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TNF-α enhances TGF-β-induced endothelial-to-mesenchymal transition via TGF-β signal augmentation

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Abstract

The tumor microenvironment (TME) consists of various components including cancer cells, tumor vessels, cancer-associated fibroblasts (CAFs), and inflammatory cells. These components interact with each other via various cytokines, which often induce tumor progression. Thus, a greater understanding of TME networks is crucial for the development of novel cancer therapies. Many cancer types express high levels of TGF-β, which induces endothelial-to-mesenchymal transition (EndMT), leading to formation of CAFs. Although we previously reported that CAFs derived from EndMT promoted tumor formation, the molecular mechanisms underlying these interactions remain to be elucidated. Furthermore, tumor-infiltrating inflammatory cells secrete various cytokines, including TNF-α. However, the role of TNF-α in TGF-β-induced EndMT has not been fully elucidated. Therefore, this study examined the effect of TNF-α on TGF-β-induced EndMT in human endothelial cells (ECs). Various types of human ECs underwent EndMT in response to TGF-β and TNF-α, which was accompanied by increased and decreased expression of mesenchymal cell and EC markers, respectively. In addition, treatment of ECs with TGF-β and TNF-α exhibited sustained activation of Smad2/3 signals, which was presumably induced by elevated expression of TGF-β type I receptor, TGF-β2, activin A, and integrin αv, suggesting that TNF-α enhanced TGF-β-induced EndMT by augmenting TGF-β family signals. Furthermore, oral squamous cell carcinoma-derived cells underwent epithelial-to-mesenchymal transition (EMT) in response to humoral factors produced by TGF-β and TNF-α-cultured ECs. This EndMT-driven EMT was blocked by inhibiting the action of TGF-βs. Collectively, our findings suggest that TNF-α enhances TGF-β-dependent EndMT, which contributes to tumor progression.

Abbreviations: ActRII, activin type II receptor; ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CAF, cancer-associated fibroblast; CLDN5, Claudin 5; CM, conditioned medium; CSC, cancer stem cell; DMEM, Dulbecco's Modified Eagle's Medium; EC, endothelial cell; ECM, extracellular matrix; E-cadherin, epithelial-cadherin; EMT, epithelial-to-mesenchymal transition; EndMT, endothelial-to-mesenchymal transition; FGF, fibroblast growth factor; HUAEC, human umbilical artery endothelial cell; HUVEC, human umbilical vein endothelial cell; IL-1β, interleukin-1β; LAP, latency-associated peptide; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; OSCC, oral squamous cell carcinoma; qRT-PCR, quantitative RT-PCR; SM22α, smooth muscle protein 22α; TEC, tumor endothelial cell; TGF-β, transforming growth factor-β; TME, tumor microenvironment; TMEPAI, transmembrane prostate androgen-induced protein; TNF-α, tumor necrosis factor-α; TβRII, transforming growth factor-β type II receptor; αSMA, α-smooth muscle actin.

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KEYWORDS

activin, endothelial-to-mesenchymal transition, epithelial-to-mesenchymal transition, integrin αv, TGF-β, TGF-β type I receptor (ALK5), TNF-α

1 | INTRODUCTION

The tumor microenvironment (TME) is comprised of tumor cells surrounded by other components including tumor vessels, CAFs, inflammatory cells, and the extracellular matrix (ECM).[1] Interactions between TME components and malignant cancer cells, either through direct cell-cell contact or secretion of various cytokines, can regulate the characteristics of tumor tissues and affect spatiotemporal tumor progression and metastasis. Thus, a better understanding of the interaction between cancer cells and the TME is important for the development of novel therapeutic targets.

TME undergoes dynamic changes during tumorigenesis. For example, stromal cells differentiate into CAFs. However, recent lines of evidence have revealed that a subset of CAFs arises from ECs lining the tumor vessels, in a process called EndMT.[2-4] During EndMT, ECs change their characteristics and acquire a mesenchymal phenotype. They also lose their cell-cell contacts, decrease expression of endothelial cell-specific markers, eg vascular endothelial-cadherin (VE-cadherin), and increase expression of various mesenchymal cell markers, including smooth muscle protein 22α (SM22α).[2] We recently reported that transforming growth factor-β (TGF-β) induced the transition from tumor ECs (TECs) to α-smooth muscle actin (αSMA)-positive mesenchymal cells (myofibroblasts), which induced tumor formation more potently than αSMA-negative TEC-derived mesenchymal cells.[5] In addition to formation of CAFs during EndMT, loss of the endothelial barrier of the tumor vasculature can be observed, suggesting an important role of EndMT in facilitating metastatic dissemination. Thus, effective targeting of EndMT is likely to result in the development of novel anti-cancer therapies.[6]

There are various cytokines present within the TME, among which TGF-βs have been well studied. TGF-βs are mostly secreted by cancer cells and infiltrating inflammatory cells residing in the TME. As revealed by previous studies, TGF-β within the TME can induce the EMT of normal and transformed epithelial cells[7] as well as EndMT of ECs.[2] In mammals, TGF-β, belonging to the TGF-β family, is comprised of 33 members, including TGF-βs, bone morphogenetic proteins (BMPs), activins, Nodal, and others.[7] All members of the TGF-β family transduce their signals through 2 types of transmembrane receptors.[8] Ligand binding of activin, TGF-β, and Nodal to a constitutively active type II receptor kinase results in phosphorylation of the type I receptors, activin receptor-like kinases (ALK)-4, ALK-5, and ALK-7, respectively, which in turn activate downstream signal transduction cascades, including receptor-regulated Smad (R-Smad) pathways. Activated R-Smads can then form a complex with the common mediator Smad4 (co-Smad), stimulating nuclear translocation to interact with various transcription factors and regulating transcription of target genes. Various R-Smads participate in signaling cascades depending on the activating ligand, eg Smad2 and 3 transduce signals for TGF-βs and activins, while Smad1, 5, and 8 are responsible for BMP-dependent signaling.

There are 3 isotypes of TGF-β: TGF-β1, TGF-β2, and TGF-β3. They exhibit different activities and are differentially expressed in multiple cancer types. For example, glioma cells preferentially express the TGF-β2 isoform.[9,10] Of interest, our previous reports showed that TGF-β2 could induce EndMT of various types of ECs.[5,11-14] However, it remains to be elucidated how TGF-β2 expression is regulated in ECs.

TGF-β proteins are initially synthesized in the endoplasmic reticulum as precursor proteins containing growth factor and latency-associated peptide (LAP) and remain covalently linked, even after Golgi processing. Formation of this complex retains TGF-β in its inactive form. Moreover, this TGF-β complex is considered as a sensor when secreted into the ECM, since active TGF-β is only released from LAP during activation.[15] There are several mechanisms responsible for TGF-β activation. For example, integrins, especially integrin αvβ6 and integrin αvβ8, have been shown to play important roles in releasing active TGF-β through binding of RGD sequence of LAP to integrins.[16-19] In addition, other types of αv integrins, such as αvβ1, αvβ3, and αvβ5, have been suggested to interact with LAP and activate latent TGF-β.[20-22] RGD sequences are present in the LAPs of TGF-β1 and TGF-β3, but not in the LAP of TGF-β2; thus the mechanisms of activation of latent TGF-β2 remain to be elucidated.

The TGF-β-induced mesenchymal transition of endothelial and epithelial cells can be regulated by various factors. For example, our recent report showed endogenous fibroblast growth factor 2 (FGF2) to modulate TGF-β-dependent mesenchymal transition of mouse TECs.[5] Other reports have suggested an important role of inflammation in tumor progression. Inflammatory cells residing within the TME produce inflammatory cytokines to affect the EMT of cancer cells. Indeed, previous reports have shown that activated macrophages produce various proinflammatory cytokines, including tumor necrosis factor-α (TNF-α).[23] In addition, we have reported that TGF-β-induced EMT of A549 lung cancer cells can be augmented in the presence of TNF-α.[24] Specifically, A549 cells treated with both cytokines exhibited more profound loss of cell adhesive properties, while gaining migratory and

invasive abilities characteristic of mesenchymal cells. TNF-α can also directly affect ECs. Brett and colleagues have shown that TNF-α increased vascular leakage and permeability of endothelial monolayer.[25] Moreover, TNF-α activates both inhibitor of κB kinase/nuclear factor-κB (NF-κB) and mitogen-activated protein kinase/AP-1 pathways, which are essential for the expression of proinflammatory cytokines and the induction of many biological events occurring downstream of TNF-α, including decreased barrier function of the endothelium.[26]

TNF-α and another proinflammatory cytokine, interleukin-1β (IL-1β), have been reported to induce EndMT of various types of ECs.[27-29] However, the mechanisms underlying the role of TNF-α in enhancing TGF-β-induced EndMT are poorly understood. In this study, we examined the effect of TNF-α on TGF-β-induced EndMT using multiple types of human ECs. We observed a TGF-β-dependent increase in the expression of various mesenchymal cell markers, including SM22α. In addition, ECs treated with a combination of TGF-β and TNF-α showed higher expression of TGF-β type I receptor (ALK5), TGF-β2, activin A, and integrin αv compared with cells cultured in the presence of either cytokine, suggesting cooperation between TGF-β and TNF-α in augmenting TGF-β signals and enhancing EndMT. In addition, ECs that gained mesenchymal characteristics seemed to participate in the formation of a TGF-β2-rich microenvironment, which induced the EMT of oral cancer cells, suggesting a direct link between EndMT and EMT in TME.

2 | MATERIALS AND METHODS

2.1 | Cell culture and reagents

Human umbilical artery endothelial cell (HUVEC) were purchased from Cell Systems and maintained in the EBM ™-2MV Bullet Kit (cc-3202; Lonza). HUVECs were purchased from Lonza and maintained in the EBM ™ Bullet Kit (cc-3156; Lonza). HEK-Blue™ TGF-β cells (HEK293 cell-derived TGF-β responsive reporter cells) were purchased from InvivoGen and maintained in dulbecco's modified eagle's medium (DMEM) (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS; Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). HSC-4 cells were obtained from RIKEN Bioresource Center Cell Bank and maintained in DMEM supplemented with 10% FBS. TGF-β2 and TNF-α were purchased from Peprotech. IL-1β was purchased from FUJIFILM Wako Pure Chemical. Follistatin, anti-pan TGF-β neutralizing antibody (1D11) and control IgG were purchased from R&D Systems.

2.2 | RNA interference

The siRNAs for human RELA (silencer select siRNA ID #A: s11915, #B: s224527) and silencer negative control (Cat. AM4611) were purchased from Invitrogen (Carlsbad). All siRNAs were introduced into the HUAECs using Lipofectamine RNAi Max reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 2.8×10^4 cells were mixed with siRNA-Lipofectamine complexes and seeded into 6-well plates. Medium was refreshed 6 h later and cells were allowed to grow for an indicated period.

2.3 | RNA isolation and quantitative RT-PCR

Total RNA was prepared using the NucleoSpin RNA (TaKaRa Bio), according to the manufacturer's protocol, and reverse-transcribed by random priming using a PrimeScript II Kit (TaKaRa Bio). Quantitative RT-PCR (qRT-PCR) analysis was performed using the StepOne Plus Real-Time PCR System (Thermo Fisher Scientific). All expression data were normalized to β-actin expression. The primer sequences used in the present study are available in Table S1.

2.4 | Smad2/3-responsive reporter assay

Activation of Smad2/3 signals by TGF-β family members, including TGF-β1, TGF-β2, TGF-β3 and activin, was determined using a reporter assay that employed HEK-Blue™ TGF-β cells, allowing quantification of soluble alkaline phosphatase (SEAP) activity expressed under the control of Smad2/3/4-inducible elements. HUAECs were treated with TGF-β2 and TNF-α for 60 h. The culture medium was then replaced with DMEM with 1% FBS, followed by incubation for additional 16 h. The CM was then collected and added to the pre-seeded HEK-Blue™ TGF-β reporter cells, followed by incubation for 24 h. Fraction of the medium obtained from incubation with HEK-Blue cells was then mixed with QUANTI-Blue substrate (InvivoGen) and incubated for 30 min. The colorimetric change of the substrate by the released SEAP was quantified at 640 nm using a spectrophotometer.

2.5 | Immunocytochemistry

HUAECs were seeded on cover glasses pre-coated with Cellmatrix Type I-C (Nitta Gelatin) and cultured in the presence or absence of TGF-β2 and/or TNF-α for 72 h. The cells were then fixed with 4% paraformaldehyde, treated with 0.1% Triton X-100, blocked, and incubated with primary anti-SM22α (ab14106, abcam), followed by treatment with Alexa 594-conjugated secondary antibodies and Hoechst33342 for nuclear staining. Images were obtained using an All-in-One fluorescence microscope, BZ-X710 (KEYENCE).

HSC-4 cells were seeded on cover glasses and cultured with serum-free medium for 3 h. Then, the medium was changed to the CM of HUAECs cultured in the presence or absence of TGF-β2 and/or TNF-α for 72 h. Cells were then fixed with an ice-cold mixture of methanol-acetone (1:1) for 20 s, blocked with 1% BSA, and incubated with anti-epithelial-cadherin (E-cadherin) (1:200 dilution, 3195; Cell Signaling Technology) and anti-Vimentin (1:400 dilution,

ab92547; abcam) antibodies, followed by treatment with anti-mouse IgG (H+L) Alexa 488-conjugated (A-11001; Thermo Fisher Scientific) and anti-rabbit IgG (H + L) Alexa 594-conjugated (A-21207; Thermo Fisher Scientific) secondary antibodies and Hoechst33342 for nuclear staining. Images were obtained using a confocal microscope, TCS SP8 (Leica Microsystems GmbH).

2.6 | Tube formation assay

A 12-well plate was pre-coated with Matrigel (BD Biosciences) (0.4 mL/well). After polymerization of Matrigel at 37°C for 1 h, HUAECs were seeded to each well at a density of 1.7×10^5 cells/400 μL/well. After 8 h of incubation in EGM-2MV medium supplemented with 5% FBS, tube-like structures were photographed under phase-contrast microscopy (BZ-X710, KEYENCE) using a ×10 objective lens. Tube length was quantified using ImageJ software (US National Institutes of Health).

2.7 | Statistical analyses

Values are presented as mean ± standard deviation or standard error. Significant differences between means were determined using two-tailed unpaired Student *t* test or one-way ANOVA followed by the Student-Newman-Keuls test. Differences between means were considered statistically significant at $^*P < .05$; n.s., not significant.

3 | RESULTS

3.1 | TNF-α augments TGF-β signals in human ECs

To examine whether TGF-β and TNF-α could transduce signals in HUAECs, we analyzed the effect of both cytokines on the expression level of target genes related to each pathway. Treatment of HUAECs with TGF-β2 for 4 h significantly induced the expression of transmembrane prostate androgen-induced protein (TMEPAI), a faithful target of Smad2/3 signals (Figure 1A), while TNF-α alone slightly suppressed TMEPAI expression, which was further suppressed by combined treatment of TGF-β2 and TNF-α. In contrast, the expression of ICAM1, a direct target gene of TNF-α signals, was significantly increased by treatment with TNF-α but not by TGF-β2 (Figure 1B). TGF-β2 had no effect on the TNF-α-induced ICAM1 expression at 4 h.

Subsequently, we examined whether the expression of TMEPAI and ICAM1 is maintained in HUAECs that are treated with TGF-β and TNF-α for longer period. Increased expression of TMEPAI was observed in HUAECs treated with TGF-β2 for 72 h (Figure 1C). Furthermore, these changes were also observed with TNF-α alone (Figure 1C) to a lesser extent. Interestingly, a combination of TGF-β2 and TNF-α for 72 h induced an even higher level of TMEPAI expression (approximately 3-fold) than TGF-β2 alone (Figure 1C). Furthermore, following a 72-h incubation with TNF-α, HUAECs increased ICAM1 expression (Figure 1D). However, combined treatment with TGF-β2 and TNF-α for 72 h suppressed ICAM1 expression, compared with TNF-α alone (Figure 1D). These results suggested

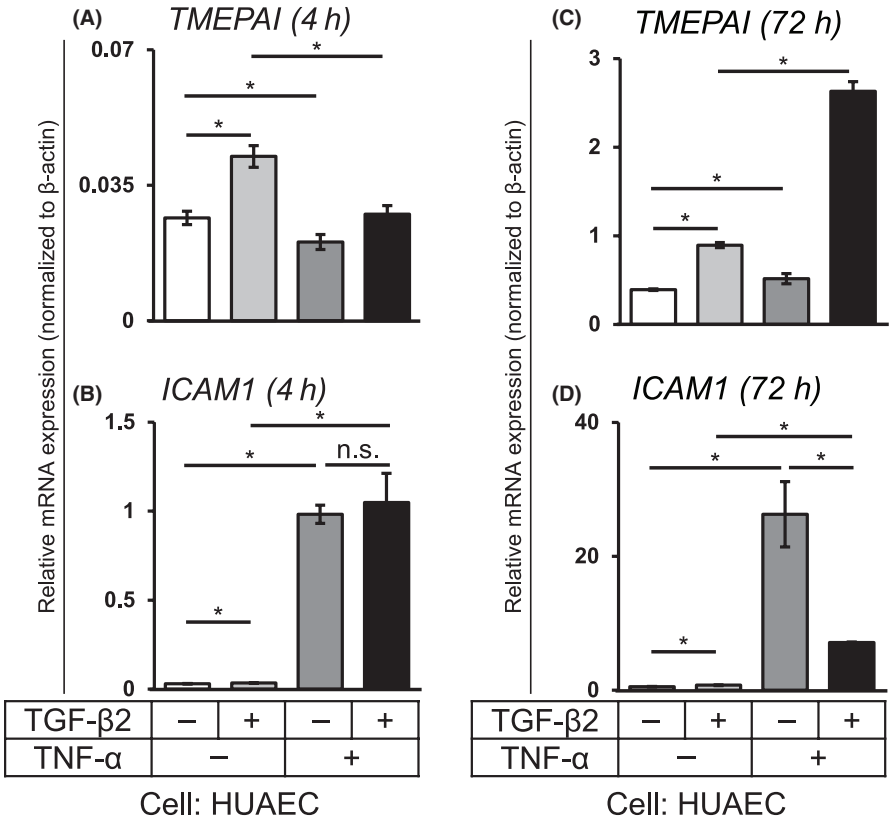


FIGURE 1 Effects of TGF-β2 and TNF-α on the expression of TGF-β2 and TNF-α target genes in HUAECs. HUAECs were cultured in the absence (–) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α for 4 h (A, B) or 72 h (C, D), followed by qRT-PCR analyses for *TMEPAI* (A, C) and *ICAM1* (B, D) expression. Error bars represent standard deviation. $^*P < 0.05$

that both TGF-β and TNF-α could transduce their signals in HUAECs and that HUAECs treated with both cytokines may acquire the characteristics needed to more potently transduce certain TGF-β signals.

3.2 | TNF-α enhances TGF-β-induced expression of mesenchymal cell markers in various types of ECs

We have previously reported that TGF-β2 could induce EndMT in multiple types of mouse ECs, including embryonic stem cell-derived ECs,[12] MS1 ECs,[13] and TECs.[5] Therefore, to examine whether TGF-β2 would elicit the same effect in human ECs, we analyzed the expression of various mesenchymal and EC markers in HUAECs treated with TGF-β2 for 72 h (Figure 2). We observed increased expression of mesenchymal cell markers, SM22α (Figure 2A) and matrix metalloproteinase 2 (MMP2) (Figure 2B), which was accompanied by decreased expression of Claudin 5 (CLDN5), an EC marker (Figure 2C), suggesting that HUAECs underwent EndMT. Further, we examined the effect of TNF-α on the TGF-β-induced EndMT (Figure 2). While treatment of HUAECs with TNF-α alone did not alter expression of mesenchymal or EC markers, TNF-α significantly enhanced TGF-β2-induced expression of SM22α (Figure 2A), MMP2 (Figure 2B) and CLDN5 (Figure 2C). Similar results were obtained using HUVECs in which TNF-α significantly enhanced TGF-β2-induced expression of SM22α, but did not significantly enhance TGF-β2-induced MMP2 expression and TGF-β2-suppressed CLDN5 expression (Figure 2D-F).

The effect of TNF-α on TGF-β2-induced expression of SM22α was also confirmed at the protein level (Figures 3 and S1). We observed changes in cell morphology and strong staining with anti-SM22α antibodies in TGF-β2-treated HUAECs (Figures 3A and S1, upper right panel). In addition, the number of SM22α-positive cells increased when cells were treated with a combination of TGF-β2 and TNF-α (Figure 3B), suggesting that TNF-α could enhance TGF-β-induced expression of mesenchymal cell markers in various types of ECs.

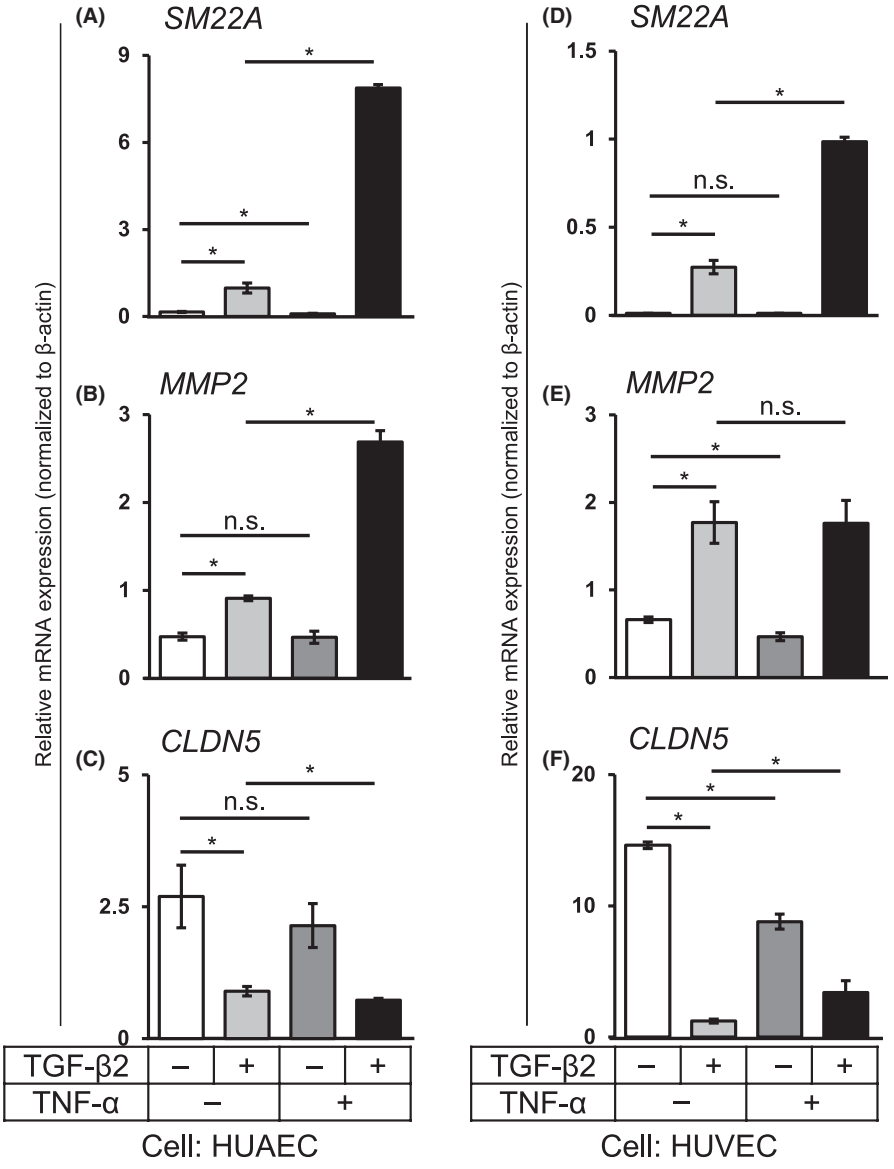


FIGURE 2 Effects of TGF-β2 and TNF-α on endothelial and mesenchymal characteristics of multiple types of endothelial cells. HUAECs (A-C) and HUVECs (D-F) were cultured in the absence (−) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α (HUAECs) and 15 ng/mL TNF-α (HUVECs), respectively, for 72 h, followed by qRT-PCR analyses for *SM22α* (A, D), *MMP2* (B, E), and Claudin 5 (*CLDN5*) (C, F) expression. Error bars represent standard deviation. **P* <0.05; n.s., not significant

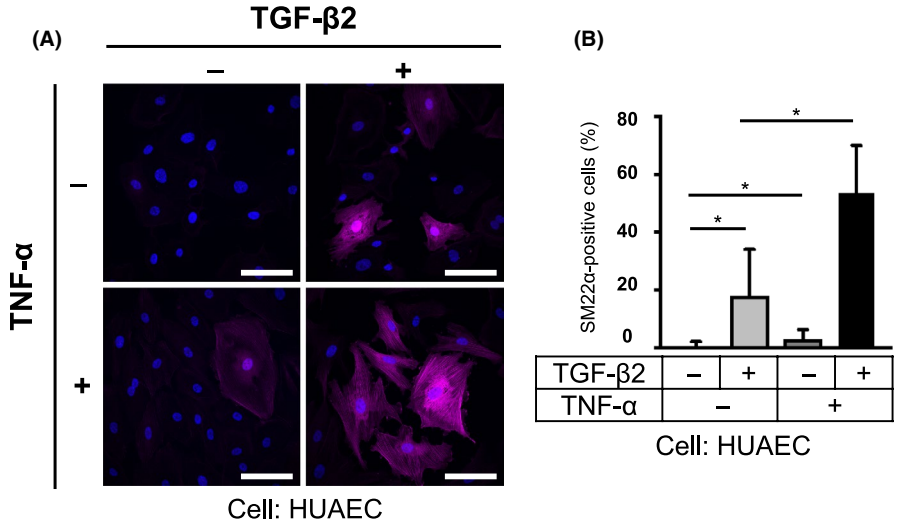


FIGURE 3 Effects of TGF-β2 and TNF-α on mesenchymal characteristics of HUAECs. HUAECs were cultured in the absence (–) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α for 72 h, followed by fluorescence immunostaining for SM22α (magenta) and nuclei (blue) (A). Scale bar, 100 μm. (B) Numbers of SM22α-positive cells were counted in 4 fields. Data are shown as the mean ± standard deviation of 4 independent experiments. *P <0.0001.

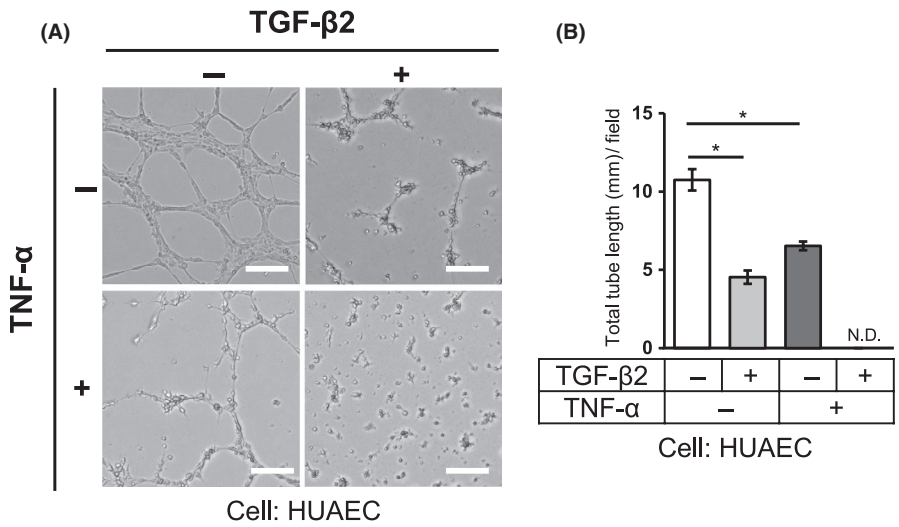


FIGURE 4 Effects of TGF-β2 and TNF-α on tube forming ability of HUAECs. HUAECs were cultured in the absence (–) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α for 72 h, followed by tube formation assay. Cells were allowed to form tube-like structures on a collagen gel for 8 h, followed by phase-contrast imaging (A) and quantification (B) of tube-like structures. Scale bars, 200 μm. Error bars represent standard deviation. *P <0.05; ND, not detected

Decreased expression of CLDN5, an EC marker, during EndMT (Figure 2C) prompted us to examine whether HUAECs lose their endothelial functions upon the treatment with TGF-β2 and TNF-α by performing tube formation assay. As shown in Figure 4A, HUAECs exhibited tube forming ability, which is characteristic of ECs. This tube forming ability was significantly decreased by treatment of HUAECs with TGF-β2 or TNF-α alone and completely abolished by the combined treatment with TGF-β2 and TNF-α (Figure 4B). Taking together the results of gene expression and tube formation assays, we concluded that HUAECs lost their endothelial phenotypes by treatment with TGF-β2 and TNF-α.

3.3 | TNF-α cooperates with TGF-β2 to induce ALK5 expression in various types of ECs

In this study, we found that TNF-α cooperates with TGF-β2 to induce expression of TMEPAI (Figure 1C) and various mesenchymal cell markers (Figures 2, 3 and S1) in HUAECs and HUVECs. To elucidate the molecular mechanisms underlying the stimulatory effect of TNF-α on TGF-β2-induced EndMT, expression of various components of the Smad2/3 signaling pathway, which regulates EndMT, was examined at 72 h after ligand stimulation. Smad2/3 signals are activated by the TGF-β/TGF-β type II receptor (TβRII)/ALK5 axis and activin/activin type II receptor (ActRIIs)/ALK4/7 and Nodal/ActRIIs/ALK7 axes. Among them, we found that the expression of ALK5, a TGF-β type I receptor, was induced by TNF-α, and further increased by TNF-α and TGF-β2 (Figure 5A). Similar results were obtained using HUVECs, which showed significant increases in ALK5 expression in the presence of TNF-α and TGF-β2 with no effect on other components of the TGF-β signaling pathway (Figure S2A). These results suggested that TNF-α enhanced TGF-β2-induced EndMT via activation of TGF-β signals through increased ALK5 expression, resulting in enhanced TMEPAI expression (Figure 1C).

3.4 | TGF-β signals increase endogenous expression of TGF-β2 and activin A, which is enhanced by TNF-α in various types of ECs

We next analyzed changes in expression of various TGF-β family ligands upon stimulation with TGF-β2, TNF-α, or both cytokines

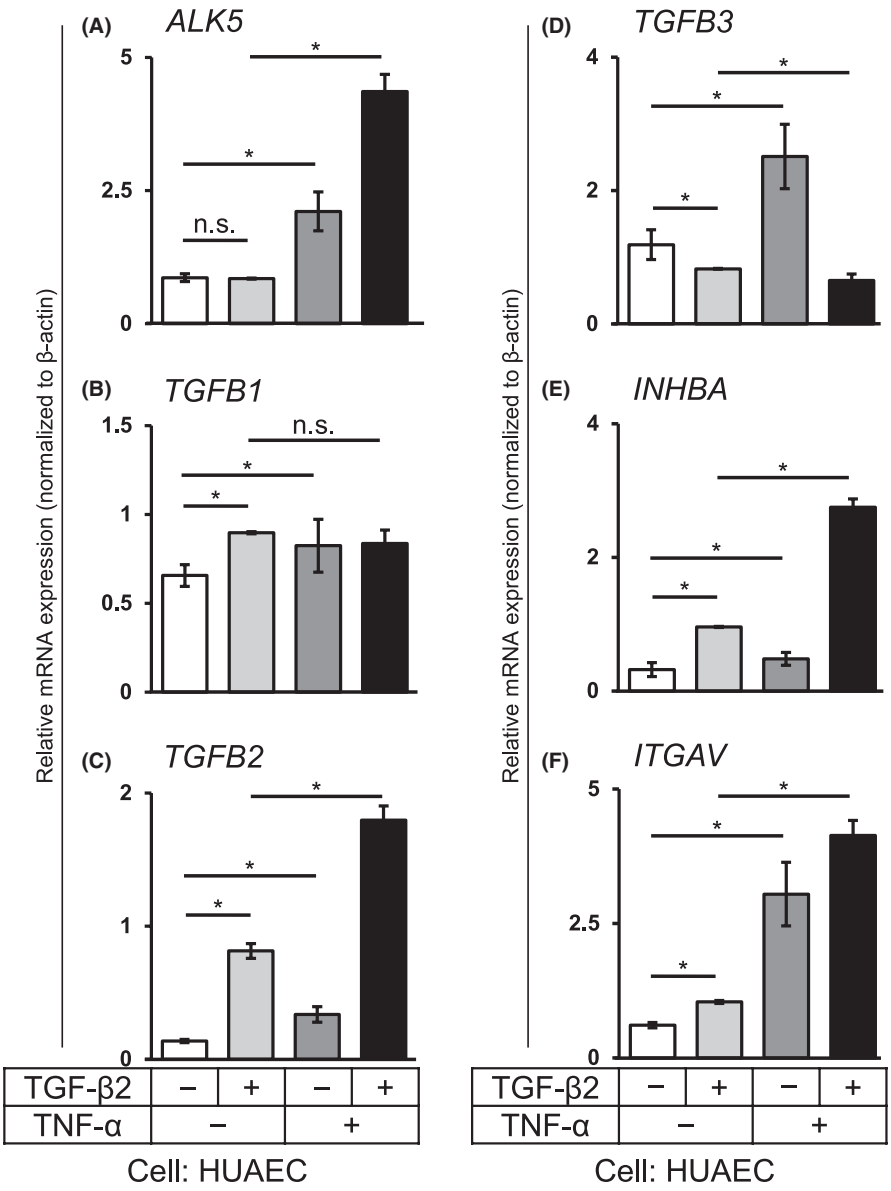


FIGURE 5 Effects of TGF-β2 and TNF-α on expression of various TGF-β signaling components in HUAECs. HUAECs were cultured in the absence (−) or presence (+) of 1 ng/mL TGF-β2, 10 ng/mL TNF-α, or a combination of both cytokines for 72 h, followed by qRT-PCR analyses for expression of *ALK5* (A), TGF-β1 (*TGFB1*) (B), TGF-β2 (*TGFB2*) (C), TGF-β3 (*TGFB3*) (D), inhibin βA (*INHBA*) (E), and integrin αv (*ITGAV*) (F). Error bars represent standard deviation. *P <0.05; n.s., not significant

(Figure 5B-E). Combined stimulation with TGF-β2 and TNF-α failed to increase expression of TGF-β1 (Figure 5B), and TGF-β3 (Figure 5D) in HUAECs. However, addition of exogenous TGF-β2 and TNF-α significantly increased expression of TGF-β2 (Figure 5C) and inhibin βA, the subunit of activin A (Figure 5E), in a similar manner to TMEPAI (Figure 1C). Furthermore, in HUVECs, very significant increase in TGF-β2 and inhibin βA transcripts was observed only upon combined treatment with TNF-α and TGF-β2 (Figure S2B-E), suggesting that TGF-β2-treated ECs secreted TGF-β2 and activin A. These changes in endogenous expression of TGF-β2 and activin A suggest that they stimulate TMEPAI expression to further enhance EndMT.

3.5 | TGF-β and TNF-α cooperate to increase integrin αv expression in various types of ECs

Various integrins have been implicated in the activation of latent forms of TGF-β1 and TGF-β3. Therefore, we examined the putative involvement of integrins in EndMT of HUAECs, induced by the combined treatment of TGF-β2 and TNF-α. For this purpose, we studied the effect of TNF-α and/or TGF-β2 on the expression of various integrin family members in HUAECs cultured for 72 h in the presence of TGF-β2, TNF-α, or a combination of both cytokines. As shown in Figures 5F and S2F, we found that integrin αv expression increased upon combined treatment with both cytokines in HUAECs and HUVECs, respectively. These results suggested that TGF-β2 and TNF-α cooperate to induce integrin αv expression, which may lead to the activation of latent TGF-β1 and TGF-β3 proteins.

3.6 | NF-κB-mediated inflammatory signals enhance TGF-β-induced EndMT

As TNF-α transmits its signals via NF-κB activation in ECs,[26] we next examined the role of NF-κB in enhancement of TGF-β2-induced EndMT by TNF-α. To examine whether the NF-κB-mediated pathway

is involved in the EndMT, we used siRNAs specific for RelA/p65, an essential signaling component of NF-κB in the TNF-α signal. Introduction of RelA siRNAs into HUAECs resulted in more than a 60% decrease in RelA expression (Figure 6A) and decreased TNF-α-induced expression of its target gene, ICAM1 (Figure 6B). Increased expression of TMEPAI by the combined treatment with TGF-β2 and TNF-α for 72 h was also suppressed by RelA knockdown (Figure 6C), suggesting that activation of TGF-β signals by TGF-β2 and TNF-α required NF-κB signals in HUAECs. Furthermore, RelA knockdown significantly suppressed the increased expression of SM22α (Figure 6D), ALK5 (Figure 6E), TGF-β2 (Figure 6F), inhibin βA (Figure 6G), and integrin αv (Figure 6H) in the HUAECs treated by the combination of TGF-β2 and TNF-α, suggesting that NF-κB is involved mechanistically in the EndMT induced by TGF-β2 and TNF-α. It is of note that the increase in the expression of SM22α and inhibin βA is largely dependent on RelA expression, while other increases in expression of others only partly depended on it.

NF-κB mediates signals mediated not only by TNF-α but also by other inflammatory cytokines including IL-1β, which is known to be involved in EndMT.[28,29] This situation further prompted us to examine the effects of simultaneous stimulation of HUAECs with TGF-β2 and IL-1β. Expression of ICAM1 was induced and sustained by IL-1β for 72 h (Figure 7A). IL-1β-induced ICAM1 expression was not suppressed by TGF-β2, indicating that IL-1β signal is differently regulated by TGF-β signal from TNF-α signal. Conversely, TGF-β2-induced expression of TMEPAI was increased by IL-1β in a similar manner as TNF-α (Figure 7B). Expression of mesenchymal cell markers, SM22α and MMP2, was also regulated by the combined treatment with TGF-β2 and IL-1β in a similar

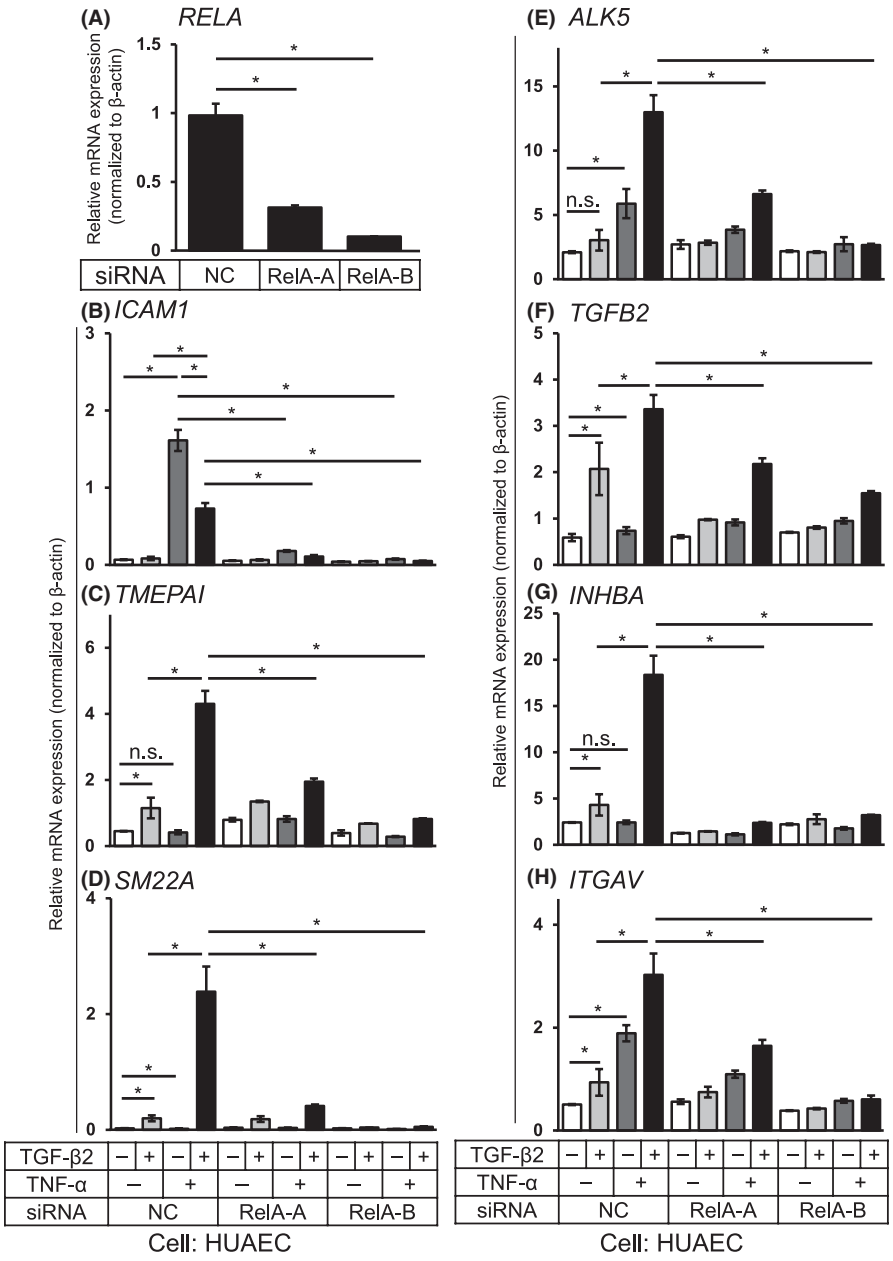


FIGURE 6 Effects of RelA on TGF-β2 and TNF-α-induced expression of various markers in HUAECs. HUAECs transfected with negative control siRNA (NC) or siRNAs for RelA (RelA-A and RelA-B) were cultured in the absence (–) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α for 72 h, followed by qRT-PCR analyses for expression of *RELA* (A), *ICAM1* (B), *TMEPAI* (C), *SM22α* (D), *ALK5* (E), TGF-β2 (*TGFB2*) (F), inhibin βA (*INHBA*) (G), and integrin αv (*ITGAV*) (H). Error bars represent standard deviation. **P* <0.05; n.s., not significant

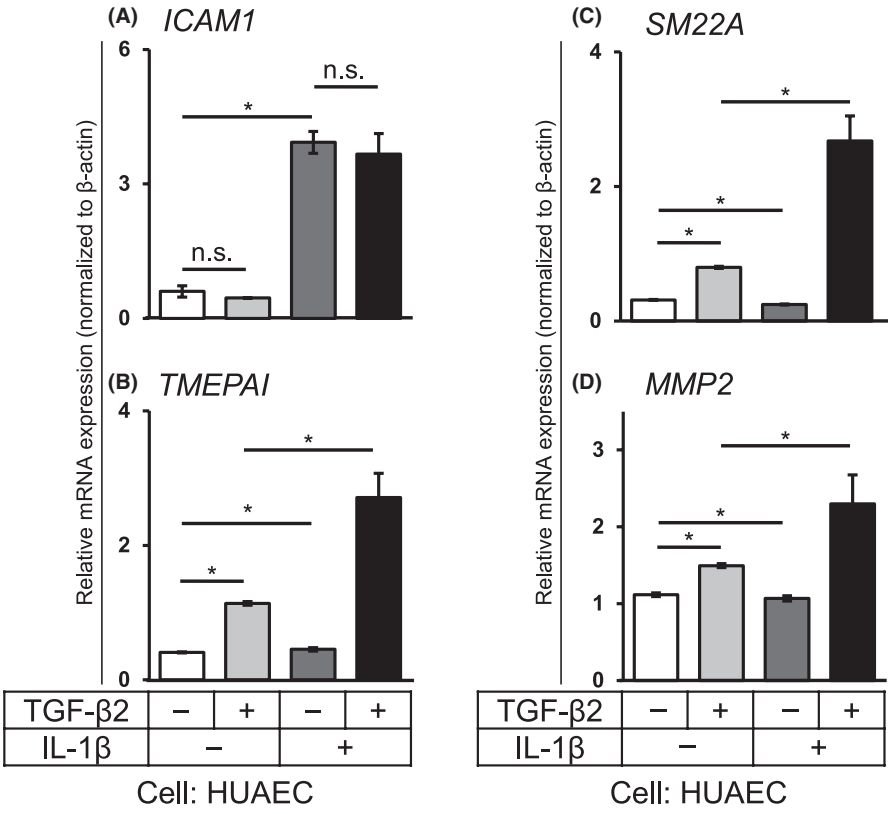


FIGURE 7 Effects of TGF-β2 and IL-1β on their target genes and mesenchymal cell markers in HUAECs. HUAECs were cultured in the absence (−) or presence (+) of 1 ng/mL TGF-β2 in combination with 3 ng/mL IL-1β for 72 h, followed by qRT-PCR analyses for expression of *ICAM1* (A), *TMEPAI* (B), *SM22α* (C), and *MMP2* (D). Error bars represent standard deviation. **P* <0.05; n.s., not significant

manner as that for TNF-α (Figure 7C,D). Taken together, IL-1β had a similar effect as TNF-α on TGF-β-induced EndMT.

3.7 | TGF-β and TNF-α cooperate to increase production of humoral factor activating Smad2/3 signal

We hypothesized that during EndMT induced by TGF-β2 and TNF-α, HUAECs produced "humoral factor(s) activating Smad2/3 signal" (Smad2/3 activating factor(s)). To test this hypothesis, a TGF-β signal-responsive HEK-Blue reporter cell system was used to monitor TGF-β-induced Smad signaling.[30] HUAECs were cultured in the presence of TGF-β2, TNF-α, or a combination of both cytokines for 60 h to allow accumulation of the putative Smad2/3 activating factor(s). The HEK-Blue reporter cells were then grown in HUAEC CM for 24 h, and activity of the "Smad2/3 activating humoral factors" was examined (Figure 8A). The CM derived from HUAECs cultured in the presence of TGF-β2 could activate TGF-β signals in HEK-Blue reporter cells as compared with the untreated control (Figure 8B). Interestingly, this effect was enhanced when CM derived from HUAECs treated with TGF-β2 and TNF-α was used (Figure 8B). Our data suggested that the presence of exogenous TGF-β2 and TNF-α induced EndMT in HUAECs by activating the Smad pathway and increasing secretion of "Smad2/3 activating humoral factors."

We further examined the physiological roles of TGF-β2 (Figure 5C) and activin A (Figure 5E) produced by HUAECs in the activation of Smad2/3 signals by using HEK-Blue reporter cells. When the CM from HUAECs treated with TGF-β2 and TNF-α was added to HEK-Blue cells in combination with follistatin, an inhibitor of activin (Figure S3A), the increased activation of Smad2/3 signals seen upon incubation with the CM was decreased significantly, suggesting that endogenous activin A was responsible for activation of Smad2/3 signals (Figure 8C). However, anti-TGF-β neutralizing antibody (1D11) (Figure S3B) failed to abrogate the activation of Smad2/3 signals by the CM from HUAECs treated with TGF-β2 and TNF-α (Figure 8D). These results suggested that EndMT induced by TGF-β2 and TNF-α increased the production of endogenous activin A from HUAECs, which led to the sustained activation of Smad2/3 signals.

3.8 | Oral squamous cell carcinoma-derived cells undergo EMT in response to humoral factors produced by TGF-β and TNF-α-treated ECs

In the TME, cancer cells are affected by various cytokines produced by stromal cells. TGF-β signals are well known inducers of the mesenchymal transition of OSCCs.[31] We previously reported that TGF-β and TNF-α cooperate to induce EMT of lung cancer cells.[24] Furthermore, activin signals have been shown to induce the EMT of OSCC lines.[32] The OSCC line HSC-4 was used in our study to examine whether cytokines, including TGF-β2, activin, and TNF-α, produced by mesenchymal cells originating from ECs could affect cancer cells. Treatment of HSC-4 cells with

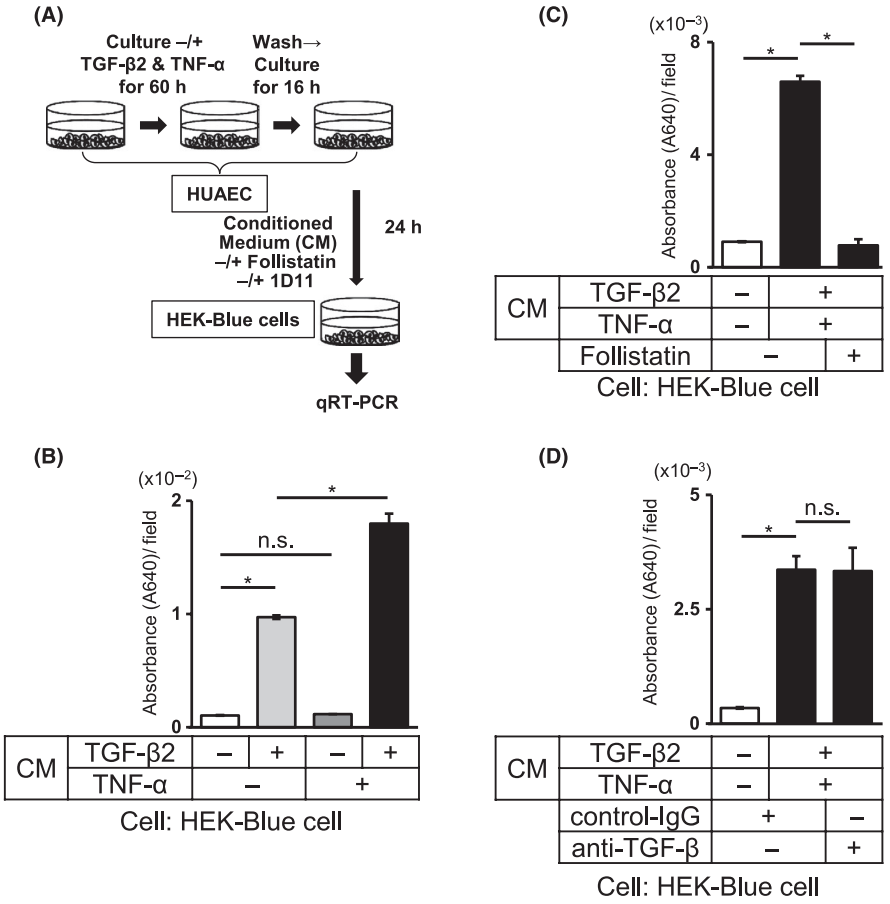
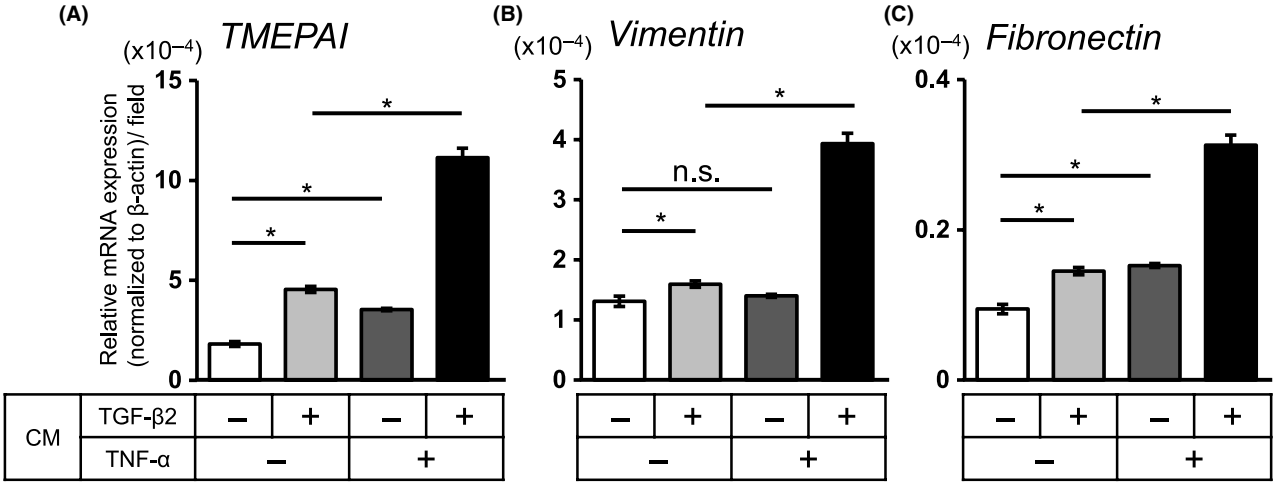


FIGURE 8 Quantification of Smad2/3-activating factors in conditioned medium (CM) of HUAECs using HEK-Blue TGF-β reporter cells. (A) HUAECs were cultured in the absence (–) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α for 60 h, followed by DMEM with 1% FBS, replacement and further culture for 16 h. (B) HEK-Blue TGF-β cells were cultured in the CM derived from HUAECs cultured for 16 h, followed by the measurement of absorbance at 640 nm, which represented colorimetric change of HEK-Blue substrate by secreted alkaline phosphatase induced by Smad2/3 signal. (C, D) HEK-Blue TGF-β cells were cultured in the CM derived from HUAECs, in the absence (–) or presence (+) of 50 ng/mL of follistatin (C), control IgG (50 μg/mL) and anti-TGF-β (1D11: 50 μg/mL) (D), followed by the measurement of the absorbance at 640 nm representing colorimetric change of HEK-Blue substrate by SEAP alkaline phosphatase induced by Smad2/3 signals. Values were normalized to the number of HUAECs responsible for secretion of Smad2/3-activating humoral factors. Error bars represent standard deviation. *P <0.05; n.s., not significant

TGF-β1 increased expression of TMEPAI, a target of TGF-β signals (Figure S4A), vimentin (Figure S4B,D), and fibronectin (Figure S4C), markers of mesenchymal cells, and decreased E-cadherin expression (Figure S4D), suggesting that TGF-β signals induced EMT of HSC-4 cells. HUAECs were cultured in the presence of TGF-β2, TNF-α, or a combination of both cytokines for 60 h. HSC-4 cells were then grown in HUAEC CM for 72 h, followed by qRT-PCR analysis for expression of TMEPAI and various mesenchymal cell markers (Figure 9). Consistent with results using HEK-Blue cells (Figure 8B), TMEPAI expression in HSC-4 cells was induced by the CM derived from TGF-β2-treated HUAECs (Figure 9A). This effect was further increased by the CM obtained from TGF-β2 and TNF-α (Figure 9A). Furthermore, we found increased expression of vimentin (Figure 9B) and fibronectin (Figure 9C) when HSC-4 cells were cultured in the CM derived from TGF-β2-treated HUAECs. Moreover, significantly increased levels of both transcripts were observed in HSC-4 cells exposed to the CM obtained from TGF-β2 and TNF-α-treated HUAECs (Figure 9B,C).

To examine whether TGF-β2 (Figure 5C) and activin A (Figure 5E) produced by HUAECs play a role in induction of EMT

FIGURE 9 Effects of HUAEC conditioned medium (CM) on the expression of TMEPAI and mesenchymal cell markers in HSC-4 oral cancer cells. (A-C) HUAECs were cultured in the absence (–) or presence (+) of 1 ng/mL TGF-β2 in combination with 10 ng/mL TNF-α for 60 h, followed by replacement with DMEM supplemented with 1% FBS and further culture for 16 h. HSC-4 cells were cultured in the CM derived from HUAECs for 72 h, followed by qRT-PCR analyses for expression of *TMEPAI* (A), vimentin (B), and fibronectin (C). (D-F) HSC-4 cells were cultured in the CM derived from HUAECs, in the absence (–) or presence (+) of 50 ng/mL follistatin, control IgG (50 μg/mL), and anti-TGF-β neutralizing antibody (1D11: 50 μg/mL) for 72 h, followed by qRT-PCR analysis for expression of vimentin (D, E) and fluorescence immunostaining for E-cadherin (green), vimentin (red) and nuclei (blue) (F). Error bars represent standard deviation. *P <0.05; n.s., not significant. Scale bar, 25 μm


(A)
TMEPAI
(x10−4)
Relative mRNA expression (normalized to β-actin)/ field
15
10
5
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(B)
Vimentin
(x10−4)
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4
3
2
1
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n.s.
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(C)
Fibronectin
(x10−4)
0.4
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CM
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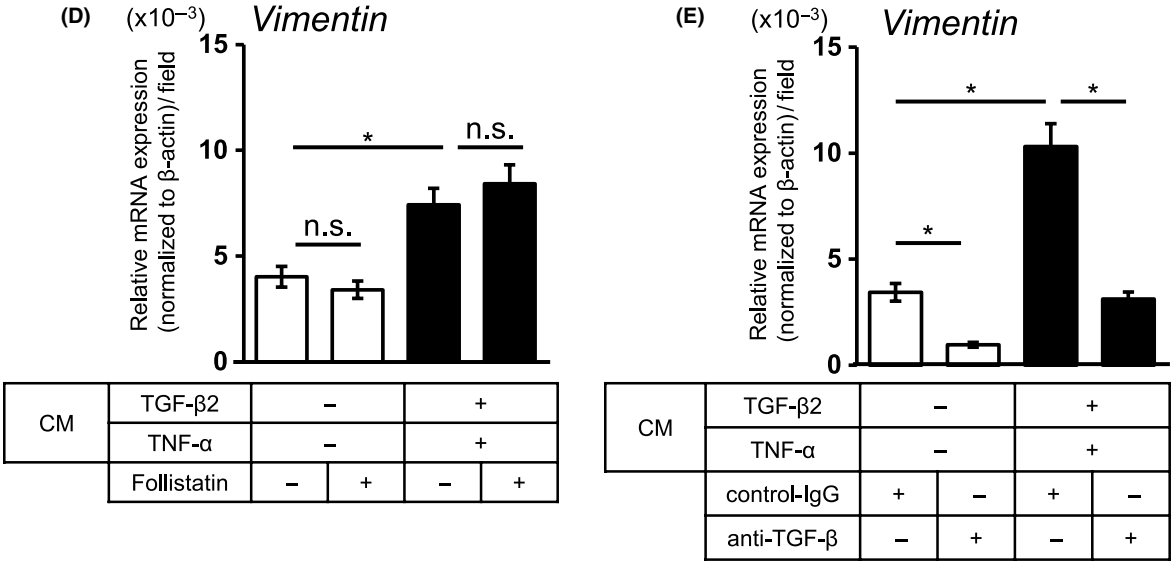
(D)
Vimentin
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Relative mRNA expression (normalized to β-actin)/ field
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Relative mRNA expression (normalized to β-actin)/ field
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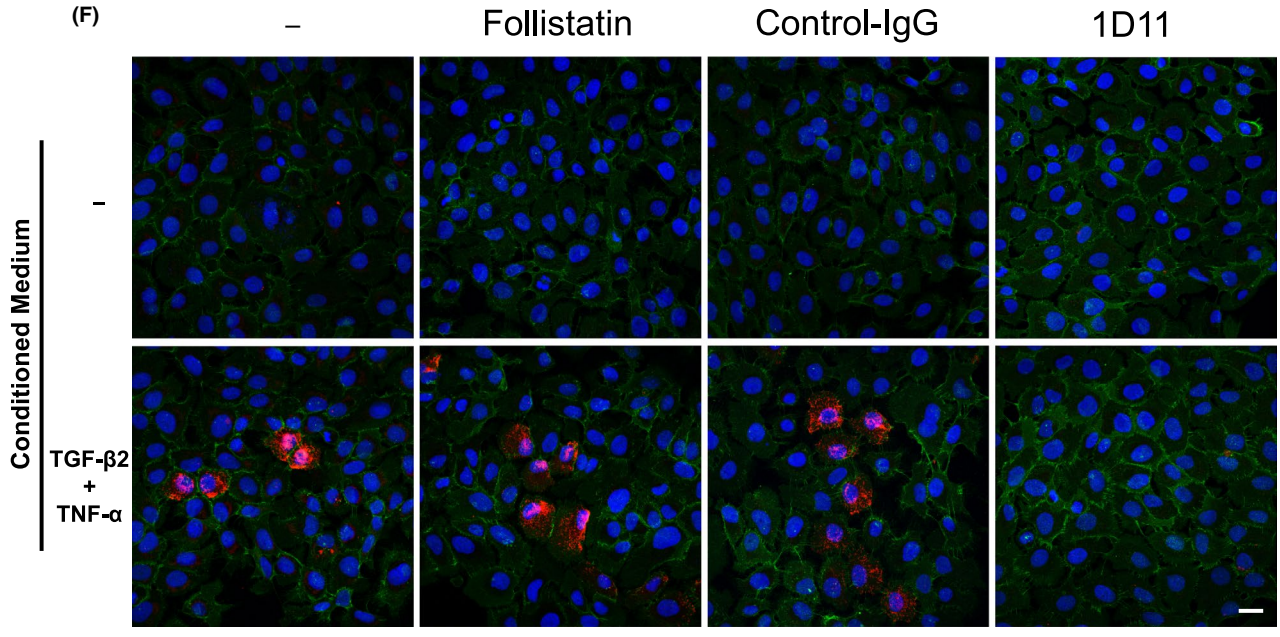
(F)
–
Follistatin
Control-IgG
1D11
Conditioned Medium
–
TGF-β2
+
TNF-α

of HSC-4 cells, we cultured HSC-4 cells in the CM from HUAECs treated with TGF-β2 and TNF-α in combination with follistatin or anti-TGF-β neutralizing antibody, followed by qRT-PCR analysis for expression of vimentin. We found that increased expression of vimentin by the CM derived from HUAECs treated with TGF-β2 and TNF-α was not affected by follistatin (Figure 9D) but it was significantly decreased by anti-TGF-β neutralizing antibody (Figure 9E). We also confirmed these results by performing immunocytochemical analyses for the expression of E-cadherin and vimentin, epithelial and mesenchymal cell markers, respectively (Figure 9F). Together with the data of Smad2/3 activation (Figure 8), these results suggested that the CM derived from ECs, treated with exogenous TGF-β2 and TNF-α, stimulated secretion of active TGF-β ligands by interaction with HSC-4 cells. Our data also suggest that the TGF-β ligands played a major role in the induction of the EMT of HSC-4 cells compared with the secreted activin A.

4 | DISCUSSION

In this study, we revealed that the EndMT was induced by TGF-β2 and further enhanced by TNF-α in various types of human ECs. While all of these stimulatory effects of TNF-α on TGF-β2-induced EndMT were not observed in the mouse TECs isolated from human melanoma xenografts in nude mice (data not shown), the differential effects of TNF-α might have been caused by the specific characteristics of mouse TECs which often have genomic instability[33] and epigenetic alterations.[34] Effects of TNF-α on TGF-β2-induced EndMT in various types of ECs need to be studied more extensively in the future. Furthermore, we found that activation of the autocrine signal loop mediated by endogenous secretion of TGF-β2 and activin A from ECs undergoing EndMT was likely to have caused the irreversible and long-lasting effects induced by exogenous TGF-β2 and TNF-α, leading to the EMT of epithelial cancer cells.

We observed upregulated TMEPAI expression in HUAECs, even at 72 h post-stimulation (Figure 1C), suggesting sustained activation of Smad2/3 signals. This effect was likely mediated by increased production of activin A in response to treatment with TGF-β2 and TNF-α (Figure 5), resulting in activation of an autocrine signaling loop. Furthermore, we found that TGF-β2 and TNF-α upregulated ALK5 (Figure 5A) and integrin αv (Figure 5F) expression, which can augment TGF-β signals. Our data suggested that autocrine TGF-β and activin signals are stabilized once Smad2/3 signals triggered by exogenous TGF-β are activated.

Furthermore, we showed that TNF-α could enhance the EndMT induced by TGF-β likely to be through a direct effect on the TGF-β signaling pathway. Liu and colleagues reported that ALK5 expression could be affected by inflammatory cytokines. In their study, treatment of lung carcinoma cells with a mixture of TNF-α, IL-1β, and interferon-γ upregulated ALK5 expression.[35] Consistent with their results, ALK5 expression in our study was primarily induced by TNF-α, and the effect of TNF-α was further enhanced by TGF-β. TGF-β could not enhance TNF-α-induced ICAM1 expression at 4 h post-stimulation (Figure 1B), but suppressed it at 72 h post-stimulation. During acute inflammation in the body, while TNF-α functions as a proinflammatory cytokine in the onset phase, TGF-β functions as an anti-inflammatory cytokine in the resolution phase.[36] For effective response in acute inflammation, TGF-β signals need to be suppressed by TNF-α at the onset phase and, in turn, the TNF-α signal needs to be suppressed by TGF-β at the resolution phase.[37] ECs may thus have an internal system to regulate these signals for acute inflammation. Consistent with previous reports that have revealed crosstalk between TNF-α and TGF-β signaling pathways in a variety of cells,[38,39] the present study also highlighted the roles of RelA, a component of NF-κB complex of TNF-α signals in the regulation of TGF-β-induced expression of putative EndMT-related molecules including SM22α as well as a TGF-β target gene, *TMEPAI* (Figure 6C). This study suggested that cooperation between these cytokines in ECs occurred at the transcription level of each signaling molecule. Whether the expression of these genes is regulated by the crosstalk between Smad and NF-κB signals needs to be elucidated in future studies.

In this study, we found that expression of TGF-β2, but not TGF-β1 or TGF-β3, was upregulated by combined treatment with TGF-β2 and TNF-α (Figure 5). The expression of the human LDS4 gene encoding TGF-β2 is known to be regulated by multiple promoter-region-specific AP-1, AP-2, SP-1, and ATF-2 transcription factor binding elements. The regulation also involves TATA box and cAMP-responsive elements activated by ATF-1.[40,41] In addition, induction of TGF-β2 expression by TGF-β1 or all-*trans* retinoic acid involves direct activation of RhoA/ROCK signaling in multiple types of cells.[42-44] Therefore, the precise mechanism underlying the regulation of TGF-β2 expression in ECs remains to be elucidated.

Multiple lines of evidence have suggested that TGF-β2 is overexpressed in various cancer types, including glioma, and that its expression is correlated with tumor progression.[45] In addition, in human pancreatic cancer, all 3 isoforms of TGF-β (TGF-β1, 2, and 3) were expressed at higher levels compared with the normal pancreas.[46] However, there was a correlation with advanced tumor stage and a more aggressive metastasizing tumor phenotype when the TGF-β2 isoform was overexpressed in tumor tissue or plasma, as proven both by animal studies and some clinical observations in humans.[46,47] In our study, we found that ECs, representing an important constituent of the TME, became the source of TGF-β2 and activin (Figure 5). Cancer stem cells (CSCs) are tumor cells that have the characteristics of self-renewal ability, tumor initiation capacity, and drug resistance.[48] CSCs reside in niches, which are distinct regions within the TME, preserving the properties of CSCs at least partially by the cytokines secreted by the niche components. Multiple lines of evidence have suggested that ECs in the TME establish a "vascular niche" by secreting various cytokines.[49] Taken together with the notion that EMT activation is linked to the acquisition of stem cell properties of cancer cells,[50] TGF-β2 secreted by ECs undergoing EndMT is likely to mediate the crosstalk between tumor

and stromal cells, promoting tumor progression via induction of the EMT of cancer cells.

While anti-TGF-β neutralizing antibody (1D11) abrogated the induction of EMT of HSC-4 oral cancer cells by the CM from HUAECs treated with TGF-β2 and TNF-α (Figure 9E,F), activation of Smad2/3 signals by the CM was not inhibited in the presence of the antibody (Figure 8D). TGF-β is produced as a latent form, which can be activated in vitro and in vivo only under certain conditions. Sato and colleagues reported that latent TGF-β was activated in co-culture of ECs and smooth muscle cells.[51] As anti-TGF-β neutralizing antibody targets activated TGF-βs, it may have failed to deplete TGF-βs from the CM of HUAECs and thus we observed activation of Smad2/3 signals in HEK-Blue reporter cells (Figure 8D). The present finding that the antibody suppressed the CM to induce the EMT of HSC-4 oral cancer cells (Figure 9E,F) suggested that latent TGF-βs in the CM may be activated via interaction with multiple components of HSC-4 cells. As ECs and cancer cells interact with each other in TME, it is crucial to understand the mechanisms of how endothelial cell-derived latent TGF-βs are activated by cancer cells to establish novel therapeutic strategies targeting the TME.

Several attempts to inhibit TGF-β2 in tumor tissues have been reported. TGF-β2 antisense compound has been clinically evaluated in patients with high-grade glioma, pancreatic and colorectal cancers, and melanoma, resulting in the reversal of tumor-induced immune suppression and inhibition of tumor growth, invasion, and metastasis.[52-54] The present study identified multiple target molecules, including activin and integrin αv, which showed increased expression in ECs undergoing EndMT. Importantly, it also raised the possibility that the inhibition of TGF-β signals is significantly effective to suppress the EndMT-driven EMT. The efficacy of the targeting strategies needs to be studied in vivo using tumor models in which EndMT plays important roles in tumor progression. Furthermore, as the EndMT has been implicated in other types of human diseases, including organ fibrosis,[55-57] atherosclerosis,[58] pulmonary arterial hypertension,[59] and cerebral cavernous malformation,[10] these molecules are expected to be good targets for prevention and treatment of pathological conditions caused by EndMT.

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DISCLOSURE

The authors declare no competing interests.

REFERENCES

1. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. *Nat Rev Cancer*. 2008;8:56-61.
2. Yoshimatsu Y, Watabe T. Roles of TGF-β signals in endothelial-mesenchymal transition during cardiac fibrosis. *Int J Inflam*. 2011;2011:724080.
3. Xiao L, Dudley AC. Fine-tuning vascular fate during endothelial-mesenchymal transition. *J Pathol*. 2017;241:25-35.
4. Zeisberg EM, Potenta S, Xie L, Zeisberg M, Kalluri R. Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. *Cancer Res*. 2007;67:10123-10128.
5. Akatsu Y, Takahashi N, Yoshimatsu Y, et al. Fibroblast growth factor signals regulate transforming growth factor-β-induced endothelial-to-myofibroblast transition of tumor endothelial cells via Elk1. *Mol Oncol*. 2019;13:1706-1724.
6. Gasparics A, Rosivall L, Krizbai IA, Sebe A. When the endothelium scores an own goal: endothelial cells actively augment metastatic extravasation through endothelial-mesenchymal transition. *Am J Physiol Heart Circ Physiol*. 2016;310:H1055-1063.
7. Miyazono K, Katsuno Y, Koinuma D, Ehata S, Morikawa M. Intracellular and extracellular TGF-β signaling in cancer: some recent topics. *Front Med*. 2018;12:387-411.
8. Morikawa M, Derynck R, Miyazono K. TGF-β and the TGF-β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harb Perspect Biol*. 2016;8:a021873.
9. Bodmer S, Strommer K, Frei K, et al. Immunosuppression and transforming growth factor-β in glioblastoma. Preferential production of transforming growth factor-β2. *J Immunol*. 1989;143:3222-3229.
10. Maddaluno L, Rudini N, Cuttano R, et al. EndMT contributes to the onset and progression of cerebral cavernous malformations. *Nature*. 2013;498:492-496.
11. Katsura A, Suzuki HI, Ueno T, et al. MicroRNA-31 is a positive modulator of endothelial-mesenchymal transition and associated secretory phenotype induced by TGF-β. *Genes Cells*. 2016;21:99-116.
12. Kokudo T, Suzuki Y, Yoshimatsu Y, Yamazaki T, Watabe T, Miyazono K. Snail is required for TGF-β-induced endothelial-mesenchymal transition of embryonic stem cell-derived endothelial cells. *J Cell Sci*. 2008;121:3317-3324.
13. Mihira H, Suzuki HI, Akatsu Y, et al. TGF-β-induced mesenchymal transition of MS-1 endothelial cells requires Smad-dependent cooperative activation of Rho signals and MRTF-A. *J Biochem*. 2012;151:145-156.
14. Suzuki HI, Katsura A, Mihira H, Horie M, Saito A, Miyazono K. Regulation of TGF-β-mediated endothelial-mesenchymal transition by microRNA-27. *J Biochem*. 2017;161:417-420.
15. Sato Y, Rifkin DB. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of a latent transforming growth factor-β 1-like molecule by plasmin during co-culture. *J Cell Biol*. 1989;109:309-315.

16. Hynes RO. Integrins: bidirectional, allosteric signaling machines. *Cell*. 2002;110:673-687.
17. Kitamura H, Cambier S, Somanath S, et al. Mouse and human lung fibroblasts regulate dendritic cell trafficking, airway inflammation, and fibrosis through integrin $\alpha_v\beta_8$-mediated activation of TGF-β. *J Clin Invest*. 2011;121:2863-2875.
18. Peng ZW, Ikenaga N, Liu SB, et al. Integrin $\alpha_v\beta_6$ critically regulates hepatic progenitor cell function and promotes ductular reaction, fibrosis, and tumorigenesis. *Hepatology (Baltimore, MD)*. 2016;63:217-232.
19. Song KH, Cho SJ, Song JY. $\alpha_v\beta_1$ integrin as a novel therapeutic target for tissue fibrosis. *Ann Transl Med*. 2016;4:411.
20. Reed NI, Jo H, Chen C, et al. The $\alpha_v\beta_1$ integrin plays a critical in vivo role in tissue fibrosis. *Sci Transl Med*. 2015;7:288ra79.
21. Patsenker E, Popov Y, Stickel F, et al. Pharmacological inhibition of integrin $\alpha_v\beta_3$ aggravates experimental liver fibrosis and suppresses hepatic angiogenesis. *Hepatology (Baltimore, MD)*. 2009;50:1501-1511.
22. Tatler AL, John AE, Jolly L, et al. Integrin $\alpha_v\beta_5$-mediated TGF-β activation by airway smooth muscle cells in asthma. *J Immunol*. 2011;187:6094-6107.
23. Wang T, He C, Yu X. Pro-inflammatory cytokines: new potential therapeutic targets for obesity-related bone disorders. *Curr Drug Targets*. 2017;18:1664-1675.
24. Kawata M, Koinuma D, Ogami T, et al. TGF-β-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells. *J Biochem*. 2012;151:205-216.
25. Brett J, Gerlach H, Nawroth P, Steinberg S, Godman G, Stern D. Tumor necrosis factor/cachectin increases permeability of endothelial cell monolayers by a mechanism involving regulatory G proteins. *J Exp Med*. 1989;169:1977-1991.
26. Wu Y, Zhou BP. TNF-α/NF-κB/Snail pathway in cancer cell migration and invasion. *Br J Cancer*. 2010;102:639-644.
27. Mahler GJ, Farrar EJ, Butcher JT. Inflammatory cytokines promote mesenchymal transformation in embryonic and adult valve endothelial cells. *Arterioscler Thromb Vasc Biol*. 2013;33:121-130.
28. Maleszewska M, Moonen JR, Huijkman N, van de Sluis B, Krenning G, Harmsen MC. IL-1β and TGF-β2 synergistically induce endothelial to mesenchymal transition in an NFκB-dependent manner. *Immunobiology*. 2013;218:443-454.
29. Sánchez-Duffhues G, García de Vinuesa A, van de Pol V, et al. Inflammation induces endothelial-to-mesenchymal transition and promotes vascular calcification through downregulation of BMPR2. *J Pathol*. 2019;247:333-346.
30. van Noort V, Scholch S, Iskar M, et al. Novel drug candidates for the treatment of metastatic colorectal cancer through global inverse gene-expression profiling. *Cancer Res*. 2014;74:5690-5699.
31. Saito D, Kyakumoto S, Chosa N, et al. Transforming growth factor-β1 induces epithelial-mesenchymal transition and integrin α3β1-mediated cell migration of HSC-4 human squamous cell carcinoma cells through Slug. *J Biochem*. 2013;153:303-315.
32. Kita A, Kasamatsu A, Nakashima D, et al. Activin B regulates adhesion, invasiveness, and migratory activities in oral cancer: a potential biomarker for metastasis. *J Cancer*. 2017;8:2033-2041.
33. Hida K, Hida Y, Amin DN, et al. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res*. 2004;64:8249-8255.
34. Maishi N, Ohba Y, Akiyama K, et al. Tumour endothelial cells in high metastatic tumours promote metastasis via epigenetic dysregulation of biglycan. *Sci Rep*. 2016;6:28039.
35. Liu X. Inflammatory cytokines augments TGF-β1-induced epithelial-mesenchymal transition in A549 cells by up-regulating TβR-I. *Cell Motil Cytoskeleton*. 2008;65:935-944.
36. Sugimoto MA, Sousa LP, Pinho V, Perretti M, Teixeira MM. Resolution of inflammation: what controls its onset? *Front Immunol*. 2016;7:160.
37. Al-Mulla F, Leibovich SJ, Francis IM, Bitar MS. Impaired TGF-beta signaling and a defect in resolution of inflammation contribute to delayed wound healing in a female rat model of type 2 diabetes. *Mol Biosyst*. 2011;7:3006-3020.
38. Takahashi E, Nagano O, Ishimoto T, et al. Tumor necrosis factor-α regulates transforming growth factor-β-dependent epithelial-mesenchymal transition by promoting hyaluronan-CD44-moesin interaction. *J Biol Chem*. 2010;285:4060-4073.
39. Bitzer M, von Gersdorff G, Liang D, et al. A mechanism of suppression of TGF-β/SMAD signaling by NF-κB/RelA. *Genes Dev*. 2000;14:187-197.
40. Roberts AB, Kim SJ, Noma T, et al. Multiple forms of TGF-β: distinct promoters and differential expression. *Ciba Found Symp*. 1991;157:7-15.
41. Kingsley-Kallesen ML, Kelly D, Rizzino A. Transcriptional regulation of the transforming growth factor-β2 promoter by cAMP-responsive element-binding protein (CREB) and activating transcription factor-1 (ATF-1) is modulated by protein kinases and the coactivators p300 and CREB-binding protein. *J Biol Chem*. 1999;274:34020-34028.
42. Shimada H, Staten NR, Rajagopalan LE. TGF-β1 mediated activation of Rho kinase induces TGF-β2 and endothelin-1 expression in human hepatic stellate cells. *J Hepatol*. 2011;54:521-528.
43. Namachivayam K, MohanKumar K, Arbach D, et al. All-trans retinoic acid induces TGF-β2 in intestinal epithelial cells via RhoA- and p38α MAPK-mediated activation of the transcription factor ATF2. *PLoS ONE*. 2015;10:e0134003.
44. Pervan CL, Lautz JD, Blitzer AL, Langert KA, Stubbs EB Jr. Rho GTPase signaling promotes constitutive expression and release of TGF-β2 by human trabecular meshwork cells. *Exp Eye Res*. 2016;146:95-102.
45. Kjellman C, Olofsson SP, Hansson O, et al. Expression of TGF-β isoforms, TGF-β receptors, and SMAD molecules at different stages of human glioma. *Int J Cancer*. 2000;89:251-258.
46. Friess H, Yamanaka Y, Buchler M, et al. Enhanced expression of transforming growth factor β isoforms in pancreatic cancer correlates with decreased survival. *Gastroenterology*. 1993;105:1846-1856.
47. Bellone G, Smirne C, Mauri FA, et al. Cytokine expression profile in human pancreatic carcinoma cells and in surgical specimens: implications for survival. *Cancer Immunol Immunother*. 2006;55:684-698.
48. Plaks V, Kong N, Werb Z. The cancer stem cell niche: how essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell*. 2015;16:225-238.
49. Butler JM, Kobayashi H, Rafii S. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nat Rev Cancer*. 2010;10:138-146.
50. Lu W, Kang Y. Epithelial-mesenchymal plasticity in cancer progression and metastasis. *Dev Cell*. 2019;49:361-374.
51. Sato Y, Tsuboi R, Lyons R, et al. Characterization of the activation of latent TGF-β by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J Cell Biol*. 1990;111:757-763.
52. Schlingensiepen KH, Schlingensiepen R, Steinbrecher A, et al. Targeted tumor therapy with the TGF-β2 antisense compound AP 12009. *Cytokine Growth Factor Rev*. 2006;17:129-139.
53. Schlingensiepen KH, Jaschinski F, Lang SA, et al. Transforming growth factor-β 2 gene silencing with trabedersen (AP 12009) in pancreatic cancer. *Cancer Sci*. 2011;102:1193-1200.
54. Oettle H, Seufferlein T, Luger T, et al. Final results of a phase I/II study in patients with pancreatic cancer, malignant melanoma, and colorectal carcinoma with trabedersen. *J Clin Oncol*. 2012;30:4034-4034.
55. Zeisberg EM, Potenta SE, Sugimoto H, Zeisberg M, Kalluri R. Fibroblasts in kidney fibrosis emerge via endothelial-to-mesenchymal transition. *J Am Soc Nephrol*. 2008;19:2282-2287.

56. Li J, Qu X, Bertram JF. Endothelial-myofibroblast transition contributes to the early development of diabetic renal interstitial fibrosis in streptozotocin-induced diabetic mice. *Am J Pathol*. 2009;175:1380-1388.
57. Widyantoro B, Emoto N, Nakayama K, et al. Endothelial cell-derived endothelin-1 promotes cardiac fibrosis in diabetic hearts through stimulation of endothelial-to-mesenchymal transition. *Circulation*. 2010;121:2407-2418.
58. Chen PY, Qin L, Baeyens N, et al. Endothelial-to-mesenchymal transition drives atherosclerosis progression. *J Clin Invest*. 2015;125:4514-4528.
59. Jimenez SA, Piera-Velazquez S. Endothelial to mesenchymal transition (EndoMT) in the pathogenesis of Systemic Sclerosis-associated pulmonary fibrosis and pulmonary arterial hypertension. Myth or reality? *Matrix Biol*. 2016;51:26-36.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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