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ORIGINAL ARTICLE

TGF-β Signaling Induces the Expression of OPN in Blood Vessel Endothelial Cells

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SUMMARY

Background: **The mechanism of blood vessel formation and degeneration still remains unclear. Transforming growth factor-β1 (TGF-β1) signaling is a critical pathway in this progression and can induce multiple biological effects. Osteopontin (OPN) is involved in mineral metabolism and the inflammatory response associated with vascular calcification.**
Methods: **To identify the relationship between TGF-β signaling pathway and OPN, we stimulated human vascular endothelial cells (HVECs) and human aortic endothelial cells (HAECs) using various concentration of TGF-β1 *in vitro*.**
Results: **As assessed by flow cytometry and western blots, apoptosis levels were significantly increased with TGF-β1 treatment. We also demonstrated that OPN increased *in vitro* with TGF-β signaling by western blot and quantitative real time polymerase chain reaction (qRT-PCR) analyses. The inhibitory phosphorylation of endothelial nitric-oxide synthase (eNOS) (Thr495) was also up-regulated by TGF-β signaling. Meanwhile, the anti-inflammatory factor Nrf2 and the activating phosphorylation of eNOS (Ser1177) were down-regulated.**
Conclusions: **Taken together, our findings demonstrate that TGF-β signaling can induce the expression of OPN, which may play an important role in the dysfunction of the vascular wall.**
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KEY WORDS

OPN, TGF-β, endothelial cells, nitric-oxide synthase

LIST OF ABBREVIATIONS

TGF-β1 - Transforming growth factor-β1
OPN - Osteopontin
VECs - vascular endothelial cells
qRT-PCR - quantitative real time polymerase chain reaction
eNOS - endothelial nitric-oxide synthase
VEGF - vascular endothelial growth factor
FGF - fibroblast growth factor
EGF - epidermal growth factor
TNF-α - tumor necrosis factor-α
IL-8 - interleukin-8
BMP - bone morphogenetic protein

ALK1 - activin receptor-like kinase
TGFbRI - transforming growth factor b type I receptor
LDS - Loeys-Dietz
ICAM1 - Intercellular Adhesion Molecule 1

INTRODUCTION

Blood vessels are a part of the circulatory system and microcirculation and play essential roles in the transport of oxygen, nutrients, waste products, and circulating cells in a normal organism. In most cases, blood vessels are quiescent in adults, but under some conditions, the growth of blood vessels is pivotal to organ development and tissue regeneration [1,2]. For example, angiogenesis consists of sprouting new vessels from the old ones and the eventual fusion of these with other new sprouts or old blood vessels to produce new vascular connections. This process of vessel growth is usually promoted and regulated by angiogenic factors, which can be divided into two groups: (1) Activators, for examples, EGF (epidermal growth factor), FGF (fibroblast growth factor) 2, and VEGF (vascular endothelial growth factor), which induce proliferation and migration of vascular endothelial cells [1,3]; and (2) maturation factors, including some members of the TGF-β (transforming growth factor-β) family, TNF-α (tumor necrosis factor-α), and IL-8 (interleukin-8), which inhibit the proliferation of endothelial cells, reestablish basal membrane, and recruit mural cells [4, 5]. Among them, BMP (bone morphogenetic protein) 9, a member of TGF-β family, can selectively activate ALK1 (activin receptor-like kinase)/Tgfbr1 (transforming growth factor b type I receptor), which is a serine-threonine kinase type I TGF-β receptor in endothelial cells [6]. Next, the activated ALK1 triggers the phosphorylation of some Smads, including Smad1, 5, and 8, which in turn forms an active complex with Smad4. This active Smad complex translocates to the nucleus, and induces the expression of many genes, such as ID, endoglin, and Tmem100 [7-9]. As a critical factor in tumor progression and metastasis, TGF-β1 induces downstream biological effects through Tgfbr1 and Tgfbr2 receptors [10,11]. Once activated, TGF-β1 and its receptor activate Smad2/3. The phosphorylated Smad2/3 then binds to Smad4 and translocates into the nucleus to express target genes [12]. Many studies demonstrated that TGF-β signaling pathway was related with tumor progression and prognosis [13,14]. It has been reported that TGF-β signaling plays an important role in breast carcinoma vascularization [15] as well as in normal vascular and cardiac development [16]. Genetically engineered mice lacking Tgfbr1/Alk5 exhibit severe defects in vascular development [17]. In addition, endothelial cells (ECs) from Tgfbr1 deficient mice also show enhanced cell proliferation, improper migratory behavior, and decreased fibronectin expression. Moreover, it was reported that several human syndromes, including Marfan and Loeys-Dietz (LDS), were linked with certain defects or misregulation in the TGF-β pathway. However, the molecular mechanisms underlying vascular abnormalities in these patients remains unclear.

OPN (Osteopontin) is a type of pleiotropic cytokine and inflammatory factor and expressed widely in many tissues. OPN is involved in the attachment of osteoclasts to the mineralized bone matrix and can induce self-reactive T cells and regulate immune responses in mammals. It has been reported that OPN also plays an important role in the development and participates in the progression of hypertensive vascular remodeling [18]. OPN is an inductor of immune responses which can affect the balance of Th1/Th2 and upregulates expression of interferon-gamma, interleukin-10, and interleukin-12 [19]. However, little is known about the relationship between OPN and TGF-β signaling in the dysfunction of blood vessels. Therefore, we speculated that OPN might be an important factor in those processes of clinical patients.

In this study, we investigated the correlation between TGF-β signaling and OPN. We also explored the effect of TGF-β1 on OPN expression in VECs by molecular and cell biology assays and found OPN could be a key factor in the dysfunction of blood vessels exposed to TGF-β signaling.

MATERIALS AND METHODS

Cell lines and culture

Human VECs (ScienCell Research Laboratories, USA) and human AECs (Thermo Fisher, USA) were cultured in endothelial cell complete medium with 1% endothelial cell growth supplement. The cells were maintained in a humidified incubator with 5% CO_2 at 37°C and digested for passage using 0.1% trypsin every 2 - 4 days.

Antibodies and other reagents

Human TGF-β1 (Cat#240-B/CF) was purchased from R&D Systems (Minneapolis, MN, USA). Antibodies for GAPDH (sc-25,778), phospho-Smad2, phospho-p38, and p38MAPK(sc-81,621) were purchased from Cell Signaling Technology (Danvers, MA, USA); α-Tubulin and Smad2 were purchased from BD Biosciences (San Jose, CA, USA); Ki67 was purchased from Abcam. Goat anti-Rabbit IgG (H + L)-Horseradish Peroxidase (HRP) and goat anti-Mouse IgG (H + L)-HRP secondary antibodies were purchased from BIO-RAD Laboratories (Hercules, CA, USA). Anti-ICAM-1 and anti-Nrf2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were then incubated with the blot, and antibody complexes were detected by chemiluminescence (Engreen, Beijing, China).

Analysis of apoptosis by flow cytometry

After treatment of TGF-β1, cells were washed with PBS and digested by 0.25% trypsin without EDTA (Thermo Fisher Scientific) for 3 minutes. After centrifuging, pel-

leted cells were obtained. Annexin V Binding Solution was then used to stain the resuspended cells with Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI). Cells were cultured in the dark for 15 minutes at room temperature and counted by flow cytometry (Coulter, USA).

Western blotting

Cultured cells were harvested for protein extraction. Total proteins were separated by SDS-PAGE and then transferred to a PVDF membrane. Specific proteins were detected using the following antibodies: anti-cleaved caspase3 (Cell Signaling Technology); anti-OPN and anti-tubulin (Sigma-Aldrich; St Louis, MO, USA). Bovine serum albumin (5%) in tris-based buffer Tween20 was used as blocking and washing solution.

RNA extraction and the quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted by Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol and quantified by using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific, Germany). cDNAs were produced from the mRNAs using Genestar ScriptRT (Genestar, China). qRT-PCR was performed in a Bio-Rad CFX-96 System (Bio-Rad, USA) using the SYBR Premix (Genestar). The specific forward (F) and reverse (R) primers used were:
eNOS: F: 5'-TGATGGCGAAGCGAGTGAAG-3',
R: 5'-ACTCATCCATACACAGGACCC-3';
OPN: F: 5'-GAAGTTTCGCAGACCTGACAT-3',
R: 5'-GTATGCACCATTCAACTCCTCG-3';
ICAM1: F: 5'-TGCCACCATCACTGTGTATTCG-3',
R: 5'-ATTCCCACGGAGCAGCACTACT-3';
Nrf2: F: 5'-CAGCACATCCAGACAGACACCA-3',
R: 5'-TATCCAGGGCAAGCGACTCAT-30;
β-actin: F: 5'-AGCCATGTACGTAGCCATCC-30,
R: 50-CTCTCAGCTGTGGTGGTGAA-3'.
Relative mRNA expression levels were normalized to the levels of the endogenous reference gene, β-actin.

Statistical analysis

All data are expressed as means ± standard error and were compared by ANOVA and Tukey post hoc test (using SPSS 19.0). Differences were considered significant at $p < 0.05$.

RESULTS

Evaluation of apoptosis in response to TGF-β1

To identify the relationship between TGF-β signaling and VEGF in blood vessels, we used TGF-β1 to stimulate human vascular endothelial cells (HVECs) and human aortic-endothelial cells (HAECs). Flow cytometry and western blot analyses showed that apoptosis levels increased with TGF-β1 treatment (Figure 1A). In addition, as an indicator of apoptosis, the level of cleaved caspase-3 was significantly increased in the TGF-β groups (Figure 1B). These findings demonstrated the relationship between apoptosis and TGF-β1 in HVECs and HAECs.

Expression of osteopontin (OPN) and related molecules in response to TGF-β1

To further analyze the effect of TGF-β signaling on HVECs and HAECs, we detected the changes in the mRNA and protein expression levels of OPN and other related inflammatory factors. Q-PCR analyses demonstrated that the mRNA expression level of OPN significantly increased *in vitro* with TGF-β1 stimulation. OPN protein levels were also significantly up-regulated in the 2 and 5 μg/mL. TGF-β1 groups compared with the control group. Similar increases were observed in the expression of ICAM1 (Figure 2). Interestingly, Nrf2 was significantly decreased by high TGF-β1 (Figure 2). These results suggested that OPN expression was induced by TGF-β signaling. This was in accordance with the changes of inflammation-related factors and appeared to be related to the TGF-β signaling pathway in HVECs and HAECs.

Elevated OPN is related to the eNOS in HVECs and HAECs

We investigated the factors affected by TGF-β signaling (eNOS, p-eNOS, and OPN). Both mRNA and protein levels of OPN were upregulated by TGF-β signaling, while the mRNA and protein levels of total eNOS remained unchanged in all treatment groups (Figure 3A and 3B). However, the inhibitory phosphorylation of endothelial nitric-oxide synthase (eNOS) (Thr495), an important marker of endothelial function, was upregulated by TGF-β1(Figure 3B and 3C). In contrast, the activating phosphorylation of eNOS (Ser1177) was downregulated (Figure 3B and 3C). Together, these findings demonstrate that TGF-β signaling can induce the expression of OPN, which may be a pivotal factor in the dysfunction of the vascular wall, accompanied by misregulation of phosphorylation of eNOS.

TGF-β1 promotes OPN secretion in VECs via Tgfbr2/Smad3 signaling pathway

Tgfbr2 is the most relevant in tumor development and progression among the three TGF-β receptors. We next investigated whether Tgfbr2 is involved in the process of OPN regulation in HVECs and HAECs treated with TGF-β1. In our study, the expression of OPN was significantly reduced by treatment with 10 ng/mL Tgfbr2 neutralizing antibody in HVECs and HAECs (Figure 4A). Furthermore, western blot showed that p-Smad3 was increased after TGF-β1 (Figure 4B and C). However, expression of OPN was significantly decreased as well when Smad3 was inhibited by SIS3, a specific Smad3 inhibitor (Figure 4D). These results suggested that TGF-β1 promotes OPN secretion in HVECs and HAECs via Tgfbr2/Smad3 signaling pathway.

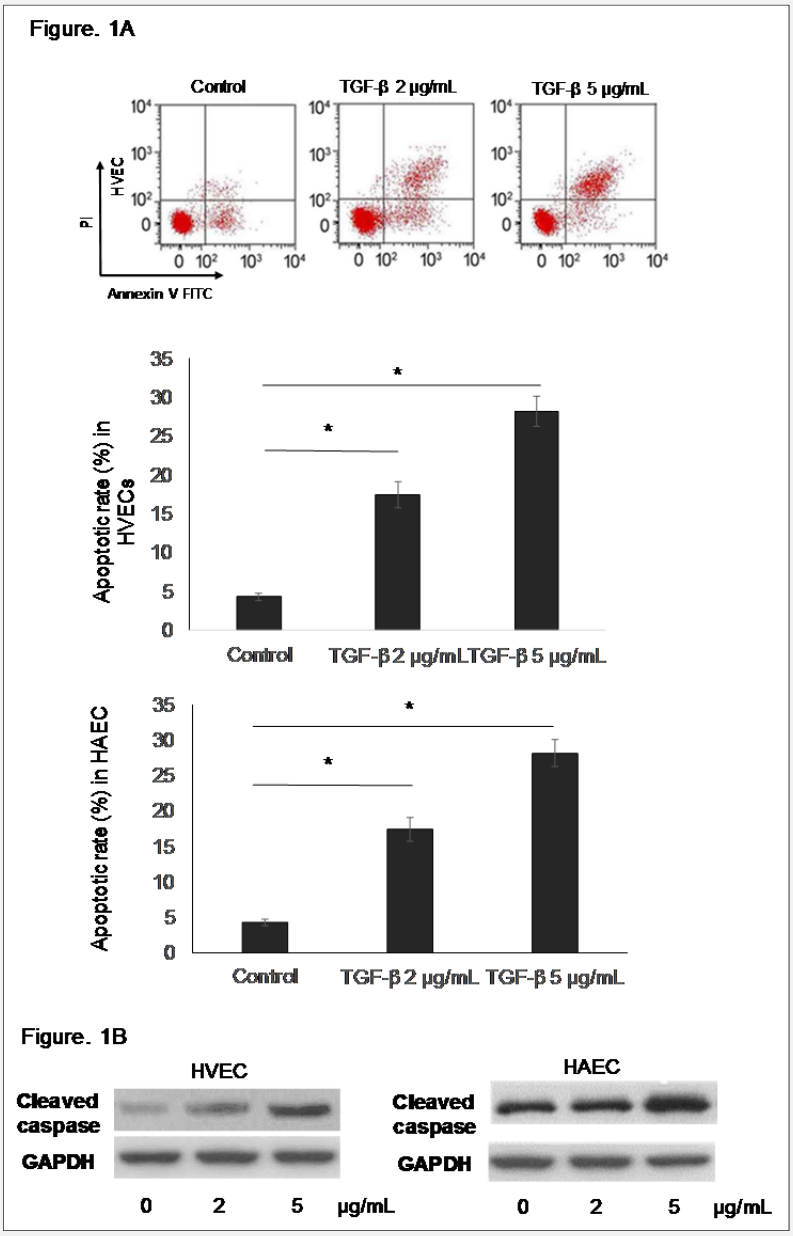

Figure 1. Evaluation of apoptosis in response to TGF-β1.

(A) Apoptosis was assessed in HVECs and HAECs using annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) flow cytometry. The percentage of apoptotic cells is shown in each histogram. (B) Cleaved caspase-3 was analyzed by western blot. *p < 0.05 vs. 0 mM.

DISCUSSION

Angiogenesis is an important biological process and pivotal in both physiological and pathological condition. Abnormal angiogenesis facilitates tumor invasion and aggressiveness [20,21]. In this study, TGF-β signaling induced apoptosis of HVECs and HAECs, and OPN expression was induced by TGF-β signaling. These results suggested that HVECs and HAECs are more susceptible to stimulation by various cytokines because these cells lie on the vessel intima.

It has been well reported that vascular calcification is an

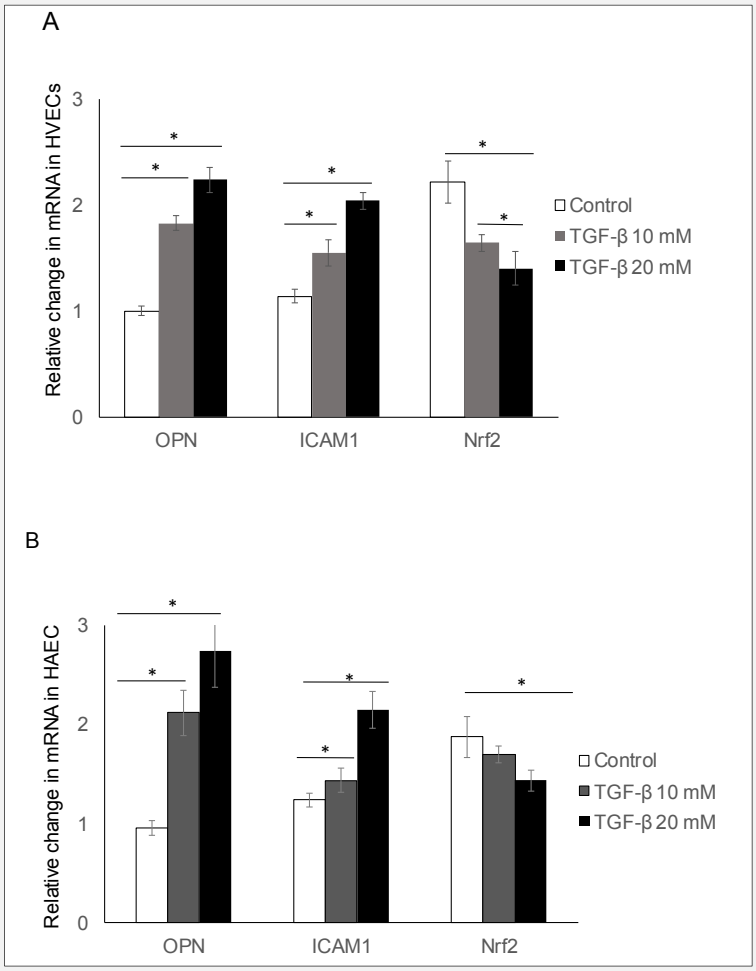

Figure 2. Expression of osteopontin (OPN) and related molecules in HVECs and HAECs exposed to TGF-β signaling.

Quantitative real-time polymerase chain reaction analyses of the gene transcription in HVECs (A) and HAECs (B). Cells were cultured in standard medium (control), or with 10 or 20 mM TGF-β1 for 12 hours * $p < 0.05$, $^{\#}$ $p > 0.05$.

important process during which vascular cells secrete numerous bone matrix proteins (collagen, biglycan) involved in the calcification events [6,7,15]. Notably, OPN expression is prominent in calcification regions of abnormal blood vessels such as degenerative and atherosclerotic vascular disease [17,19]. In this paper, we demonstrated that OPN increased with TGF-β signaling levels *in vitro*, which indicates that OPN is involved in the mineralization process and apoptosis induced by TGF-β signaling. Importantly, we also found that the mRNA and protein expression levels of OPN increased with the TGF-β treatment. Together, the results suggest that OPN is upregulated by TGF-β signaling and might have a close relationship with vascular calcification and apoptosis.

During the process of vascular calcification, inflammation of blood vessels also plays an essential role. It has been well known that OPN is a multifunctional factor that is involved in leukocyte recruitment, cell survival, and inflammation [20]. Here, there were synergetic effects of OPN on calcification-related proteins in VECs treated with various TGF-β signaling. ICAM1 is normally expressed at low level on cell surface, but increased in inflammatory diseases to mediate intercellular adhesion [21-23]. In this study, our data shows that the expression of ICAM1 is increased significantly by TGF-β signaling.

Nrf2 is a transcription factor which regulates the expression of many genes. In the promoters of those genes, there are antioxidant response elements [12]. Thus, Nrf2

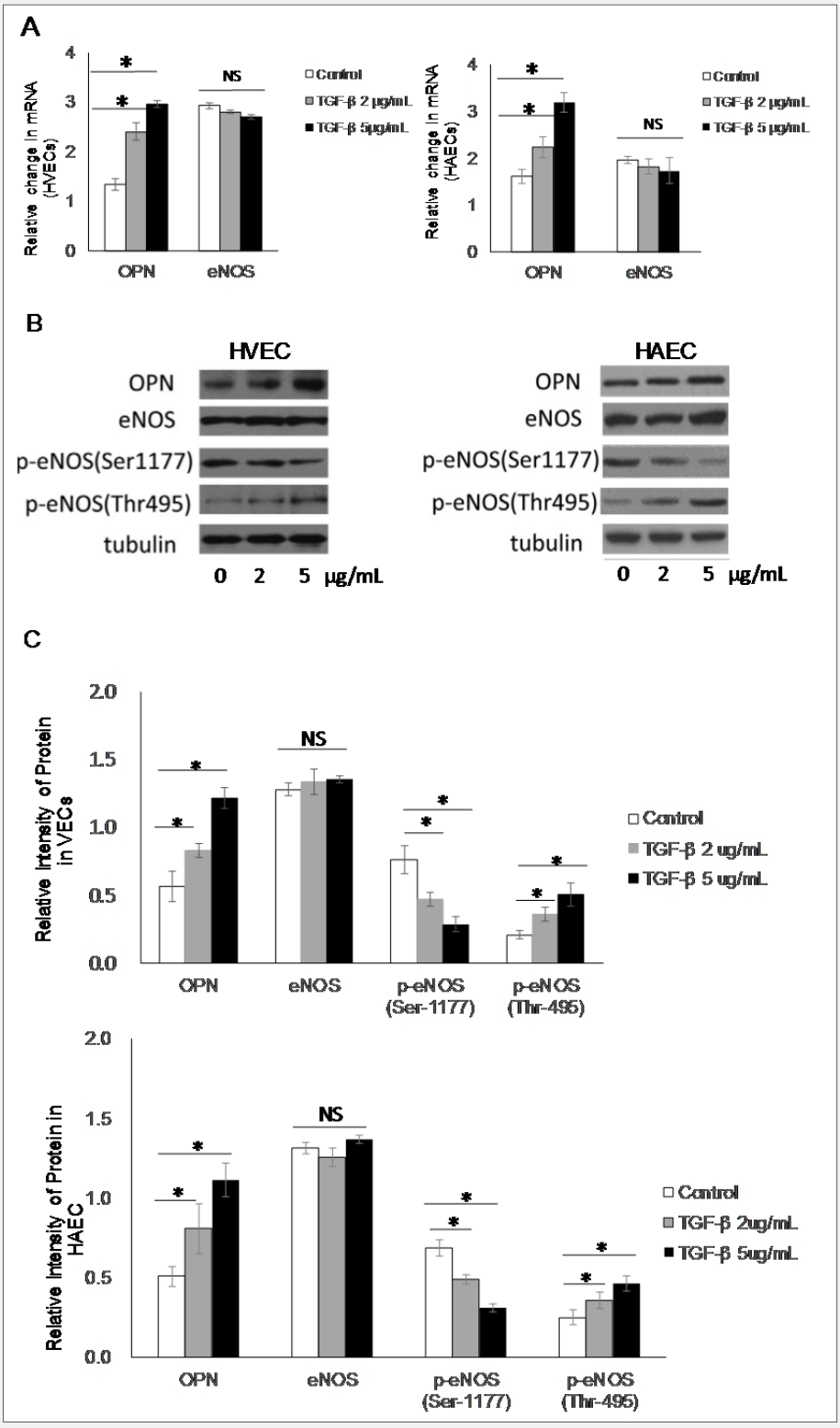

Figure 3. Elevated OPN is related to the eNOS in HVECs and HAECs.

(A) Quantitative real-time polymerase chain reaction analyses of the gene transcription in HVECs and HAECs. (B, C) Western blot analyses of protein translation in HVECs and HAECs. * $p < 0.05$, $^{\#}$ $p > 0.05$.

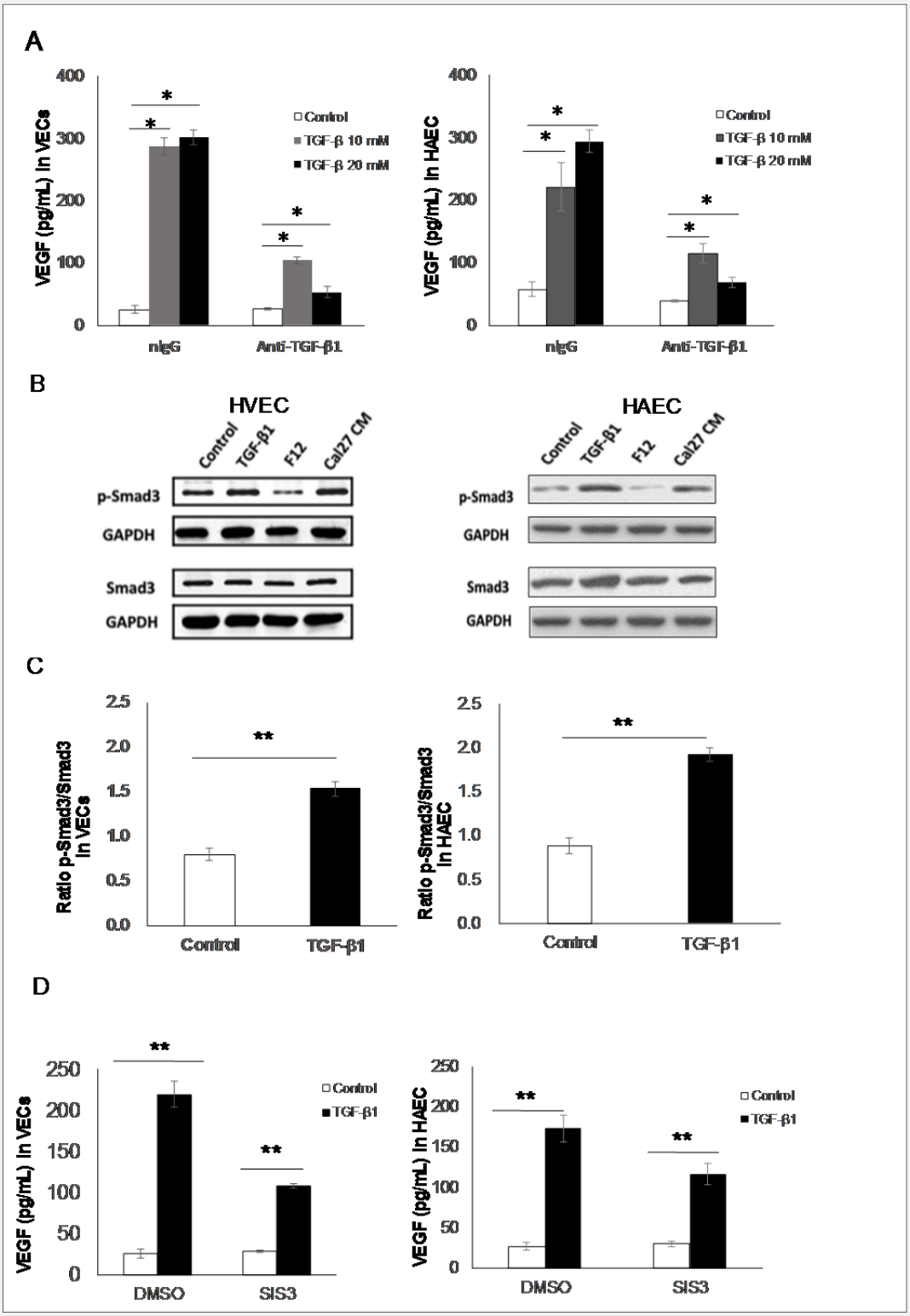

Figure 4. TGF-β1 promotes VEGF secretion in HVECs and HAECs via TbRII/Smad3 signaling pathway.

(A) TbRII neutralizing antibody reduced OPN expression, $p < 0.05$, compared to IgG, $p < 0.001$. Smad3 inducing OPN expression in HVECs and HAECs. (B, C) TGF-β1 could significantly improve Smad3 phosphorylation, compared to control, $p < 0.001$. (D) SIS3 could block the effect of TGF-β1 on promoting OPN expression, compared to DMSO, $p < 0.01$.

expression is regulated in response to external stimuli [13]. Our results showed that the expression of Nrf2 is also significantly downregulated by TGF-β signaling *in vitro* and *in vivo*.

Overall, the cooperation between the increased ICAM1 levels and decreased Nrf2 expression leads to an imbalance of physiological functions in blood vessels. Together with upregulated OPN, it could promote the calcification of vascular walls responsible for TGF-β signaling activation. eNOS catalyzes the production of nitric oxide, a characteristic molecule in VECs that maintains endothelial functions. eNOS activity is mainly regulated by posttranslational modifications such as phosphorylation. Two of the most common phosphorylation sites are Ser1177 (activation) and Thr495 (inhibition) [24]. Our results showed that TGF-β signaling does not affect the expression of eNOS, but regulates eNOS activity through altered phosphorylation at Ser1177 and Thr495. Therefore, cooperation between abnormal eNOS phosphorylation and excessive OPN expression disturbs normal endothelial function and causes a series of lesions.

Notably, TGF-β1 plays two opposite roles in the process of tumorigenesis and development: in the early stage of tumorigenesis, a small amount of TGF-β1 is expressed in tumor cells, and TGF-β1 can be a tumor suppressor; but with the progress of tumors, more tumor cells express TGF-beta 1; at this time, TGF-beta 1 becomes a substance to promote the growth of tumors. In the late stage of tumorigenesis, TGF-β1 directly acts on tumor cells and accelerates the process of tumors. Here, we reported that TGF-β1 promotes apoptosis in vascular cells, which mostly happens in the early stage of tumorigenesis.

CONCLUSION

This is the first time that OPN is reported as a crucial factor induced by TGF-β signaling in blood vessel endothelial cells. During this process, TGF-β signaling induces the expression of OPN *in vitro*, increases the expression of inflammatory factors and disturbs the function of eNOS in HVECs and HAECs. Thus, this dysfunction could be the beginning of various vascular diseases. Combined with the induction of apoptosis, the increased OPN plays a role in the mechanism of vascular formation, and suggests it may be a potential target for future therapy of blood vessel disease.

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Declaration of Interest:
The authors declare that they have no competing interests.

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