

EOFAZ inhibits endothelial-to-mesenchymal transition through downregulation of KLF4

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Received July 11, 2019; Accepted March 11, 2020

DOI: 10.3892/ijmm.2020.4572

Abstract. Essential oil from *Alpinia zerumbet* rhizome (EOFAZ), which is termed Yan shanjiang in China, is extensively used as an herbal medicine in the Guizhou area and has been shown to protect against the damaging effects of cardiovascular injury *in vitro* and *in vivo*. In the present study, it was hypothesized that the protective effects of EOFAZ on transforming growth factor (TGF)- β 1-induced endothelial-to-mesenchymal transition (EndMT) in human umbilical vein endothelial cells (HUVECs) were mediated by inhibition of Krüppel-like factor 4 (KLF4). Cell motility was assessed using wound healing and Transwell assays. The expression of endothelial markers and mesenchymal markers were determined by reverse transcription-quantitative PCR, immunofluorescence staining and western blotting, and additionally, phosphorylated NF- κ B p65 expression was determined by western blotting. Furthermore, the involvement of KLF4 in EndMT was determined using RNA interference to knockdown the expression of KLF4. TGF- β 1 treatment significantly promoted EndMT, as evidenced by downregulation of vascular endothelial-cadherin and upregulation of α -smooth muscle actin in HUVECs, and by enhancing cell migration. Small interfering RNA-mediated knockdown of KLF4 reversed TGF- β 1-induced EndMT. Additionally,

treatment with EOFAZ inhibited TGF- β 1-induced EndMT in a dose-dependent manner. These results suggest that TGF- β 1 may induce EndMT through upregulation of KLF4, and this may be reversed by EOFAZ. Therefore, EOFAZ was shown to inhibit TGF- β 1-induced EndMT through regulation of KLF4.

Introduction

The vascular wall consists of endothelial cells, smooth muscle cells, fibroblasts and extracellular matrix, and is considered an active and integrated organ (1). The vasculature is sensitive to a range of stimuli, several of which may contribute to pathophysiological changes in response to stresses that result in proliferation of the vasculature (2). The initiating event in vascular remodeling is endothelial cell damage. Alterations in the presence of vasoactive substances following endothelial cell dysfunction are involved in the development and progress of cardiovascular diseases. A vicious circle is caused by further injury of endothelial cells following the onset of cardiovascular diseases (3). This dynamic process characterizes vascular remodeling, and is defined as any enduring change in the size and/or composition of an adult blood vessel that allows blood vessels to adapt and heal (4). Vascular remodeling underlies the pathogenesis of major cardiovascular disorders, including pulmonary arterial hypertension (PAH) and atherosclerosis, as well as wound healing (5). Vascular remodeling is involved in cells progressing through four cellular processes: Differentiation, death, migration, and production or degradation of extracellular matrix (3,6). Endothelial cells are pivotal in the initiation of vascular remodeling. The increase in mesenchymal or smooth muscle (SM)-like phenotypes originates from increased endothelial cell transformation (7). Endothelial cells are the first barrier of blood vessels and recently they have been regarded as contributors to vascular remodeling through endothelial-to-mesenchymal transition (EndMT) (8).

EndMT is the process by which endothelial cells undergo phenotypic changes and acquire a mesenchymal/SM-like phenotype (9). Under pathophysiological conditions, such as transplant atherosclerosis and restenosis, endothelial-derived mesenchymal cells may accelerate the development of

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Key words: essential oil from *Alpinia zerumbet* rhizome, human umbilical vein endothelial cells, transforming growth factor- β 1, endothelial-to-mesenchymal transition, Krüppel-like factor 4

neointimal lesions (10). Evidence for EndMT has been demonstrated in the context of cardiac and vascular development, wound healing and various dysfunctions, including tissue fibrosis and diabetic nephropathy (11). Thus, EndMT is regarded as an important mechanism for the generation of SM-like cells. Endothelial cells facilitate vascular development and dysfunction, which act through direct and indirect roles in the vascular remodeling process (12). A previous study has demonstrated that EndMT is involved in the pathogenesis of vascular remodeling (13). EndMT contributes to neointimal formation in interpositional vein grafts (14). Similarly, EndMT contributes to intimal hyperplasia and early-lesion formation in atherosclerosis (15). Furthermore, EndMT contributes to progression of PAH, atherosclerosis and wound healing (16). Therefore, in the present study, it was hypothesized that EndMT may accelerate the pathogenesis of vascular remodeling. However, the role of EndMT and the mechanisms regulating cell phenotype adaptation during vascular remodeling are poorly understood. Studies have reported that mouse and human endothelial cells are induced by transforming growth factor (TGF)- β 1 to transform into SM-like cells in which the expression of α -smooth muscle actin (α -SMA) is upregulated (17,18). Under the synergistic action of TGF- β 1 and the Wnt signaling pathway, EndMT may be induced. Therefore, in the present study, TGF- β 1 was used to develop an *in vitro* model of EndMT (19).

Zingiberaceae belongs to the ginger family, which consists of a large number of aromatic perennial species shown to exhibit therapeutic potential in a number of cardiac-related therapies (20). *Alpinia zerumbet* (Pers.) Burt et Smith, termed Yan Shanjiang in China, is an aromatic plant originating in the East Indies and is widely used as an herbal medicine in South America, Oceania and Asia (21,22). It contains several bioactive constituents and possesses a broad spectrum of pharmacological properties, such as antihypertensive, anti-inflammatory and cardiovascular protective effects, as well as antioxidant effects (23,24), and is widely used as a local Miao herbal medicine in the Guizhou province. Previous studies have shown that essential oil from *Alpinia zerumbet* rhizome (EOFAZ) is effective against the vasoconstriction induced by release of norepinephrine and KCl (25). The mechanisms mediating the beneficial effects of EOFAZ may include inhibition of oxidative stress and inflammation, and induction of apoptosis (26). However, the effects of EOFAZ on EndMT in a range of stress situations has not been investigated, particularly in relation to TGF- β 1 signaling (22).

In the present study, a cell based *in vitro* model treated with TGF- β 1 was established to verify the hypothesis that EOFAZ protects against TGF- β 1-induced EndMT, and to determine the underlying molecular mechanism by which EOFAZ exerts its beneficial effects. The present study identified a novel molecular mechanism by which EOFAZ exerts its effects and also provides a theoretical basis for use of EOFAZ treatment for cardiac disorders.

Materials and methods

Extraction of EOFAZ. The essential oil was extracted from the fruit of *Alpinia zerumbet*, which was collected in Zhenfeng County, Guiyang, China, in October 2018. The

fruit was identified by Professor Qingde Long (Department of Pharmacognosy and Medico-botany, Guizhou Medical University, Guiyang, China) and a voucher specimen (no. 20181029) was deposited to the Key Laboratory of Optimal Utilization of Natural Medicinal Resources, Guizhou Medical University. The method of extraction/isolation and the compounds of EOFAZ were identified according to our previous study (22).

Cell culture. Human umbilical vein endothelial cells (HUVECs) were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in Endothelial Cell Medium (ScienCell Research Laboratories, Inc.) supplemented with 5% FBS (ScienCell Research Laboratories, Inc.) and 100 U/ml penicillin and streptomycin, and maintained in an incubator at 37°C with 5% CO₂. Cells were sub-cultured and seeded into 6- or 24-well plates for subsequent experiments as required. The cells were pretreated with EOFAZ and LY2109761 (Selleck Chemicals) for 2 h at 37°C, and TGF- β 1 (Peprotech EC Ltd.) was added for 72 h. HUVECs were trypsinized with 0.25% trypsin and collected for analysis.

Cell morphological assessment. The HUVECs were plated in 6-well plates at a density of 3x10⁵ cells/well and were incubated overnight. Following treatment with 10 ng/ml TGF- β 1 for 72 h at 37°C, the cultured plates were examined and photographs were obtained using an inverted light microscope (magnification, x100).

Western blot analysis. Cells were lysed in RIPA lysis buffer containing protease inhibitors (Beyotime Institute of Biotechnology). The protein concentration was determined by BCA assay. For western blotting, a total of 40 μ g protein/lane from lysed cells was separated by 10% SDS-PAGE. The proteins were transferred to a PVDF membrane and the membrane was blocked with 5% non-fat dry milk at room temperature (25°C) for 2 h. The membrane was then incubated with the following primary antibodies overnight at 4°C: Rabbit anti-Krüppel-like factor 4 (KLF4; 1:1,000; cat. no. 12173S), anti-vascular endothelial (VE)-cadherin (1:1,000; cat. no. 2500), anti- α -SMA (1:1,000; cat. no. 19245), anti-snail (1:1,000; cat. no. 5276) and anti-NF- κ B phosphorylated (p)-p65 (1:1,000; cat. no. 3033T). All primary antibodies were from Cell Signaling Technology, Inc. and all were diluted in TBS and 0.2% Tween-20. Subsequently, the membranes were washed and incubated with a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Inc.; 1:10,000; cat. no. 7076) for 90 min at room temperature. Signals were visualized using an ECL kit (GE Healthcare). The expression levels of protein were calculated by using ImageJ V1.8.0.112 software (National Institutes of Health). Protein signals were normalized to β -actin (Cell Signaling Technology, Inc.; 1:1,000; cat. no. 3700).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted using the TransZol Up Plus RNA kit (Sangon Biotech Co., Ltd.). Total RNA was purified with 75% ethanol and its concentration was determined using spectrophotometry. cDNA was synthesized from the purified RNA (200 ng per sample) using a reverse transcription kit (cat. no. DRR037A;

Takara Bio, Inc.), according to the manufacturer's protocol. Subsequently, qPCR was performed on an ABI 7300 Real-Time PCR system SYBR Premix ExTaq (Takara Bio, Inc.). The thermocycling conditions were as follows: Pre-incubation at 94°C for 30 sec followed by 39 cycles of amplification at 94°C for 5 sec and 56°C for 30 sec. The sequences of the primers were: Vascular endothelial (VE)-cadherin forward, 5'-CTT CACCCAGACCAAGTACACA-3' and reverse, 5'-AGGGCT CATGTATCGGAGGT-3'; α -SMA forward, 5'-ACCATCGGG AATGAACGCTT-3' and reverse, 5'-CTGTCAGCAATGCCT GGGTA-3'; snail forward, 5'-GAAGATGCACATCCGAA GCC-3' and reverse, 5'-TTCACATCCGAGTGGGTCTG-3'; and GAPDH forward, 5'-TGTAACGGATTTGGCCGTA-3' and reverse, 5'-GATGGTGTGGGTTTCCCGT-3'. GAPDH was used as the loading control.

Immunofluorescence staining. Cells at a density of 1×10^4 /ml were seeded onto slides and fixed with 4% paraformaldehyde (200 μ l/well) for 10 min, and washed twice with PBS, 10 min each time. The cell membrane was treated with 0.1% Triton X-100 in PBS (200 μ l/well) at room temperature for 10 min, and then washed twice with PBS, 10 min each time. The plates were agitated gently several times by hand to drain the liquid and cells were then blocked with 4% goat serum in PBS at room temperature for 30 min. The liquid was gently removed, anti-VE-cadherin and anti- α -SMA primary antibodies were added, and the cells were incubated at 37°C for 1 h or at 4°C overnight. The primary antibodies used were: Anti-VE-cadherin (1:100; Cell Signaling Technology, Inc.; cat. no. 2500) and anti- α -SMA (1:100, Cell Signaling Technology, Inc.; cat. no. 19245). DAPI (1:1,000; Sigma-Aldrich; Merck KGaA; cat. no. AC23192) was used to stain the nuclei at room temperature for 10 min. Subsequently, the cells were washed three times with PBS and then incubated with fluorescein-labeled secondary antibodies (Cell Signaling Technology, Inc.; 1:100; cat. nos. 4416 and 12877) at 37°C for 60 min. Following washing with 0.01 M PBS three times, 5 min per wash, anti-fade reagent was used to mount the slides at 4°C. Finally, cells were imaged using a fluorescence microscope (magnification, x200).

Wound healing assay. HUVECs were seeded in 24-well plates and grown overnight to confluence. For EndMT induction, cells were treated with 10 ng/ml TGF- β 1, 2 μ M LY21097612 and 0.25, 1 or 4 μ g/l EOFAZ for 72 h at 37°C. The monolayer of cells was scratched using a 20- μ l pipette tip to create the wound. Floating cells were removed by washing twice with PBS and serum-free Endothelial Cell Medium was added. Wound closure rate was assessed using images captured with a light microscope (Leika DM3000k magnification, x100) after 24 h. The motility index=(Mean of 0 h migration distance-mean of 12 h migration distance)/mean of 0 h migration distance.

Transwell migration assay. Cells were trypsinized, centrifuged at 150 x g for 5 min at room temperature, washed with PBS 1-2 times and resuspended in serum-free culture medium containing BSA. A cell suspension of 100 μ l (2×10^4 cells) was added to the upper chamber of the Transwell insert, and 400 μ l medium containing 10% FBS was added to the lower chamber.

The cells were cultured for 12 h, after which the Transwell chamber was removed, the medium was discarded, and cells were washed twice with PBS and fixed with paraformaldehyde for 15 min at room temperature. Subsequently, the cells were stained for 10 min with 0.1% crystal violet at room temperature. The unmigrated cells in the upper layer were gently removed with a cotton swab and the chamber was then washed three times with PBS. Cells were observed under an inverted light microscope (magnification, x100) and the number of cells in five randomly chosen fields were counted. The number of migrated cells was quantified by manual counting, and the motility index was determined using the following formula: Motility index=the number of migrated cells in the experimental group/the number of cells that migrated in the control group.

Immunoprecipitation. HUVECs were harvested 72 h after treatment with TGF- β 1 or EOFAZ at 37°C for 2 h, the supernatant was removed, the cells were lysed with NP-40 lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors, and the lysate was centrifuged on ice at 12,000 x g for 30 min at 4°C. A small quantity of lysate was used for western blotting, and 1 μ g antibody (anti-KLF4; 1:200; Cell Signaling Technology, Inc.; cat. no. 12173S) was added to the remaining cell lysate and incubated overnight at 4°C with gentle agitation. A total of 10 μ l protein A agarose beads were washed with lysis buffer and centrifuged at 300 x g for 3 min at 4°C. The washed protein A agarose beads were then added to the cell lysate-antibody solution and incubated at 4°C with gentle agitation for 2-4 h. Following immunoprecipitation, agarose beads were pelleted by centrifugation at 300 x g for 3 min at 4°C. The supernatant was aspirated, and the agarose beads were washed 3-4 times with 1 ml lysis buffer. Finally, 15 μ l 2x SDS sample buffer was added, and the samples were boiled for 5 min. Samples were subsequently subjected to western blotting, as previously described.

Small interference (si)RNA transfection. HUVECs were plated in 6-well plates at a density of 2×10^6 cells/well and transfected with siRNA. Briefly, 5 μ l KLF4 and control siRNA was mixed with RPMI-1640 media (Wisent, Inc.). Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was mixed with the RPMI-1640 media and the mixture was left to stand for 20 min at room temperature. The Lipofectamine[®]-siRNA mixture was then added to each well containing cells and the cells were incubated for 6 h. Medium was then replaced with antibiotic-free Endothelial Cell Medium supplemented with 5% FBS for 24 h, after which cells were used for subsequent experiments. The KLF4 siRNA sequences were: Sense 5'-AGAGTTCCCATC TCAAGGCA-3' and antisense, 5'-GTCAGTTCATCTGAC CGGG-3'. The sequences of the control non-specific siRNA were: Sense 5'-CGUUGUUCGCUUCCUGAGTT-3' and antisense, 5'-CUCAGGAAGCGAACAAACGTG-3'.

Statistical analysis. All data are expressed as the mean \pm standard deviation of at least three repeats. SPSS v.21.0 (IBM, Corp.) was used for statistical analysis. Comparisons among multiple groups were analyzed using one-way analysis of variance followed by Bonferroni's post hoc test. Comparisons between two groups were analyzed using a

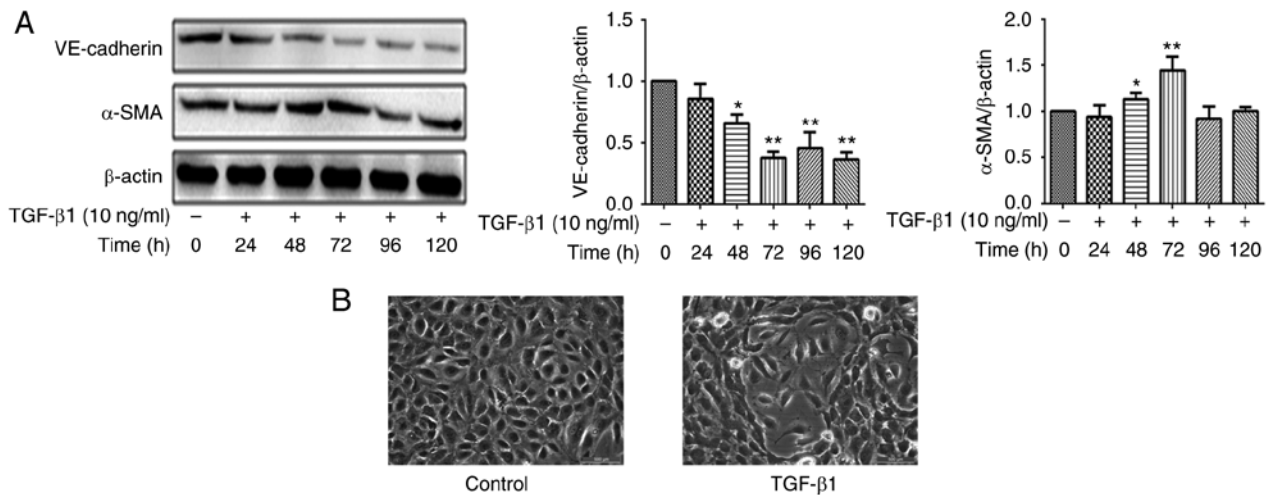


Figure 1. EndMT is induced by TGF- β 1 in HUVECs. (A) Effect of 10 ng/ml TGF- β 1 on cell phenotypic markers at different times. (B) Untreated HUVECs exhibited a cobble-stone like morphology under the light microscope and TGF- β 1-treated HUVECs acquired a fibroblast-like morphology. Magnification, $\times 400$. $n=3$. * $P<0.05$, ** $P<0.01$ vs. control. EndMT, endothelial-to-mesenchymal transition; TGF- β 1, transforming growth factor- β 1; HUVEC, human umbilical vein endothelial cell; EndMT, endothelial-to-mesenchymal transition; VE-cadherin, vascular endothelial-cadherin; α -SMA, α -smooth muscle actin.

two-tailed Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EndMT is induced by TGF- β 1 in HUVECs. TGF- β 1 is a powerful factor for induction of EndMT, although the effect depends of the treatment time and dose in different models (17). Relative VE-cadherin and α -SMA expression levels were determined by western blotting. After cells were treated in Endothelial Cell Medium with or without 10 ng/ml of TGF- β 1, the expression levels of VE-cadherin and α -SMA changed in a time-dependent manner. As presented in Fig. 1A, the HUVECs were treated with 10 ng/ml TGF- β 1 for various durations, and the VE-cadherin and α -SMA levels were most significantly changed at 72 h, thus treatment with 10 ng/ml TGF- β 1 for 72 h was selected to establish the EndMT model in following experiments. In addition, the morphological EndMT-associated changes in HUVECs by TGF- β 1 treatment for 72 h were observed using an inverted microscope (Fig. 1B). Cells in the control group demonstrated a cobble-stone like morphology and the junctions between the cells were dense. The cells induced by TGF- β 1 were primarily fibroblast in morphology, the distance between cells was increased, and additionally the presence of filamentous pseudopod protrusions was observed. Taken together, TGF- β 1 induced morphological and phenotypic changes in HUVECs to a more mesenchymal-like phenotype, suggesting the cells had undergone EndMT and an EndMT model had been established.

EOFAZ inhibits TGF- β 1-induced EndMT in HUVECs. Subsequently, HUVECs were exposed to 10 ng/ml TGF- β 1 for 72 h for EndMT induction. Additionally, the cells were pretreated with EOFAZ and LY2109761 (a TGF- β 1 inhibitor) for 2 h and then stimulated with TGF- β 1. The results of RT-qPCR and western blotting analysis demonstrated that EOFAZ, like LY2109761, significantly reversed the TGF- β 1-induced increase in α -SMA and Snail expression and the TGF- β 1-induced

decrease in VE-cadherin expression (Fig. 2A and B). Similar changes were observed by immunofluorescence (Fig. 2C).

EOFAZ inhibits the expression of EndMT markers in cell migration. During the EndMT process initiated in endothelial cells, the cellular migratory ability is promoted (14). A wound healing assay was used to observe the effects of EOFAZ on the migration of endothelial cells treated with TGF- β 1. The results demonstrated that the cell migration rate significantly increased in the TGF- β 1-treated group compared with the control group. In addition, scratch immunofluorescence staining demonstrated that VE-cadherin was downregulated, while α -SMA was upregulated in the migrating cells after TGF- β 1 treatment. The number of migrated cells was significantly reduced in cells pre-treated with LY2109761 and EOFAZ, and the expression of VE-cadherin was increased and the expression of α -SMA was decreased compared with the cells only treated with TGF- β 1 (Fig. 3A and B). These results suggest that the TGF- β 1-induced EndMT is associated with the enhanced migratory capacity of mesenchymal cells, whereas EOFAZ inhibits this migratory capacity.

Effect of EOFAZ on the expression of KLF4 and NF- κ B p-p65, and on the binding between KLF4 and NF- κ B induced by TGF- β 1 in HUVECs. As presented in Fig. 3C and D, KLF4 and NF- κ B p-p65 expression levels were upregulated in the TGF- β 1-induced cells compared with the control. The p-p65/p65 ratio is appropriate to evaluate the activation of NF- κ B (27). The results demonstrated that EOFAZ and LY2109761 significantly decreased the TGF- β 1-induced increase in p-p65/p65 ratio, which indicated the inhibitory effects of EOFAZ on NF- κ B nuclear translocation and activation. The TGF- β 1-induced increase in KLF4 was also decreased in the presence of EOFAZ or LY2109761 (Fig. 3C and D). These results suggested that EOFAZ inhibits TGF- β 1-induced KLF4 and NF- κ B activation. A previous study reported that NF- κ B is one of key regulators during EndMT in endothelial cells; activation or inhibition of the NF- κ B signaling pathway

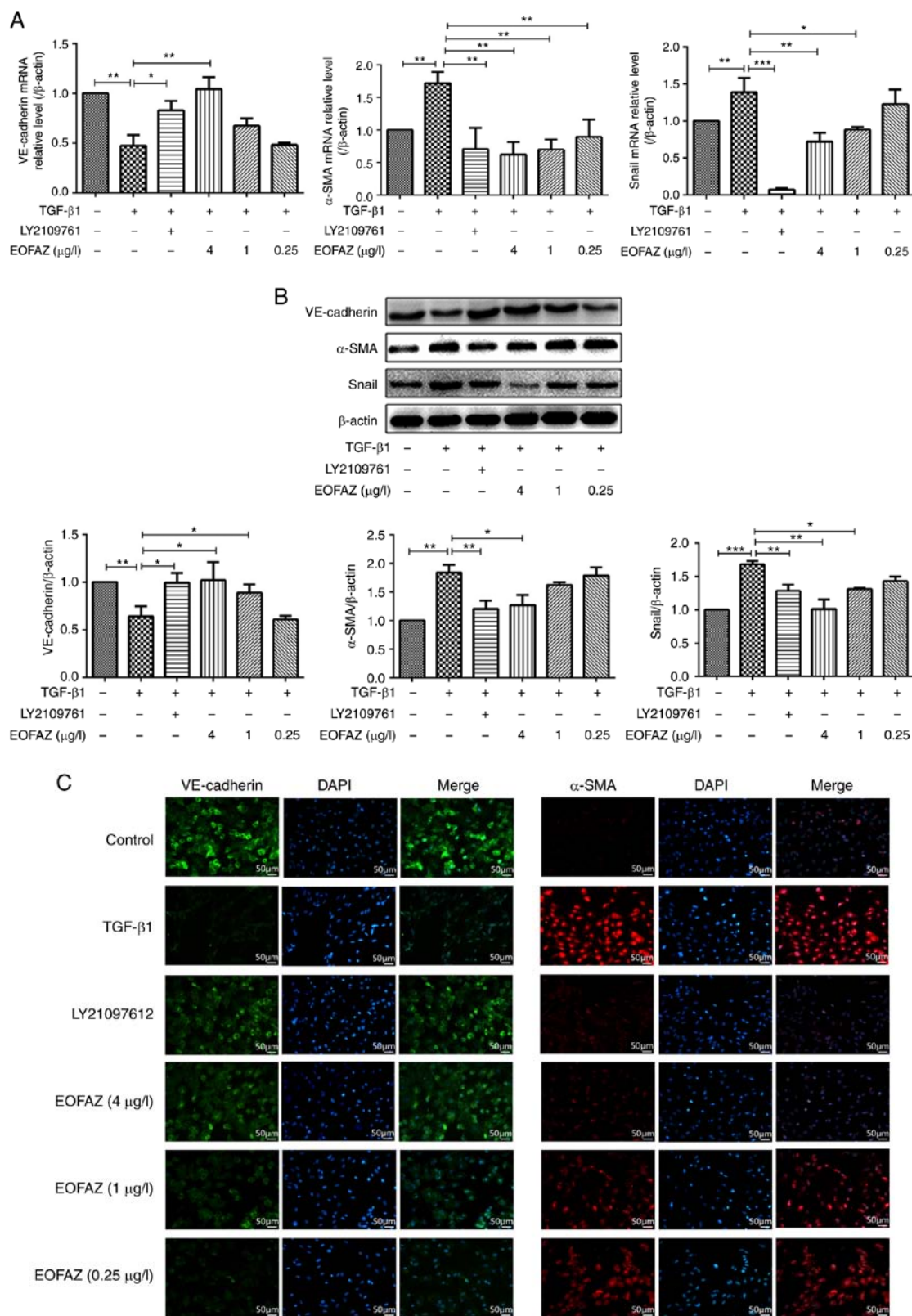


Figure 2. Eofaz inhibits TGF-β1-induced EndMT in HUVECs. (A) Effect of Eofaz on the mRNA expression levels of VE-cadherin, α-SMA and Snail, as measured by RT-qPCR and normalized to β-actin. n=5. (B) Western blots of VE-cadherin, α-SMA and Snail. β-actin was used as the loading control. (C) Immunofluorescence of endothelial marker protein VE-cadherin (green fluorescence), mesenchymal marker protein α-SMA (red fluorescence) and nuclei (DAPI, blue). *P<0.05, **P<0.01, ***P<0.001. Eofaz, essential oil from *Alpinia zerumbet* rhizome; EndMT, endothelial-to-mesenchymal transition; TGF-β1, transforming growth factor-β1; HUVEC, human umbilical vein endothelial cell; α-SMA, α-smooth muscle actin; VE-cadherin, vascular endothelial-cadherin.

resulted in EndMT reversal (28). In addition, it has been reported that NF-κB is essential for both the induction and maintenance of EndMT (29).

A previous study suggested that KLF4 inhibits TNF-α-mediated induction of vascular cell adhesion protein 1 (VCAM1) expression by blocking the binding of p65 to the

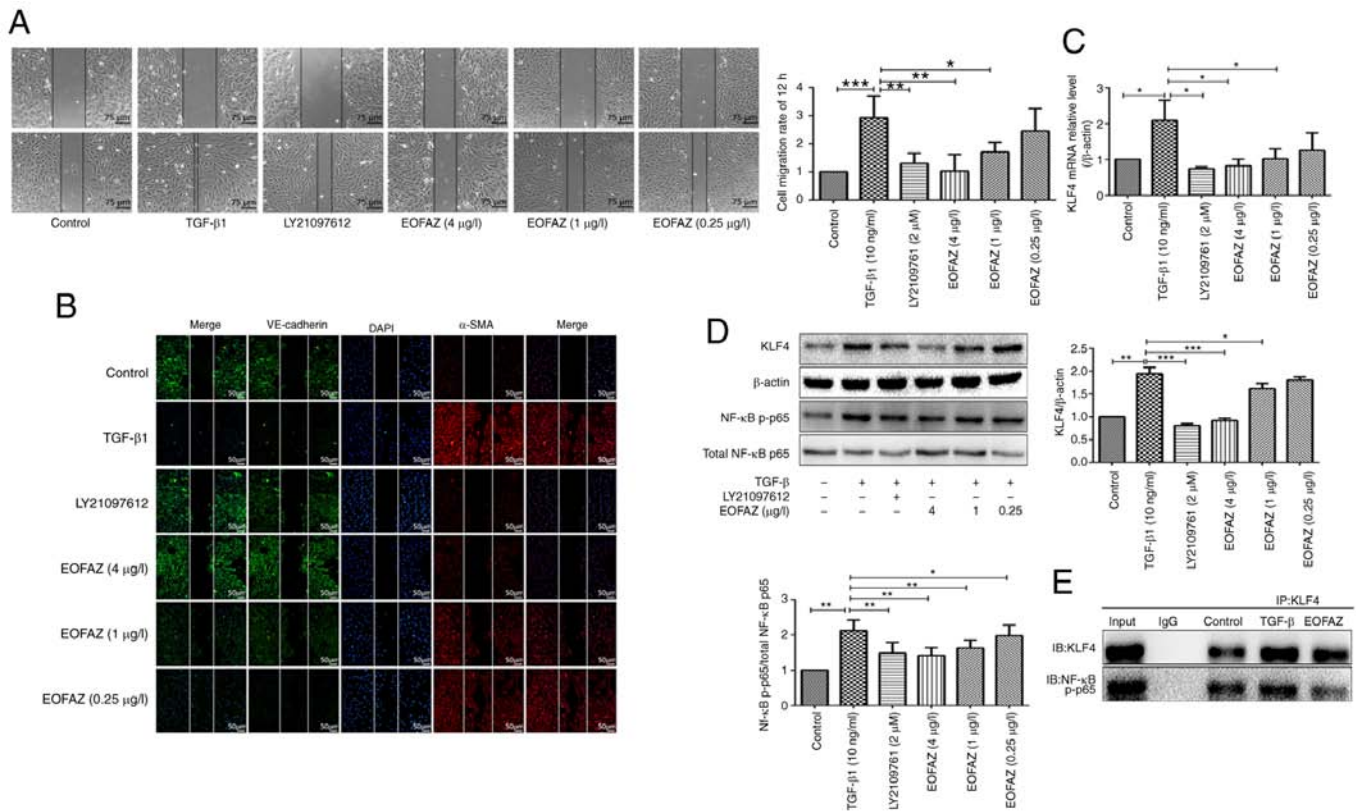


Figure 3. EOFAZ inhibits HUVECs migration and regulates biomarkers in TGF-β1-induced EndMT. (A) Wound healing assay of HUVECs treated with TGF-β1 and EOFAZ for the indicated time intervals. (B) A wound healing assay was used to observe the effects of EOFAZ on the migration of endothelial cells. Endothelial marker protein VE-cadherin (green fluorescence), mesenchymal marker protein α-SMA (red fluorescence) and nuclei (DAPI, blue). (C) Reverse transcription-quantitative PCR analysis of the mRNA expression of KLF4 in HUVECs treated with TGF-β1 and EOFAZ. (D) Western blot analysis was performed with treated HUVECs to analyze the expression of KLF4 and NF-κB p-p65. β-actin was used as the loading control. (E) Detection of the interaction between KLF4 and NF-κB by co-immunoprecipitation. *P<0.05, **P<0.01, ***P<0.001. EOFAZ, essential oil from *Alpinia zerumbet* rhizome; EndMT, endothelial-to-mesenchymal transition; TGF-β1, transforming growth factor-β1; HUVEC, human umbilical vein endothelial cell; p-, phosphorylated; KLF4, Krüppel-like factor 4; α-SMA, α-smooth muscle actin; VE-cadherin, vascular endothelial-cadherin.

VCAM1 promoter through the physical association between KLF4 and p65 (30-32). Therefore, the present study hypothesized that KLF4 similarly regulates the activation of NF-κB in the TGF-β1-induced EndMT process. To confirm binding between KLF4 and NF-κB in HUVECs treated with TGF-β1 in the absence or presence of EOFAZ, co-immunoprecipitation studies were performed. The results demonstrated that TGF-β1 enhanced the binding between KLF4 and NF-κB. However, EOFAZ treatment reduced the TGF-β1-induced interaction between KLF4 and NF-κB, although it was not determined if this effect was direct or indirect (Fig. 3E).

Effect of KLF4 siRNA transfection on KLF4 expression. Previously, it has been suggested that KLF4 serves an important role in EndMT, which is involved in the development and progression of cerebral cavernous malformations. However, to the best of our knowledge, whether the effect of EOFAZ on EndMT is mediated by KLF4 in HUVECs has not been assessed. To investigate the relationship between EOFAZ and KLF4, a KLF4-specific siRNA was utilized to knockdown the expression of KLF4 (Fig. 4A and B). The results demonstrated that the green fluorescence-labeled siRNA was successfully transferred into HUVECs, and western blotting analysis demonstrated that KLF4 expression was significantly reduced following transfection with KLF4 siRNA.

Effect of EOFAZ on TGF-β1-induced HUVEC migration following KLF4-knockdown. To evaluate HUVEC migration following transfection with KLF4 siRNA, wound healing and Transwell assays were performed. The KLF4 expression was significantly downregulated by siRNA interference (Fig. 4A and B). As shown in Fig. 4C, KLF4-knockdown significantly reduced TGF-β1-induced cell migration. Transwell assays revealed that the number of migrated cells was significantly decreased in the KLF4-knockdown cells compared with the TGF-β1 group, and EOFAZ treatment decreased the number of migrated cells under TGF-β1 stimulation (Fig. 4D). These results suggest that EOFAZ inhibition of TGF-β1-mediated EndMT induction in HUVECs may occur through downregulation of KLF4 expression.

EOFAZ inhibits TGF-β1-induced EndMT via KLF4. To determine the effect of KLF4 siRNA on EOFAZ-mediated inhibition of TGF-β1-induced EndMT, the expression of α-SMA and VE-cadherin was determined by immunofluorescence analysis. As presented in Fig. 5A, VE-cadherin expression was downregulated and α-SMA expression was upregulated in the TGF-β1 group. However, following KLF4-knockdown and EOFAZ treatment, the effects on VE-cadherin and α-SMA expression were reversed compared with the TGF-β1-treated group. Furthermore, the expression

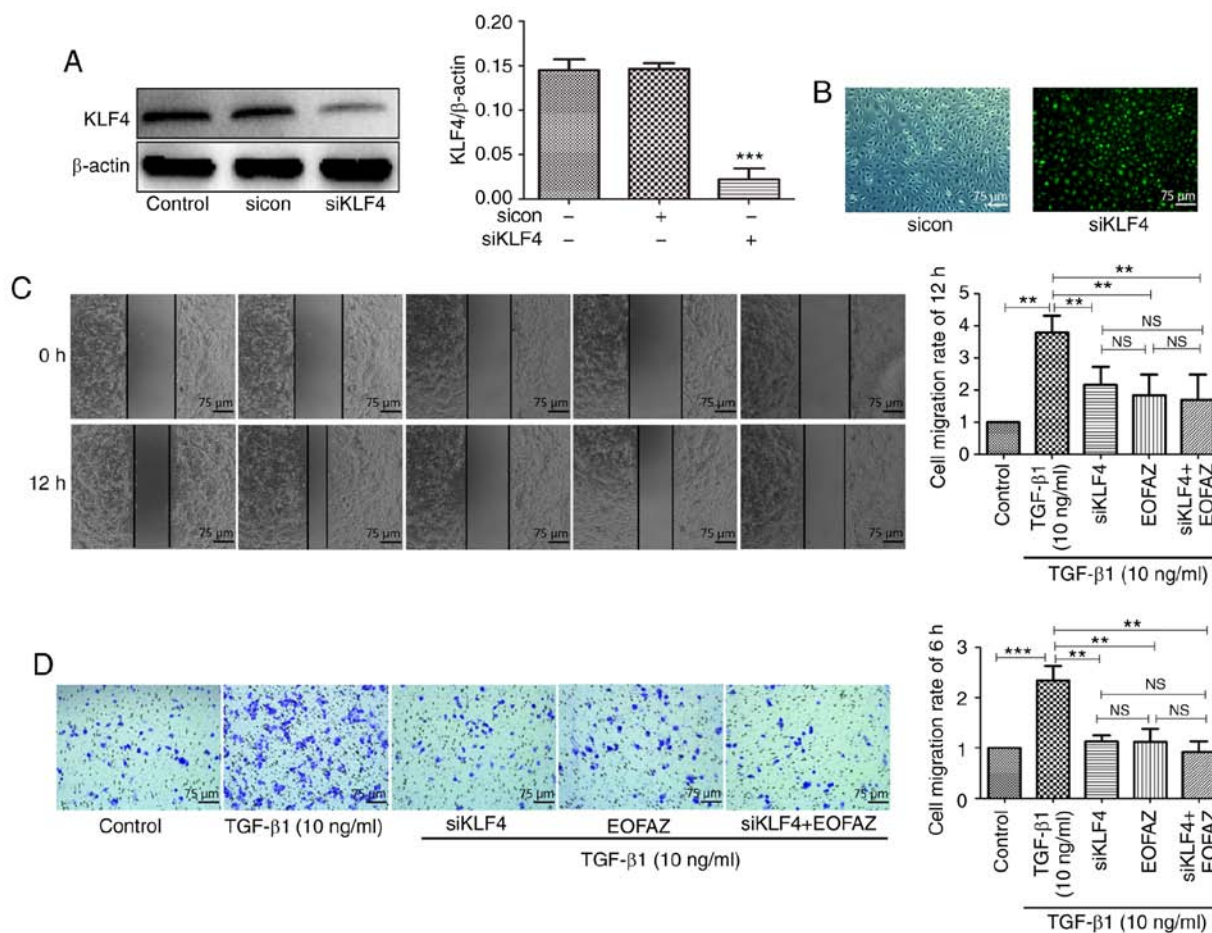


Figure 4. Effect of KLF4-knockdown on the migration in HUVECs. (A) The efficacy of siKLF4 transfection was measured by western blotting. (B) Fluorescence microscopy was used to observe whether green fluorescence-labeled siKLF4 was effectively transfected into the cells. Magnification, $\times 100$. (C) Wound healing assay of HUVECs treated with TGF- β 1, EOFAZ and siKLF4. (D) Transwell assay of HUVECs treated with TGF- β 1, EOFAZ and siKLF4. $n=5$. ** $P<0.01$, *** $P<0.001$. NS, not significant; TGF- β 1, transforming growth factor- β 1; HUVEC, human umbilical vein endothelial cell; siRNA, small interfering; KLF4, Krüppel-like factor 4; sicon, control small interfering RNA; siKLF4, KLF4 small interfering RNA; EOFAZ, essential oil from *Alpinia zerumbet* rhizome.

levels of α -SMA, Snail, VE-cadherin and NF- κ B p-p65/p65 ratio were determined by western blotting, and α -SMA, Snail, VE-cadherin were analyzed by RT-qPCR. Similar results were observed for the changes in VE-cadherin and α -SMA expression. In addition, the Snail expression and NF- κ B p-p65/p65 ratio levels were significantly repressed when exposed to siKLF and EOFAZ (Fig. 5B). Alterations in mRNA expression levels of VE-cadherin, α -SMA and Snail were consistent with the western blotting (Fig. 5C). Together, these results demonstrated that KLF4-knockdown inhibited EndMT, and there were no significant differences between EOFAZ treatment and siKLF4 treatment. These results suggest that EOFAZ regulates EndMT via KLF4.

Discussion

Endothelial cells are highly sensitive to stimuli and physiological cues in the immediate environment (33). Thus, injury of the vascular endothelium is an initial step in the pathogenesis of vascular remodeling and contributes to vascular remodeling as a source of SM-like cells, as endothelial cells can acquire a fibro-proliferative mesenchymal phenotype through EndMT under specific pathological conditions, including shear stress, oxidative stress and atherosclerosis (34). The present study

demonstrated that endothelial cells differentiate towards a SM-like phenotype via EndMT trans-differentiation when treated with TGF- β 1, and that the KLF4 signaling pathway was involved in this process. Additionally, it was shown that EOFAZ could inhibit TGF- β 1-induced EndMT via downregulation of KLF4.

EndMT is a newly recognized, fundamental biological process involved in development and tissue regeneration, as well as pathological processes, including certain complications of diabetes, fibrosis, PAH, atherosclerosis (35-38), tumor metastasis and development of fibrotic lesions in certain vital organs (32). EndMT is tightly controlled by a series of signaling networks, similar to the epithelial-to-mesenchymal transition (38). EndMT is involved in intima formation and pulmonary vascular angiogenesis (16) and may contribute to cardiac fibrosis (39); similar results have been observed in diabetes mellitus-induced cardiac fibrosis (40) and further studies confirmed that EndMT contributes to fibrogenesis associated with exposure to TGF- β 1 (41).

Consistent with these previous results, the present study demonstrated that HUVECs underwent phenotypic and biological behavioral transitions following TGF- β 1 exposure consistent with fibrogenesis. TGF- β 1 is involved in a wide range of pathological conditions, including a number of

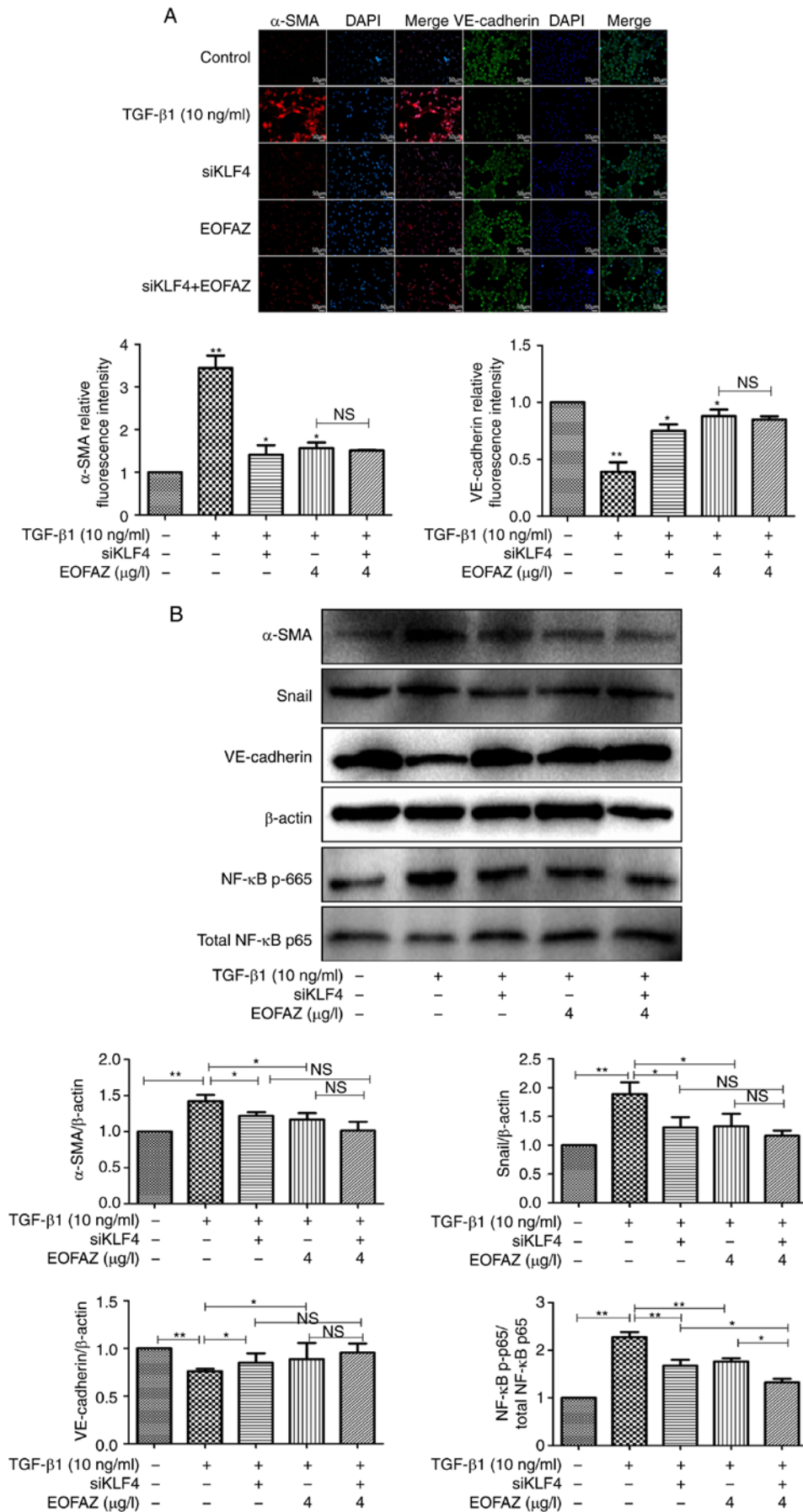


Figure 5. Effect of KLF4 knockdown on TGF- β 1-induced EndMT. (A) Effect of KLF4-knockdown on the expression levels of VE-cadherin and α -SMA was assessed using immunofluorescence. Endothelial marker protein VE-cadherin (green fluorescence), mesenchymal marker protein α -SMA (red fluorescence) and nuclei (DAPI, blue). Magnification, x200. (B) Effect of KLF4-knockdown on the expression of VE-cadherin, α -SMA, NF- κ B p-p65 and Snail were evaluated by western blotting. β -actin was used as the loading control.

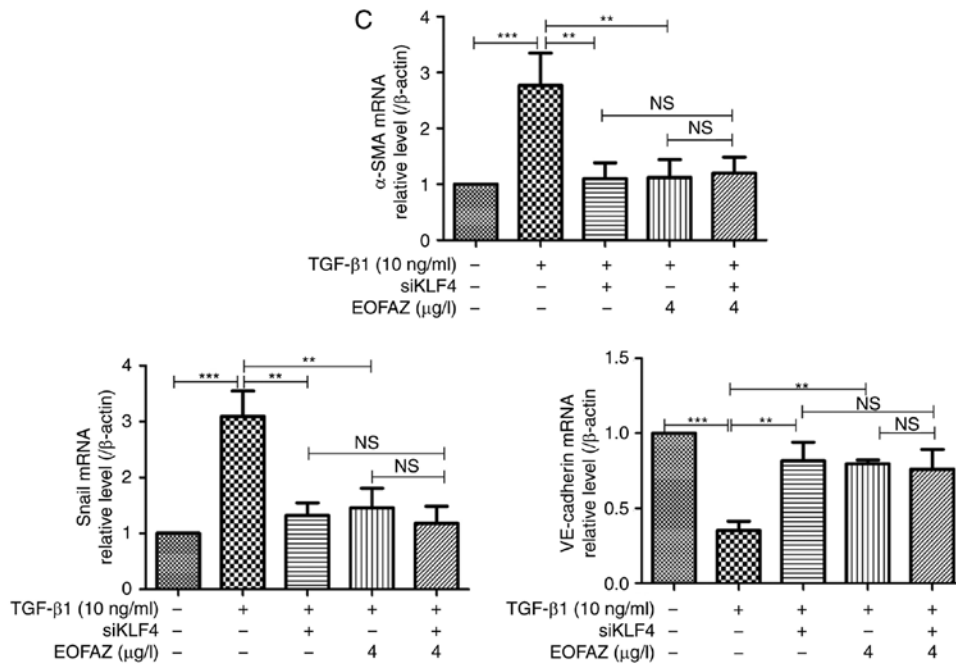


Figure 5. Continued. (C) Effect of KLF4-knockdown on mRNA expression levels of VE-cadherin and α -SMA, normalized to β -actin. n=5. *P<0.05, **P<0.01, ***P<0.001. NS, not significant; siKLF4, KLF4 small interfering RNA; p-, phosphorylated; KLF4, Krüppel-like factor 4; EOFAZ, essential oil from *Alpinia zerumbet* rhizome; N.S., not significant; TGF- β 1, transforming growth factor- β 1; α -SMA, α -smooth muscle actin; VE-cadherin, vascular endothelial-cadherin.

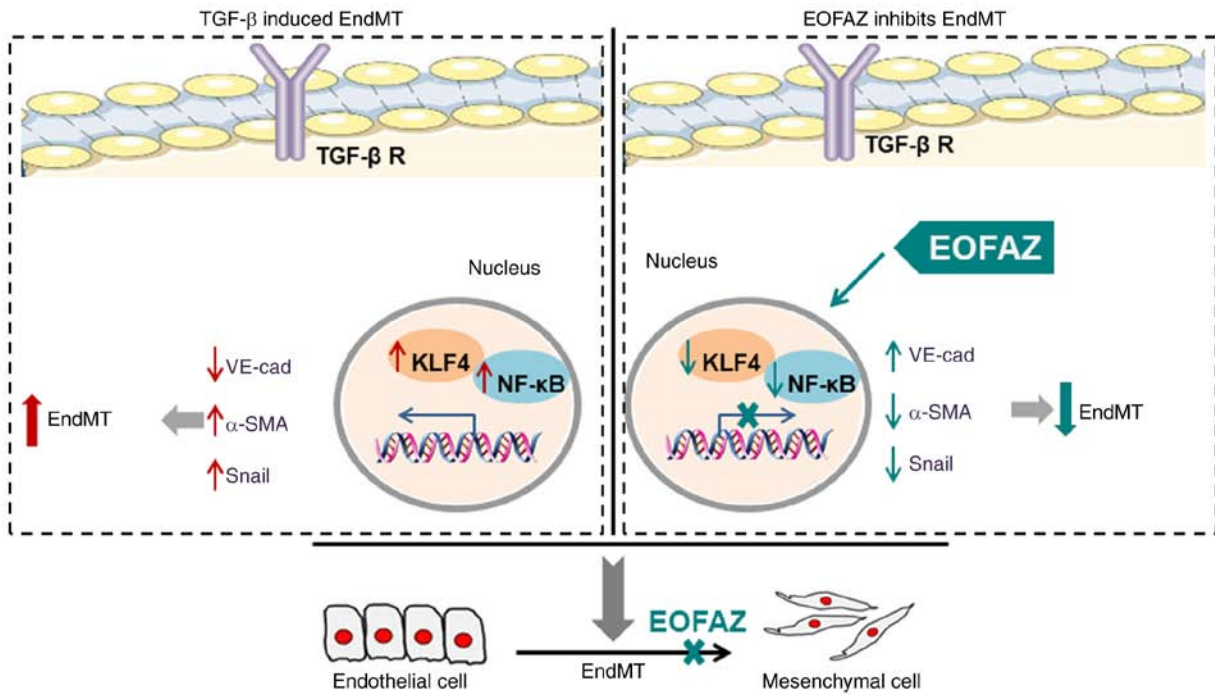


Figure 6. The inhibition effect of EOFAZ on TGF- β 1-induced EndMT. As a crucial factor for induction of EndMT, TGF- β 1 induces downregulation of the endothelial marker VE-cadherin and upregulation of the mesenchymal marker α -SMA and Snail. In addition, TGF- β 1 enhances the binding between KLF4 and NF- κ B. The present study identified that EOFAZ inhibits the migration of HUVECs upon TGF- β 1-induced EndMT via upregulating α -SMA and Snail, and downregulating VE-cadherin. Furthermore, the mechanism research demonstrated that EOFAZ could decrease KLF4 expression and subsequently prevent the combination between KLF4 and NF- κ B, which could suppress the activation of NF- κ B, as well as the stimulation of TGF- β 1. EOFAZ, essential oil from *Alpinia zerumbet* rhizome; TGF- β 1, transforming growth factor- β 1; α -SMA, α -smooth muscle actin; VE-cadherin, vascular endothelial-cadherin; HUVEC, human umbilical vein endothelial cell; EndMT, endothelial-to-mesenchymal transition; TGF- β R, transforming growth factor- β receptor.

different types of cancer (42), myocardial infarction (43), cerebral cavernous malformations (44), vascular remodeling and different types of organ fibrosis (40,41). During this process, TGF- β 1 activates EndMT via multiple pathways

including the canonical Smad-dependent and non-canonical Smad-independent pathways, and the bFGF, Wnt and Notch signaling pathways (45). A previous study has demonstrated the contribution of TGF- β 1 to EndMT in the regulation of

cardiovascular diseases (46). Therefore, any potential target to inhibit or reduce TGF- β 1/Snail signaling may exhibit potential as an anti-proliferative reagent. The present study demonstrated that TGF- β 1 treatment increased the expression of Snail, α -SMA and NF- κ B p-p65/p65 ratio, and reduced the expression of VE-cadherin, suggesting that TGF- β 1 can induce EndMT. Treatment with EOFAZ inhibited TGF- β 1-mediated induction of EndMT. Additionally, the increases in Snail and α -SMA expression and NF- κ B p-p65/p65 ratio in TGF- β 1-treated cells were significantly decreased following treatment with EOFAZ. These results suggest that EOFAZ may be a promising agent for ameliorating EndMT-related diseases.

KLF4 is an evolutionarily conserved zinc finger-containing transcription factor and is widely expressed in a range of tissues where it regulates a range of cellular processes, including cell growth, proliferation and differentiation (47). KLF4 has a critical regulatory effect on a range of functions, including maintaining intestinal epithelial homeostasis, proliferation, migration and tube formation in human retinal microvascular endothelial cells, and inhibiting NF- κ B transcriptional activity and the expression of downstream prothrombotic, proinflammatory genes (48-51). KLF4 mediates an increase in the lesions and the pseudo-normal vasculature of endothelial-specific CCM1-knockout mice (32). KLF4 also regulates VE-cadherin expression and endothelial barrier function, angiogenesis via the Notch signaling pathway, vascular tone and permeability, apoptosis, inflammation and atherothrombosis (50). KLF4 can contribute to epithelial-mesenchymal transition during the development and progression of cancer (51). Additionally, previous studies have shown that KLF4 is involved in EndMT in cerebral cavernous malformations (47). In the present study, the results demonstrated that KLF4 was excessively activated during TGF- β 1 exposure, which resulted in the increase in the degree of EndMT in HUVECs. KLF4-knockdown inhibited EndMT and decreased the protein and mRNA expression levels of Snail and α -SMA, whilst increased VE-cadherin expression. Following treatment with EOFAZ, the processes associated with EndMT were reversed. These results suggest that EOFAZ may inhibit TGF- β 1-induced EndMT through inhibition of KLF4.

In conclusion, EOFAZ significantly inhibited the extent of EndMT following TGF- β 1 exposure, a protective effect that was mediated through reduction in KLF4 activity. In sum, a graphical abstract is proposed and presented in Fig. 6, which summarizes the whole investigation. The results of the present study contribute to the understanding of the molecular mechanisms underlying EndMT and reveal novel targets, which may be the basis of potential therapeutic strategies.

Acknowledgements

The authors wish to thank Dr Di Pan (The State Key Laboratory of Functions and Applications of Medicinal Plants, School of Pharmaceutical Sciences, Guizhou Medical University, Guiyang, China) for their kind linguistic advice and technical assistance.

Funding

This study was supported by the National Natural Science Foundation of China (grant nos. 81560811, 81760725 and

U1812403-4-4), the Guizhou Provincial Key Technology R&D Program [grant no. (2020)4Y093], the Scientific and Technological Founding of Guizhou Province [grant no. QKHJ (2016)1128], the Foundation of Science and Technology Department Cooperation Program of Guizhou Province (grant no. 2016-7358) and Guizhou Graduate Research (grant no. 0711007020259).

Availability of data and materials

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

Authors' contributions

YZ performed part of the experiments and wrote the manuscript. CL performed the experiments. YH designed the study and analyzed the data. SZ performed the experiments. YX and YC analyzed the data. FJ and LT extracted the essential oil. XS designed the experiment and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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