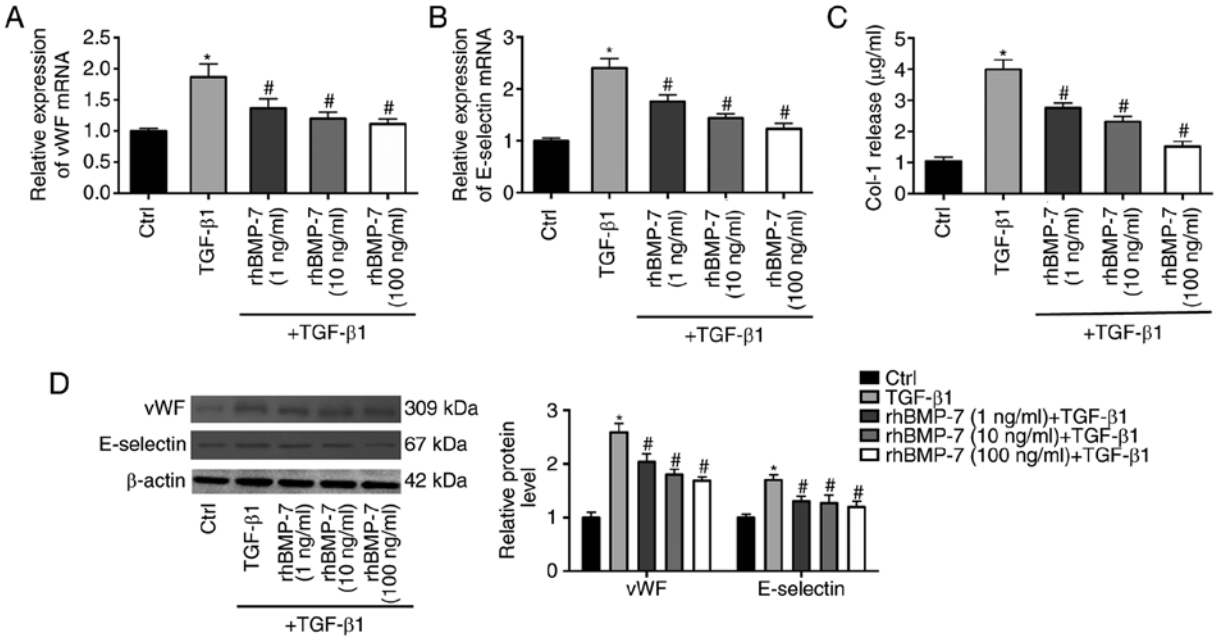

Figure 2. rhBMP-7 attenuates TGF-β1-induced endothelial cell injury. Endothelial cells were treated with TGF-β1 or TGF-β1 plus different concentrations of rhBMP-7. Reverse transcription-quantitative PCR was performed to detect the mRNA expression levels of (A) vWF and (B) E-selectin in endothelial cells. (C) Col-1 level was examined by ELISA. (D) Protein levels of vWF and E-selectin were detected by western blot analysis in endothelial cells. n=3; *P<0.05 vs. Ctrl group; #P<0.05 vs. TGF-β1 group. Col-1, type I collagen; Ctrl, control; rhBMP-7, recombinant human bone morphogenetic protein 7; TGF, transforming growth factor; vWF, von Willebrand factor.

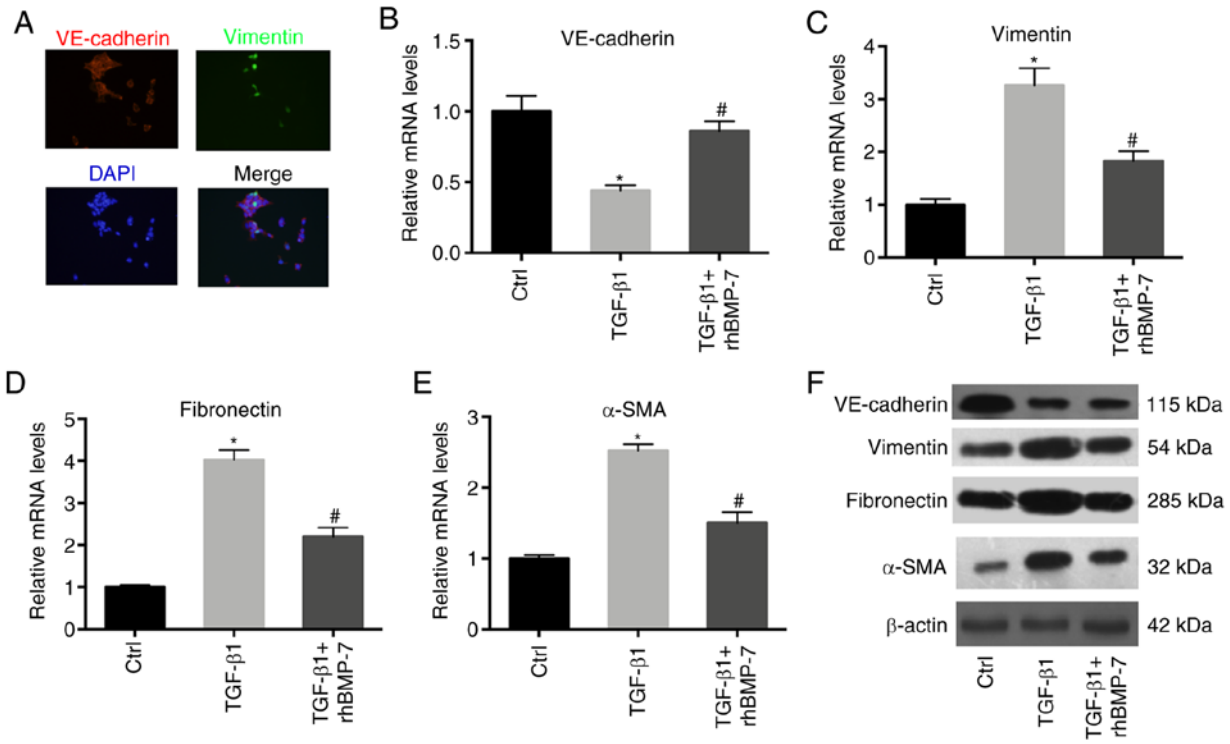

Figure 3. rhBMP-7 inhibits TGF-β1-induced endothelial to mesenchymal transition. (A) Immunofluorescence assay for VE-cadherin and vimentin (magnification, x100). Endothelial cells were treated with TGF-β1 or TGF-β1 + rhBMP-7. The mRNA expression levels of (B) VE-cadherin, (C) vimentin, (D) fibronectin and (E) α-SMA were detected in these cells. (F) Protein levels of VE-cadherin, vimentin, fibronectin and α-SMA were detected in these cells. n=3; *P<0.05 vs. Ctrl group; #P<0.05 vs. TGF-β1 group. α-SMA, α smooth muscle actin; Ctrl, control; rhBMP-7, recombinant human bone morphogenetic protein 7; TGF, transforming growth factor; VE, vascular endothelial.

Smad2 and Smad3 expression was mediated by rhBMP-7 and Smad5. As demonstrated in Fig. 5, rhBMP-7 mitigated TGF-β1-induced Smad2 and Smad3 expression. Conversely, Jab-1 increased the expression of Smad2 and Smad3 inhibited by rhBMP-7. These data suggested that the Smad5 antagonist reversed rhBMP-7-induced inhibition of Smad2/3 expression.

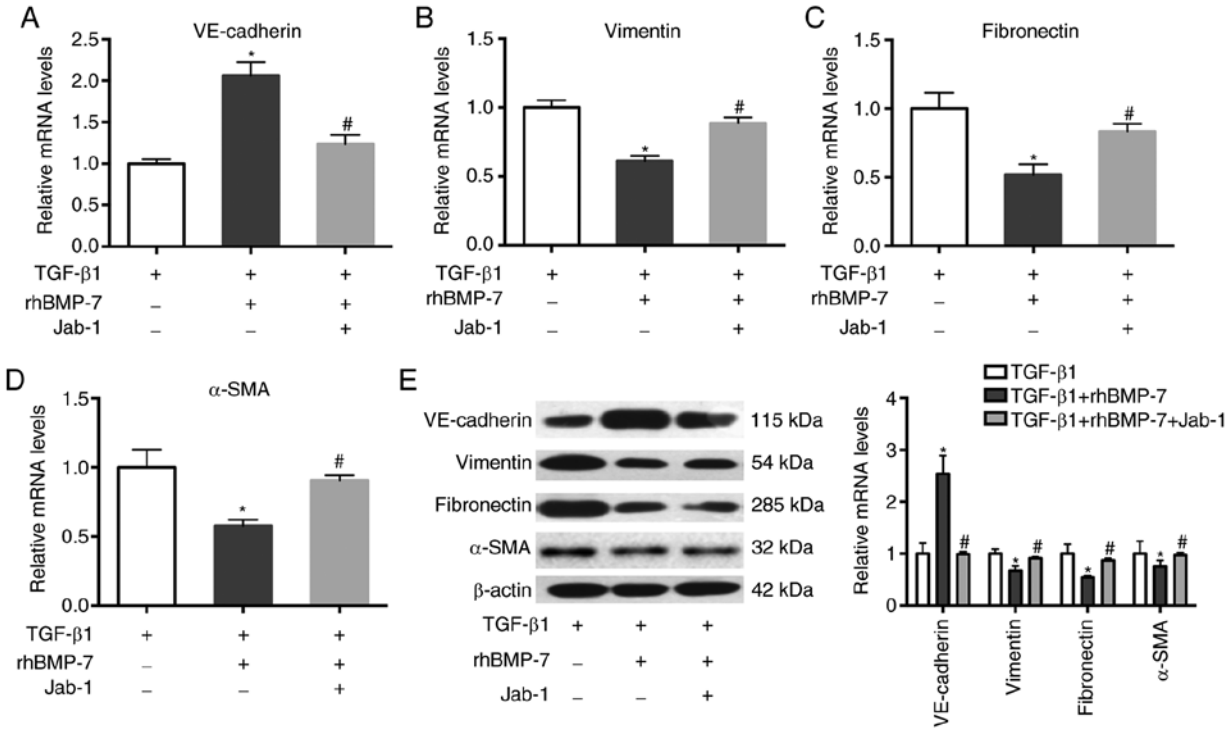

Figure 4. Smad5 antagonist reverses the effects of rhBMP-7 on TGF-β1-induced endothelial to mesenchymal transition. Endothelial cells were treated with TGF-β1, TGF-β1 + rhBMP-7 or TGF-β1 + rhBMP-7 + Jab-1. The mRNA expression levels of (A) VE-cadherin, (B) vimentin, (C) fibronectin and (D) α-SMA were detected in these cells. (E) Protein levels of VE-cadherin, vimentin, fibronectin and α-SMA were detected in these cells. n=3; *P<0.05 vs. TGF-β1 group; #P<0.05 vs. TGF-β1 + rhBMP-7 group. α-SMA, α smooth muscle actin; rhBMP-7, recombinant human bone morphogenetic protein 7; TGF, transforming growth factor; VE, vascular endothelial.

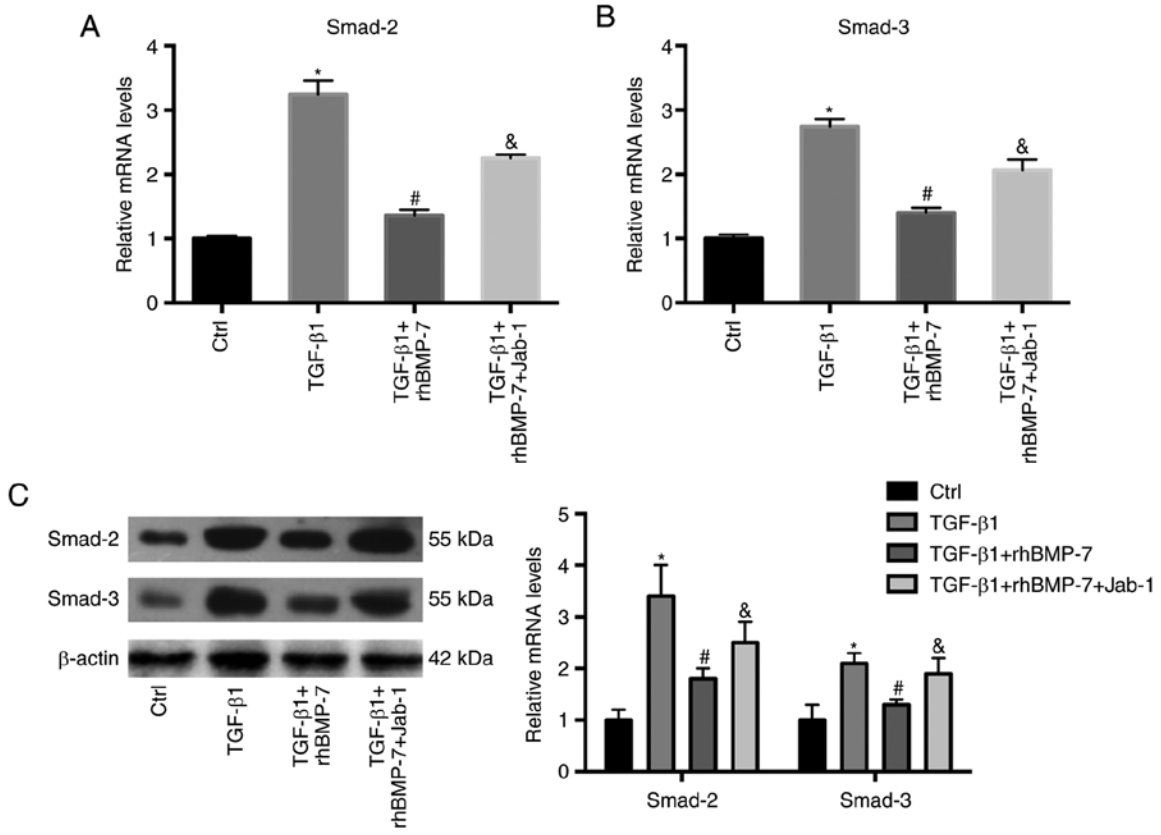

Figure 5. Smad5 antagonist upregulates rhBMP-7-inhibited Smad2/3 expression. Endothelial cells were treated with TGF-β1, TGF-β1 + rhBMP-7 or TGF-β1 + rhBMP-7 + Jab-1. The mRNA expression levels of (A) Smad2 and (B) Smad3 were detected in these cells. (C) Protein levels of Smad2 and Smad3 were detected in these cells. n=3; *P<0.05 vs. Ctrl group; #P<0.05 vs. TGF-β1 group; &P<0.05 vs. TGF-β1 + rhBMP-7 group. Ctrl, control; rhBMP-7, recombinant human bone morphogenetic protein 7; TGF, transforming growth factor.

Discussion

CECs are identified by morphological features and the presence of endothelial markers, including vWF, CD146 and CD31. Mounting evidence has demonstrated that high levels of CECs might indicate considerable damage to the endothelial cell layer (20,21). Additionally, endothelial dysfunction has been demonstrated to be involved in diverse cardiovascular diseases (22). Increased numbers of CECs have been observed in several cardiovascular diseases, including hypertension, heart failure, peripheral vascular disease and atrial fibrillation (10,23,24). Wang *et al* (25) identified that CEC numbers were higher in patients with acute myocardial infarction compared with healthy controls. Patients with venous thromboembolism also demonstrate higher numbers of CECs, as well as vWF and vascular cell adhesion molecule 1 (26). Woywodt *et al* (27) demonstrated that CECs can serve as markers of endothelial damage or repair in stroke. CECs are also related to the pathogenesis of other diseases. Lombardo *et al* (7) suggested that type 2 diabetes mellitus increases the count of CECs in peripheral blood. A high CEC count is also correlated with shorter overall survival and progression-free survival in patients with non-small cell lung cancer (28).

Recently, Agarwal *et al* (11) identified that CECs undergo EndMT following migration to the wound site of musculoskeletal injury, which suggests the potential of CECs as a target to prevent EndMT-related pathologies. However, there are few studies on the molecular mechanism of CECs undergoing EndMT. EndMT is a phenomenon occurring under several pathological conditions, including cardiac fibrosis (29). EndMT may be an important source of mesenchymal cells, which exhibit a high migratory potential and increased extracellular matrix production. In addition, EndMT can cause endothelial dysfunction during inflammatory conditions (5) It has been demonstrated that TGF-β and the BMP family of growth factors are the best-studied mediators of EndMT via Smad-dependent and Smad-independent pathways (12).

The present study identified that TGF-β1 induced endothelial cell injury and EndMT in CECs. TGF-β1 significantly reduced VE-cadherin expression, and induced the expression of vimentin, fibronectin and α-SMA. To further investigate the mechanism underlying TGF-β1-induced EndMT in CECs, CECs were further exposed to rhBMP-7. The present study demonstrated that rhBMP-7 inhibited TGF-β1-induced EndMT in CECs. Consistent with these results, several studies have demonstrated that BMP-7 suppresses EndMT *in vivo* and *in vitro* (19,30). Zhang *et al* (19) reported that BMP-7 inhibits hypoxia-induced EndMT in pulmonary artery endothelial cells and in experimental models of pulmonary artery hypertension. BMP-7 treatment has also been reported to have a positive impact on the severity of liver disease by attenuating EndMT (31). Furthermore, supplementation of exogenous rhBMP-7 effectively ameliorated EndMT and experimental endocardial fibroelastosis in rats (30).

Mechanistically, BMP-7 is capable of suppressing Smad2/3 phosphorylation through phosphorylating Smad1, 5 and 8. BMP-7-induced inhibition of mesenchymal markers requires Smad5 in mesangial cells (32). In the present study, the Smad5 antagonist Jab-1 was used to treat endothelial cells in the TGF-β1 + rhBMP-7 group. It was identified that the Smad5 antagonist reversed the effects of rhBMP-7 on TGF-β1-induced EndMT. Furthermore, the Smad5 antagonist reversed the inhibitory effects of rhBMP-7 on Smad2/3 expression. These data suggested that rhBMP-7 may suppress TGF-β1-induced EndMT in CECs through regulating Smad5.

In summary, this study revealed that TGF-β could induce EndMT in CECs, and rhBMP-7 could suppress this process by regulating Smad5. These data suggested a therapeutic target associated with the inhibition of EndMT in CECs for cardiovascular diseases.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WG and JJ designed and performed the research. YM, SX, TL and YL performed the cell experiments. WG analyzed the data and wrote the paper.

Ethics approval and consent to participate

The present study was approved by the ethics committee of Taizhou Hospital. Informed consent was obtained from all individuals that participated in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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