Inhibitory Effect of Essential Oil from Fructus *Alpiniae zerumbet* on Endothelial-to-Mesenchymal Transformation Induced by TGF-β_1 and down-regulating KLF4

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Research

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Abstract

Background: Endothelial Mesenchymal Transformation (EndMT) contributes to the development of cardiovascular disease. Krüpple factor 4 (KLF4) is a zinc finger transcription factor whose N-terminus can recruit acetyltransferase to promote histone acetylation, thereby affecting the transcription activation of downstream genes. Our previous studies have shown that EOFAZ has protective effects on HUVECs oxidative stress induced by TGF-β_1. However, whether EOFAZ has a protective effect on EndMT induced by TGF-β_1 and whether it is related to the regulation of downstream signals by KLF4 has not yet been elucidated.

Methods: The protective effects of EOFAZ were evaluated in TGF-β_1-treated EndMT in Human umbilical vein endothelial cells (HUVECs). Cell mobility was evaluated by wound-healing, transwell assays and angiogenesis experiment. Western blot analysis, Quantitative real-time PCR analysis (qRT-PCR) and immunofluorescence staining were utilized to determine the expression of endothelial and mesenchymal markers , KLF4, Histone 3 acetylation and Notch/Snail signaling axis. Small interfering RNA (siRNA) and adenovirus infection were used to determine the effciency of KLF4 inhibition and overexpression. Immunoprecipitation experiments were performed to analyze protein interactions.

Results: We reported that EOFAZ has a protective effect on EndMT induced by TGF-β_1. Deletion of KLF4 inhibited EndMT induced by TGF-β_1 in HUVECs. EOFAZ pretreatment and KLF4 knockout reduced the migration ability of HUVECs , and increased endothelial markers accompanied by decreased mesenchymal markers, meanwhile caused the change of Notch/Snail signal axis. In addition, TGF-β_1 upregulated the expression of KLF4, while the high expression of KLF4 promoted the acetylation of histone H3, and there was a protein interaction between the acetylated histone H3 and KLF4.

Conclusions: These results suggest that TGF-β_1 may promote the acetylation of histone H3 and activate the transduction of Notch/Snail signal axis by up-regulating the expression of KLF4, which may induce EndMT and this effect may be reversed by EOFAZ. Therefore, EOFAZ may inhibit EndMT induced by TGF-β_1 by down-regulating KLF4 expression.

1. Background

Cardiovascular diseases(CVD) have seriously endangered human health[1,2], so it is particularly important to find the appropriate drugs and treatment strategies. Studies have demonstrated that many cardiovascular risk factors first act on vascular endothelial cells(VECs). It can cause VECs function damage under the action of many kinds of stimulating factors and injury factors, and the secretion of cytokines also changes , the most typical of which is the abnormal increase of TGF-β. The function and morphology of endothelial cells are excessively transformed into fibroblasts, and EndMT occurs[3]. EndMT is a process in which endothelial cells(ECs) lose adhesion and polarity, meanwhile lose their specific markers such as vascular endothelial cell cadherin(VE-cadherin)and obtain mesenchymal cell phenotypes such as alpha-smooth muscle actin(α-SMA). As a result, ECs are transformed into

mesenchymal cells and the ability of cell migration and collagen secretion are increased[4]. EndMT process plays an important role in cardiac valve formation. Pathological EndMT can lead to congenital valvular disease. Studies have shown that the degree of coronary atherosclerosis in patients is closely related to the activation degree of EndMT and endothelial TGF-β signal[3]. When the TGF-β/Smad signaling pathway is activated, the EndMT process can be triggered[5]. After the formation of the heteropeptide Smad complex into the nucleus, the transcriptional response of the specific target gene in the Snail, Twist1, Slug nucleus can be regulated by synergistic interaction with other transcription factors[6]. EC migration ability is enhanced, which continuously induces vascular endothelial dysfunction and accelerates the occurrence and development of CVD[7,8]. Therefore, effective intervention and reversal of EndMT induced by TGF-β and inhibition of vascular remodeling are new research directions and ideas to improve cardiovascular function.

Krüpple like factor 4(KLF4) is a transcription factor containing zinc finger. KLF4 can enhance TGF-β signaling through the transforming growth factor-β receptor(TβR) , and KLF4 itself is also regulated by TGF-β[9]. It has been reported that KLF4 can directly regulate the expression of some EndMT marker proteins such as fibroblast marker protein(Fsp1) and stem cell marker(Sca1)[10], which has attracted our attention. Notch signal plays an important role in the development of many kinds of organisms. The activation of Notch signal leads to the cleavage and release of the intracellular domain and then enters the nucleus to regulate the downstream target molecules, including Snail which is related to EndMT[11], thus promoting the process of EndMT induced by TGF-β_1[12]. Among them, Snail as a zinc finger transcription factor, mainly promotes the occurrence of EndMT by inhibiting the activity of VE-cadherin. The above studies show that Notch signal is a new target closely related to EndMT and atherosclerotic process, and Snail as a downstream of Notch, is also an important "protein with EndMT switching effect". Therefore, we speculate that Notch/Snail signal axis is involved in regulating EndMT. Other studies have shown that KLF4 binds to the human Notch-1 promoter, which inhibits the expression of KLF4 in human breast cancer cells and leads to the decrease of Notch-1 expression[13]. It indicates the potential molecular cascade mechanism between KLF4 and Notch signaling pathway. Kim et al.[14] found that increasing the acetylation level of histone H3 could induce endothelial dysfunction and accelerate the progression of atherosclerotic disease. Transcription factor Snail forms a complex with histone deacetylase and inhibits its transcription in the process of pancreatic cancer[15,16], indicating that histone acetylation modification may be involved in regulating the transcription process of Snail. Studies have confirmed that the N-terminal of KLF4 contains a strong transcriptional activation domain, which can recruit p300/CBP-bingding protein-associated factor(PCAF) to specific DNA binding sites of target genes and promote the histone H3 acetylation[17], and then activate the gene transcriptional activation[18]. This suggests that the transcriptional regulation of KLF4 and Notch/Snail signal axis may be related to the histone acetylation modification.

Alpinia zerumbet (Pert) Burtt et.Smith is a prominent member of Zingiberaceae which belongs to the ginger family. It is a traditional medicine in the minority areas of Guizhou Province and listed on “The quality Standard for traditional Chinese Medicine and ethnic Medicine in Guizhou Province” published in

2003. A dry and ripe fruit of *Alpinia zerumbet* has been widely used for warm middle dryness and dampness, relieving pain by activating qi, intercepting malaria, etc[19]. The main active ingredient of *Alpinia zerumbet* is EOFAZ, which has the effects of anti-inflammation and analgesia, antioxidation, anti-atherosclerosis[20] and so on. In recent years, our research group has carried out a lot of research work on the cardiovascular pharmacological activity of EOFAZ, which proves that it plays a role in cardiovascular protection mainly through the regulation of vascular endothelial function. We have previously reported that EOFAZ inhibits inflammatory injury of Human Aortic Endothelial cells induced by lipopolysaccharide by regulating NF-κB signal[21]. In addition, EOFAZ has protective effect on HUVECs oxidative stress induced by TGF-β_1[22]. Combined with previous studies, we propose that the protective effect of EOFAZ on vascular endothelium is based on the regulation of endothelial mesenchymal transition to play a vascular activity?

In this study, we investigated the effect and underlying molecular mechanisms of EOFAZ on EndMT induced by TGF-β_1 in HUVECs. We found that EOFAZ may mediate histone H3 acetylation modification by down-regulating the expression of KLF4, and regulate the level of the Notch/Snail signal axis to inhibit EndMT induced by TGF-β_1. These results are helpful to find the molecular markers and intervention targets for the early diagnosis of the key pathological process of EndMT, and also provide an experimental support for the therapeutic use of EOFAZ for prevention and treatment of cardiovascular diseases.

2. Methods

2.1 Extraction and preparation of EOFAZ

The essential oil was extracted from the fruit of *Alpinia Zerumbet*, which was collected in Zhenfeng County, Guizhou province, China. The fruit was identified by Professor Qingde Long who worked for the department of Pharmacognosy in Guizhou Medical University .The volatile oil was obtained by Soxhlet extractor, and the extraction rate was 1.3%. Gas chromatography-mass spectrometry (GC-MS) was used to analyze the chemical constituents of volatile oil from whole fruit of medicinal materials[23,24]. The EOFAZ was exactly extracted from the analytical balance and dissolved with dimethyl sulfoxide(DMSO). The concentration of stock liquor was 1×10^7 μg·L^{-1} and the microporous filter membrane was sterilized and placed at 4°C. Different concentrations of solutions were prepared by medium in the experiment.

2.2 Reagents

Transforming growth factor-β_1 (TGF-β_1) was purchased from Peprotech (NJ,USA). Human umbilical vein endothelial cells(HUVECs) and Endothelial cell culture medium (ECM) were purchased from Sciencell (CA,USA). Transforming growth factor-β_1 inhibitor (LY2109761) was purchased from Selleck (TX,USA). KLF4 small interference RNA was purchased from Genepharma (Shanghai,China). KLF4 adenovirus transfection reagent(HBAD-EGFP, HBAD-h-KLF4) was purchased from Hanheng Biotechnology Co.,Ltd (Shanghai,China). Lipofectamine 2000 reagent was purchased from Invitrogen (CA,USA). The Transwell

chamber and Immobilon western chemiluminescence reagent were purchased from Millipore (MA,USA). Matrigel was purchased from BD Biosciences (NY,USA). TransZol Up Plus RNA Kit was purchased from Quanshijin Biotechnology Co., Ltd. (Beijing, China). Immunoprecipitation kit was purchased from Sangon Biotech (Shanghai,China). Protein quantitative kit was purchased from Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Cell Lysates(RIPA) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Antibody directed against vascular endothelial cadherin (VE-cadherin), *α*-smooth muscle actin (*α*-SMA), zinc finger transcription factor Snail, Krüpple-like factor 4 (KLF4), Notch-1, Notch-3, Histone H3, Acetyl-Histone H3 and p300 were from Cell Signaling technology (MA,USA). Antibody directed against histone deacetylase 2 (HDAC2) was from Proteintech (Hubei, China). Antibody directed against β-actinwas from Affinity Biosciences (Shenzhen,China). Antibody directed against GAPDH and horseradish peroxidase (HRP)-conjugated secondary antibody (1: 7000 dilution)were from Bioworld (Nanjing, China).

2.3 Transient transfection

siKLF4 sequence is as follows: 5'-GGACUUUAUUCUCUCCAAUTT-3' , 5'-AUUGGAGAGAAUAAAGUCCTT-3'. According to the manufacturer's instructions, when the cell fusion rate was 60%, HUVECs were transfected with Lipofectamine 2000. After 6 hours, they were replaced with 5%ECM and cultured for 24 hours. The adenovirus infection index (virus number: cell number) was 30:1. HBAD-h-KLF4 4 μL was added to the 2 mL system and changed to normal medium after 6 h, then cultured the cells for 24 hours. The knockout and overexpression efficiency of KLF4 were detected by Western blotting. The cells successfully transfected were used in subsequent experiments.

2.4 Cell Culture and Treatment

HUVECs cultured in Endothelial Cell Medium containing 5% fetal bovine serum and incubated in a humidifier at 37 °C and 5% CO_2. HUVECs at passages 4-5 were used in this study. Cells were assigned to the following experimental groups: Control group(5%ECM), TGF-β_1 group(10ng·mL^{-1}), Inhibitor of TGF-β_1 group(LY2109761, 2μmol·L^{-1} and TGF-β_1, 10ng·mL^{-1}), siKLF4+ TGF-β_1 group(siKLF4 and TGF-β_1, 10ng·mL^{-1}). Control group(5%ECM), TGF-β_1 group(10ng·mL^{-1}), siKLF4 group, siKLF4+ TGF-β_1 group(siKLF4 and TGF-β_1, 10ng·mL^{-1}), EOFAZ high dose(EOFAZ-H, 4μg·L^{-1} and TGF-β_1, 10ng·mL^{-1}), EOFAZ low dose(EOFAZ-L, 1μg·L^{-1} and TGF-β_1, 10ng·mL^{-1}). Control group(5%ECM), TGF-β_1 group(10ng·mL^{-1}), EOFAZ group(EOFAZ, 4μg·L^{-1} and TGF-β_1, 10ng·mL^{-1}). Control group(5%ECM), Ad-NC group(HBAD-EGFP, 5%ECM), Ad-KLF4 group(HBAD-h-Null-KLF4, 5%ECM). Control group(5%ECM), TGF-β_1 group(10ng·mL^{-1}), AdKLF4+TGF-β_1 group(AdKLF4 and TGF-β_1, 10ng·mL^{-1}), siKLF4+ TGF-β_1 group(siKLF4 and TGF-β_1, 10ng·mL^{-1}), EOFAZ group(EOFAZ, 4μg·L^{-1} and TGF-β_1, 10ng·mL^{-1}). Cell transfection was carried out according to the method in section 2.3. After pre-incubation with EOFAZ for 2 h, TGF-β_1 was added and incubated for 72 h to induce EndMT.

2.5 Wound-healing assay and Transwell migration assay

For wounding-healing assay, 1×10^4 $\cdot mL^{-1}$ cells were grown to confluence in 24-well plates. The cell monolayer was scratched using a 10-μl pipette tip and the drugs prepared by serum-free medium were added.Representative photographs were taken after 12hunder inverted microscope. Five marked points were randomly selected to determine the data and calculate the mobility. Mobility = (average value of 0h scratch distance-average value of 12h scratch distance) / average value of 0h scratch distance. For Transwell migration assay, 2×10^4 cells were plated into the upper chamber of Transwell inserts precoated in 24-well plates. The lower chamber was filled with ECM supplemented with 5% FBS. After incubation for 6h at 37 °C, cells that moved to the bottom surface of the chamber were stained with 0.1% crystal violet and counted under an inverted microscope.

2.6 Angiogenesis experiment

The Metrigel and serum-free ECM were diluted in a proportion of 1:1, evenly spread in 24-well plates and coagulated in a incubator for 30 minutes, then $2\times10^5\cdot mL^{-1}$ cells were inoculated in 24-well plates. Representative photos were taken and counted after 4-6 hours.

2.7 Immunofluorescence staining

The cells were inoculated in a 24-well plate with a density of 1×10^4 mL^{-1}, and the cells were fixed with 4% paraformaldehyde for 15 min after treatment as described above. The cells were incubated with anti-VE-cadherin, anti-*α*-SMA antibody (1:50 dilution; CST) or anti-Histone H3, anti-Acetyl-Histone H3(1:200 dilution;CST) followed by Alexa Fluor 488/cy3-conjugated secondary antibody. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole;1:1000 dilution; Sigma-Aldrich). Stained cells were visualized using a fluorescence microscope and the fluorescence intensity was detected by enzyme labeling instrument.

2.8 Western blotting

Cells were lysed in RIPA lysis buffer containing protease inhibitors and then loaded on SDS-polyacrylamide gels for electrophoresis before transfer to PVDF membrane. After blocking non-specific binding sites, the membranes were probed with the first antibody mentioned above at 4°C. Blots were incubated with horseradish peroxidase-conjugated secondary antibody, and detected by chemiluminescence. The immunoreactive bands were produced by the Syngene Gel Imaging system (Bio-Rad, California, USA) and quantified using ImageLab 4.0 software. All antibodies and dilution ratio were listed in Table 1.

Table 1 Specifc antibodies for Western blot

Antibody	Company	Catalogue number	Dilution
VE-cadherin	CST	2500	1:1000
α-SMA	CST	19245	1:1000
Snail	CST	3879	1:1000
KLF4	CST	4038	1:1000
Notch-1	CST	3608	1:1000
Notch-3	CST	5276	1:1000
Histone H3	CST	4499	1:1000
Acetyl-Histone H3	CST	9649	1:1000
p300	CST	86377	1:1000
HDAC2	Proteintech	12922-3-AP	1:1000
β-actin	Affinity Biosciences	T0022	1:7000
GAPDH	Bioworld	MB001	1:10000

2.9 Quantitative real-time PCR analysis（qRT-PCR）

Total RNA was extracted using TransZol Up Plus RNA Kit and reverse transcribed to cDNA followed by Real-time PCR reaction. Thermal profile conditions were as follows: preincubation at 94 °C for 30s followed by 39 cycles of amplification at 94 °C for 5s and 56 °C for 30s. VE-cadherin, α-SMA, KLF4, Snail, Notch-1 and Notch-3 transcript levels were calculated by the $2^{-\Delta\Delta CT}$ method. All primers above were synthesized by Sangon (Shanghai, China). Primer sequence is show in Table 2.

Table 2 Specifc primers for quantitative RT-PCR

Gene	Forward	Reverse
VE-cadherin	CAAGGACACTGGCGAAA	ACGCATTGAACAACCGA
α-SMA	CGTGGCTATTCCTTCGTT	ACGTTCATTTCCGATGGT
Collagen Ⅰ	GCCTCAACATCCCCTACA	CAGCCCACGAAGAACAGA
Snail	ACATCCGAAGCCACACG	TGGGGACAGGAGAAGGG
KLF4	AGGAGCCCAGCCAGAAA	TCCAGTCACAGACCCCATC
Notch-1	AGGCTCTGCCGACATCA	AGGAAGGGGTGCTCTGG
Notch-3	GCTGTTCCCCTTGACTGG	CTTTGTGGGGCTGCTGT
β-actin	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

2.10 Immunoprecipitation assay

When the cells were harvested at the end of observation, an appropriate amount of IP cleavage buffer (including protease inhibitor) was added, then cracked on ice for 30 minutes after ultrasound, and the supernatant was absorbed after centrifugation. A small amount of lysate was taken for Western blot analysis, and the remaining lysate was added 1ug antibody and 18ul protein A/G-beads to the cell lysate and incubated overnight at 4 ℃. After immunoprecipitation reaction, centrifugation for 5 minutes, protein A/G-beads was washed with lysate for 6-7 times. Finally, adding appropriate volume of loading buffer and heating at 95℃for 5 minutes, SDS-PAGE gel electrophoresis was carried out.

2.11 Statistical analysis

Statistical significance was calculated by Student's t-test. The variance homogeneity test and One-Way ANOVA analysis were used for the comparison among groups. A value of $p<0.05$ was considered significant. Datas are expressed as mean ± S.D. Each experiment was performed in triplicate.

3. Results

3.1 EndMT is induced by TGF-β_1 in HUVECs

The increase of exogenous and endogenous TGF-β is an effective inducer of EndMT[25]. The expression levels of VE-cadherin, α-SMA and Collagen Ⅰ were determined by qRT-PCR. After HUVECs was treated in endothelial cell medium with or without 10 ng·mL^{-1} TGF-β_1 for five time points (24,48,72,96 and 120 h), the expression level of VE-cadherin, α-SMA and Collagen Ⅰ were time-dependent during the period of 24 to 72 h. As shown in figure 1, when HUVECs was treated with 10 ng·mL^{-1} TGF-β_1 for different time, the changes of VE-cadherin, α-SMA and Collagen Ⅰ were the most significant at 72 h, so we chose 10 ng·mL^{-1} TGF-β_1 to incubate HUVECs for 72 h to induce EndMT model.

3.2 KLF4-knockdown inhibits TGF-β_1 induced EndMT in HUVECs

Several lines of evidence have established a link between KLF4 and EMT. When inhibiting the expression of KLF4 in prostate cancer cells, it can effectively inhibit the occurrence of EMT in vitro, and play an important role in inhibiting the migration and invasion of tumor cells[26]. To explore the role of KLF4 in the EndMT progression, We knocked down the expression of KLF4 through KLF4 specific siRNA and the efficiency is 60%(Fig.2A). In TGF-β_1-induced EndMT model, knockout of KLF4 and addition of TGF-β_1 inhibitor (LY21097651) increased the expression of endothelial marker VE-cadherin and inhibited the expression of mesenchymal marker α-SMA and "protein with EndMT switching effect" Snail (Fig.2B). We found that knockout of KLF4 gene can block the EndMT progression induced by TGF-β_1 in HUVECs.

3.3 EOFAZ and KLF4-knockdown inhibit TGF-β_1 induced EndMT

Next, we measured the changes in the levels of EndMT-related genes. Immunofluorescence analysis confirmed the upregulation of VE-cadherin in parallel with reduction of α-SMA in KLF4-depleted and EOFAZ pretreatment cells(Fig.3A). qRT-PCR and Western blot analysis consistently revealed that KLF4 silencing and EOFAZ pretreatment raised the expression of VE-cadherin and suppressed the expression of α-SMA and KLF4 in TGF-β_1 treated HUVECs(Fig.3B,C). These results suggest that KLF4 silencing and EOFAZ pretreatment block the EndMT process induced by TGF-β_1, and the protective effect of EOFAZ on EndMT may be based on the regulation of KLF4.

3.4 EOFAZ and KLF4-knockdown attenuate TGF-β_1 induced HUVECs migration

Vascular endothelial cells confer a high degree of invasiveness and migration when it loss-specific marker expression while obtaining the expression of mesenchymal cells or myofibroblasts phenotype induced by TGF-β_1. Next, we asked whether KLF4 is involved in migration induced by TGF-β_1 in HUVECs. When endogenous KLF4 was downregulated and EOFAZ pretreatment , the migration of HUVECs induced by TGF-β_1 was remarkably impaired(Fig.4A,B). In vitro angiogenesis has been used as a model to study events such as endothelial cell migration, invasion, and differentiation into capillaries. The number of tubules was significantly reduced after KLF4 silencing and EOFAZ pretreatment(Fig.4C). The results indicated that EOFAZ and silencing KLF4 could reduce the migration ability of HUVECs induced by TGF-β_1.

3.5 EOFAZ and KLF4-knockdown reverse the expression of Notch/Snail signal axis induced by TGF-β_1

Notch signal can synergistically regulate cell self-renewal and differentiation with KLF4[27]. At the same time, Notch/Snail signal axis also plays an important role in TGF-β-induced EndMT in the study of HUVECs[28]. Therefore, we proposed whether KLF4 can regulate Notch/Snail signal axis in TGF-β_1-induced EndMT. The results of Western Blot and qRT-PCR showed that after EOFAZ intervention and KLF4 silencing, the expression level of Notch-1 and its downstream target Snail were significantly reduced, and the expression level of Notch-3 was increased (Fig. 5A, B). The above results confirmed that KLF4 can be

used as upstream signal to regulate Notch/Snail signal axis, meanwhile EOFAZ down-regulated KLF4 expression and regulated Notch/Snail signal axis. It also suggested that the two members of the Notch family, Notch-1 and Notch-3, may have different molecular signaling mechanisms in EndMT induced by TGF-β_1.

3.6 KLF4 overexpression increases the level of histone H3 acetylation in HUVECs

Literature studies have shown that the N-terminal of KLF4 contains a strong transcriptional activation domain, which can recruit HATs family protein p300 to the binding site of transcription factors in the promoter region of related target genes, and promote histone H3 acetylation induced by TGF-β_1[17]. Our experiments showed that transcription factor KLF4 expression was increased and Notch/Snail signal axis was up-regulated during EndMT induced by TGF-β_1. Based on the effect of histone acetylation on gene transcription, the effect of KLF4 on the histone H3 acetylation was analyzed. In this study, the high expression of KLF4 was achieved by stable adenovirus transfection with HUVECs. The results of Western blot showed that overexpression of KLF4 significantly increased the protein expression level of Acetylated-histone H3, but had no obvious effect on the protein expression level of histone H3 (Fig. 6). The results confirmed that the high expression of KLF4 up-regulated the acetylation level of histone H3. The results suggest that KLF4 promotes histone H3 acetylation, which provides a looser chromatin environment for gene transcription, facilitates gene transcriptional regulation, and may affect the expression level of Notch/Snail signal axis.

3.7 EOFAZ reduces histone H3 acetylation in EndMT induced by TGF-β_1

The results of immunoprecipitation experiment confirmed that there was protein binding between KLF4 and acetyl-histone H3, and TGF-β_1 incubation significantly increased the expression level of KLF4 and acetyl-histone H3, while EOFAZ could reduce the binding between them (Fig. 7A). Immunofluorescence staining showed that the expression level of histone H3 itself did not change after TGF-β_1 stimulation, but the expression of acetyl-histone H3 increased significantly, while the expression of acetyl-histone H3 decreased significantly after EOFAZ treatment (Fig. 7B). The above results suggest that there is a binding mode between KLF4 and Acetyl-histone H3, and the administration of EOFAZ can reduce the acetylation level of histone H3. It also suggests that histone H3 acetylation may be a potential molecular mechanism involved in TGF-β_1-induced EndMT.

3.8 EOFAZ and KLF4-knockdown promote histone H3 deacetylation and inhibit the expression of Notch/Snail signal axis in EndMT induced by TGF-β_1

The expression levels of p300 and HDAC2 that affecting histone acetylation modification were analyzed by Western blot. As shown in fig. 8, the expression of acetyl-histone H3 and p300 were further up-regulated and the expression of HDAC2 was further down-regulated in AdKLF4+TGF-β_1 group. However, compared with TGF-β_1 group, the expression levels of acetyl-histone H3, p300 and HDAC2 were reversed after EOFAZ treatment and KLF4 silencing. Next, the effect of KLF4 on Notch/Snail signal axis was

analyzed by Western blot. The results showed that EOFAZ intervention and KLF4 silencing could significantly down-regulate the expression of Notch-1 and its downstream target molecule Snail caused by TGF-β_1 and KLF4 overexpression, and at the same time significantly up-regulate the expression level of Notch-3. The results showed that EOFAZ may promote histone H3 deacetylation by down-regulating KLF4 expression, thus regulating the level of Notch/Snail signal axis.

4. Discussion

Cardiovascular diseases are currently the main cause of human death[29]. Under the influence of internal and external damage factors, cells can be transformed into mesenchymal cells through the EndMT process[30], and the expression of endothelial specific markers is reduced, such as platelet endothelial cell adhesion molecules CD31 and Vascular endothelial cadherin (VE-cadherin) are accompanied by increased expression levels of mesenchymal cell markers such as α-smooth muscle actin (α-SMA)[31].In EndMT endothelial microenvironment, endothelial cell migration ability is significantly enhanced, accompanied by a large amount of extracellular matrix protein secretion, and its excessive deposition can further exacerbate endothelial dysfunction.Therefore, the search for EndMT-targeted drugs plays an important role in the treatment of cardiovascular system diseases.

TGF-β/Smad signal pathway is a classic signal pathway in the process of EndMT. Other signal pathways such as Wnt/β-catenin, Notch and various receptor tyrosine kinases have also been proved to activate EndMT[32]. It has been reported that Notch signal transduction is related to TGF-β, Snail, Slug and other growth factors and transcription factors[33,34]. Activation of Notch pathway can promote the expression of Snail and Slug, thereby inhibiting the expression of epithelial marker E-cadherin[35]. The above results indicate that Notch is involved in the EndMT process which is closely related to atherosclerosis. At the same time, Notch can act as an upstream to regulate the expression of "protein with EndMT switching effect" Snail, suggesting that Notch/Snail signal axis regulates endothelial cell biology which plays an important role in the EndMT process. Although Notch has determined cell fate and differentiation in a variety of cells, the molecular basis of Notch upstream regulation is still poorly understood. It is known that transcription factor Krüppel-like factor 4 (KLF4) is a key regulator of endothelial cell biology, and the increase of its transcriptional activity can promote the occurrence of EndMT in endothelial cells of cavernous cell tumor intima[10]. Studies have found that KLF4 can be used as an upstream regulator of angiogenesis to differentially regulate the expression of Notch receptors, ligands and target genes[36]. The interaction between Notch and KLF4 has been shown in mouse models and in terminal differentiation of human epithelial cell lines[37,38]. Therefore, it is interesting to understand the regulatory mechanism between KLF4 and Notch/Snail signal axis.

EOFAZ is a volatile oil extracted from the fruit of *Alpinia zerumbet*, a traditional medicine of ethnic minorities in Guizhou. It has pharmacological effects such as protecting endothelium damage and inhibiting vascular remodeling[39,40], and shows unique advantages in the treatment of cardiovascular diseases. However, the underlying mechanism of its protective effect on EndMT is currently under research. We extracted volatile oil from the mature fruits of *Alpinia zerumbet* and analyzed its chemical

composition by gas chromatography-mass spectrometry (GC-MS)[41].We have previously demonstrated that α-pinene, β-pinene, 1,8-eucalyptol, and camphene have significant protective and regulatory effects on vascular endothelial cells, while EOFAZ can protect HUVEC cell death induced by lipopolysaccharide in the order of α-pinene > 1, 8-eucalyptol > β-pinene[42]. In this study, we first found that TGF-β_1 promoted the expression of α-SMA and KLF4 and enhanced the migration ability of HUVECs, which was consistent with other reports[43], but EOFAZ blocked these effects induced by TGF-β_1 in vitro. To understand the mechanism by which EOFAZ protects TGF-β_1-induced EndMT, we knocked out KLF4. The results show that the deletion of KLF4 can inhibit the process of EndMT, and KLF4 can be used as an upstream regulator of Notch/Snail signal axis.

Histone acetylation is an important mechanism of gene epigenetic regulation, which is mainly catalyzed by histone acetylases (HATs) and histone deacetylases (HDACs). As important regulators of gene expression, HATs and HDACs control the dynamic balance of the internal environment in the body. It has been found that they can interact with some EndMT transcription factors and acetylate them[44]. As an important transcription factor of EndMT, Snail is also a downstream target molecule of Notch signal. Therefore, we speculated that histone acetylation modification may be involved in transcriptional regulation of Notch/Snail signal axis. It has been reported that KLF4 can recruit HATs family protein p300 to transcription factor binding sites in the promoter region of vascular smooth muscle cell differentiation-related genes, and promote histone H3 acetylation induced by TGF-β_1[17]. Histone H3 acetylation provides a looser chromatin environment and facilitates the activation of gene transcription[18]. Therefore, we propose whether the regulation of Notch/Snail signal axis by KLF4 is related to histone H3 acetylation. The results showed that TGF-β_1 upregulated the level of KLF4, increased the acetylation level of histone H3, activated the transduction of Notch/Snail signal axis, and increased the expression level of Snail. Our experimental study found that EOFAZ may inhibit EndMT by down-regulating the level of KLF4 and affecting the deacetylation of histone H3, thereby inhibiting the level of Notch/Snail signal axis.

In summary, in this study that siRNA technique and adenovirus transfection were used to realize the knockdown and overexpression of KLF4 in endothelial cells in vitro, and we analyzed the possible mechanism of KLF4 regulating histone H3 acetylation so as to inhibit Notch/Snail signal axis , and further clarify the effect of EOFAZ on interventing and reversing EndMT. Our results provide strong support for the use of EOFAZ in the prevention and treatment of cardiovascular disease among ethnic minorities in southern China. The precise regulation mechanism of EOFAZ on KLF4 and the active components responsible for pharmacological action of EOFAZ need to be further studied. The focus of our next work is to explore whether using a mixture or a single key ingredient of EOFAZ has better pharmacological effects.

5. Conclusions

EOFAZ may inhibit the EndMT process induced by TGF-β_1 by down-regulating the level of KLF4, promoting the deacetylation of histone H3 and regulating the transduction of Notch/Snail signal axis(Fig

9), thereby providing a theoretical basis for clinical treatment of CVD.

6. Abbreviations

CVD, cardiovascular disease; TGF-β_1, transforming growth factor-β_1; TβR, transforming growth factor-β receptor; EOFAZ, essential oil of Fructus *Alpiniae zerumbet*; HUVECs, human umblical vein endothelial cells; EndMT, endothelial-to-mesenchymal transition; EOFAZ-H, high dose of EOFAZ; EOFAZ-L, low dose of EOFAZ; Acetyl-histone H3, acetylated-histone H3; ECM, endothelial cell medium; KLF4, Krüpple-like factor 4; HATs, histoneacetylases; HDACs, histonedeacetylases; HBAD-h, HBAD-h-Null-KLF4; PCAF, p300/CBP-bingding protein-associated factor; siRNA, small interference RNA; VE-cadherin, vascular endothelial cell cadherin; α-SMA, alpha-smooth muscle actin.

Declarations

7. **Conflict of interest**

The authors declare no conflict of interest.

8. **Acknowledgements**

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9. **Author Contributions**

S.Z., Y.Z. conceived experiments and wrote and edited the manuscript. S.Z., L.H., X.S. designed experiments and analyzed data. M.H., X.T. performed research and contributed to the discussion. Y.Z. and X.S. are the guarantor of this work and, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors read and approved the fnal manuscript.

10. **Availability of data and materials**

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

11. **Consent for publication**

Not applicable.

12. **Ethics approval and consent to participate**

Not applicable.

13. **Funding**

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Tables

Table 1 Specifc antibodies for Western blot

Antibody	Company	Catalogue number	Dilution
VE-cadherin	CST	2500	1:1000
α-SMA	CST	19245	1:1000
Snail	CST	3879	1:1000
KLF4	CST	4038	1:1000
Notch-1	CST	3608	1:1000
Notch-3	CST	5276	1:1000
Histone H3	CST	4499	1:1000
Acetyl-Histone H3	CST	9649	1:1000
p300	CST	86377	1:1000
HDAC2	Proteintech	12922-3-AP	1:1000
β-actin	Affinity Biosciences	T0022	1:7000
GAPDH	Bioworld	MB001	1:10000

Table 2 Specifc primers for quantitative RT-PCR

Gene	Forward	Reverse
VE-cadherin	CAAGGACACTGGCGAAA	ACGCATTGAACAACCGA
α-SMA	CGTGGCTATTCCTTCGTT	ACGTTCATTTCCGATGGT
Collagen Ⅰ	GCCTCAACATCCCCTACA	CAGCCCACGAAGAACAGA
Snail	ACATCCGAAGCCACACG	TGGGGACAGGAGAAGGG
KLF4	AGGAGCCCAGCCAGAAA	TCCAGTCACAGACCCCATC
Notch-1	AGGCTCTGCCGACATCA	AGGAAGGGGTGCTCTGG
Notch-3	GCTGTTCCCCTTGACTGG	CTTTGTGGGGCTGCTGT
β-actin	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT

Figures

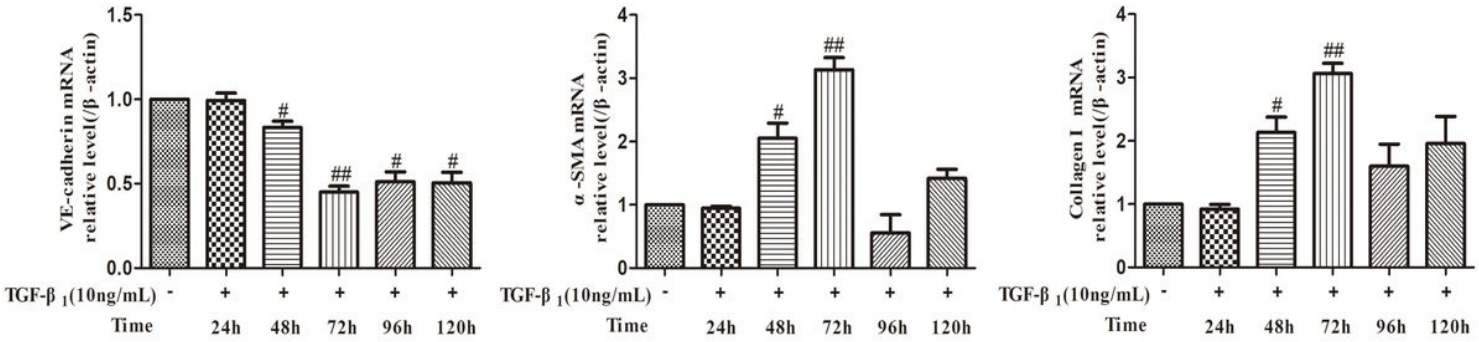

Figure 1

Effect of TGF-β1 stimulation on the expression of EndMT markers. Equal number of HUVECs treated with 10 ng·mL-1 TGF-β1 and different time. The expression of VE-cadherin, α-SMA and Collagen Ⅰ were detected by Quantitative real-time PCR analysis. All experiments were repeated at least three independent times. Data were normalized with relevant loading controls and then presented as mean ± SD. ## $p < 0.01$,# $p < 0.05$ control v.s TGF-β1.

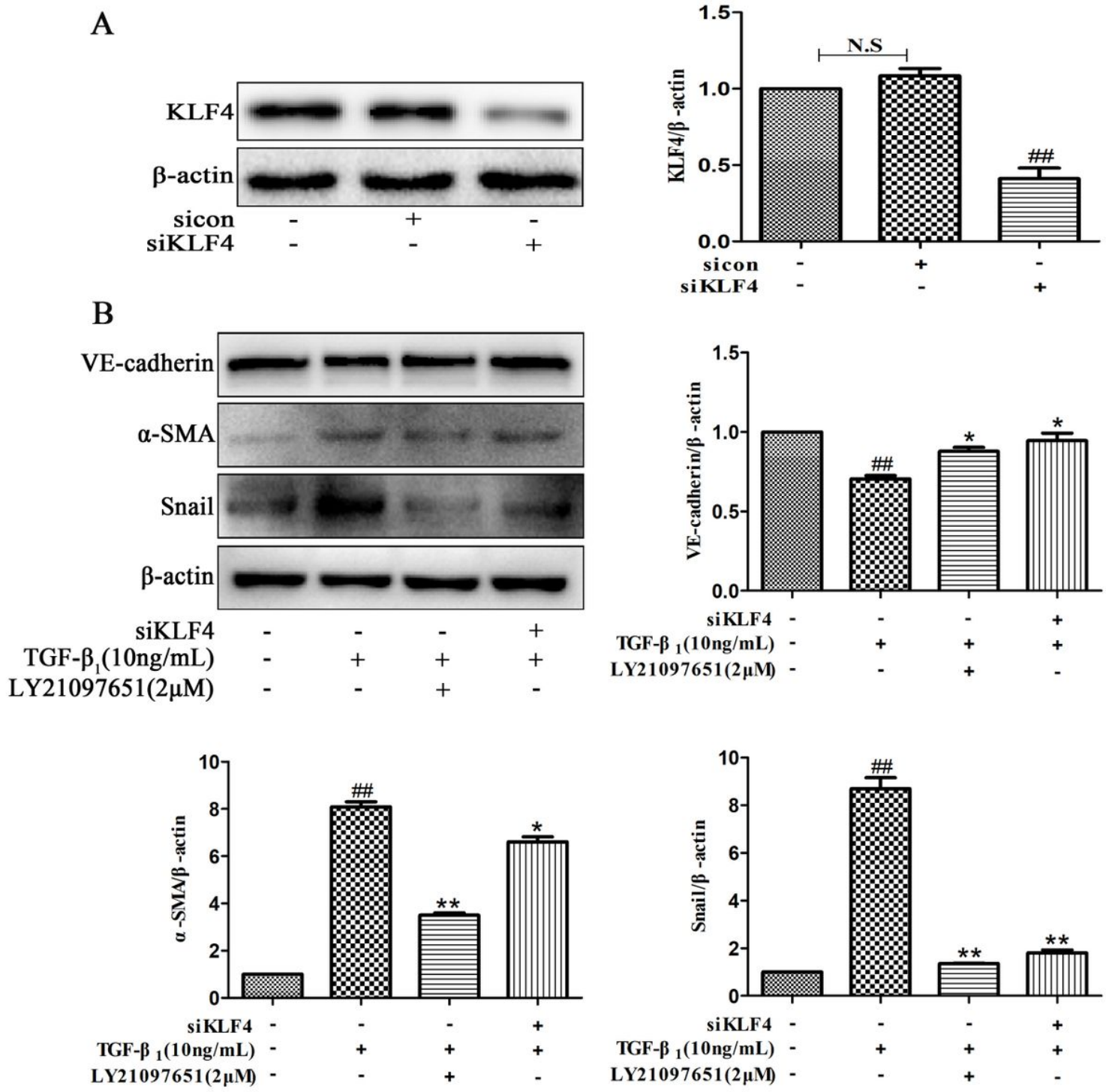

Figure 2

The role of KLF4 in EndMT induced by TGF-β1. (A)Western Blot analysis of KLF4 protein in HUVECs transfected with expression Vector. (B)HUVECs was pretreated with TGF-β1 inhibitor (LY21097651,2 μM) for 2h, and then treated with TGF-β1 for 72 h. Protein levels of VE-cadherin, α-SMA and Snail were detected by Western blot analysis. All experiments were repeated at least three independent times. Representative western blots are shown in Figure B. Densitometry was performed on western blot bands, data were normalized with relevant loading controls and then presented as mean ± SD. N.S, not

significant; ##p < 0.01 siKLF4 v.s sicon; ##p < 0.01 control v.s TGF-β1; *p < 0.05, **p < 0.01 LY21097651 and siKLF4 v.s TGF-β1.

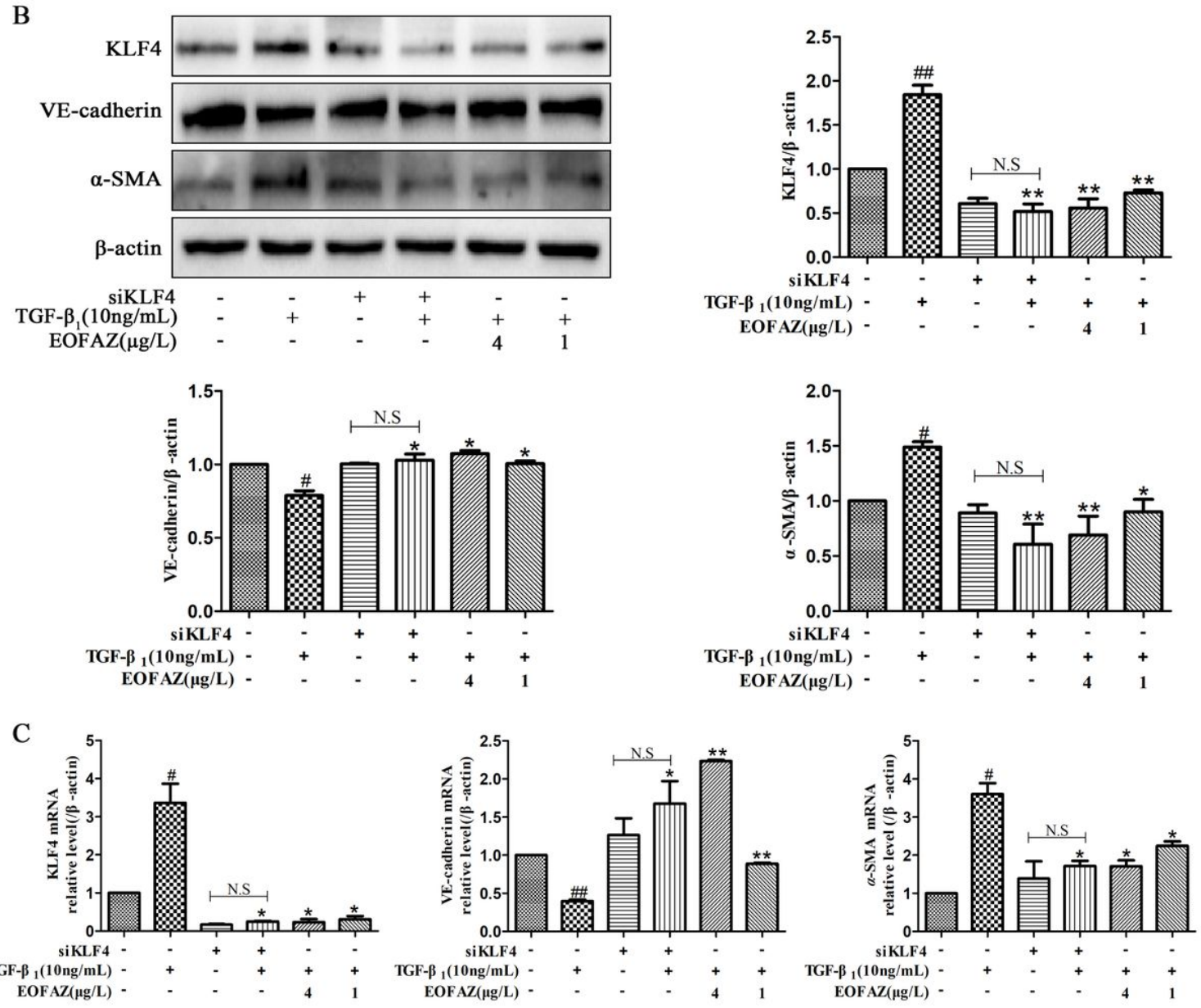

Figure 3

The role of KLF4 and EOFAZ in EndMT induced by TGF-β1. (A)Immunofluorescence analysis of VE-cadherin and α-SMA. Green fluorescence labeled endothelial marker protein VE-cadherin (fluorescent secondary antibody 488), red fluorescence labeled mesenchymal marker protein α-SMA (fluorescent secondary antibody Cy3), and the nucleus was stained blue with DAPI. (B)HUVECs was pretreated with EOFAZ(4 μg·L-1,1 μg·L-1) for 2 h, and then treated with TGF-β1 for 72 h. Protein levels of VE-cadherin, α-SMA and KLF4 were detected by Western blot analysis. Representative western blots are shown in Figure B. (C)Quantitative real-time PCR analysis of VE-cadherin, α-SMA and KLF4 mRNA levels in HUVECs. All experiments were repeated at least three independent times. Densitometry was performed on western blot bands, data were normalized with relevant loading controls and then presented as mean ± SD. N.S, not

significant; #p < 0.05, ##p < 0.01 control v.s TGF-β1; *p < 0.05, **p < 0.01 siKLF4+ TGF-β1, EOFAZ-H and EOFAZ-L v.s TGF-β1.

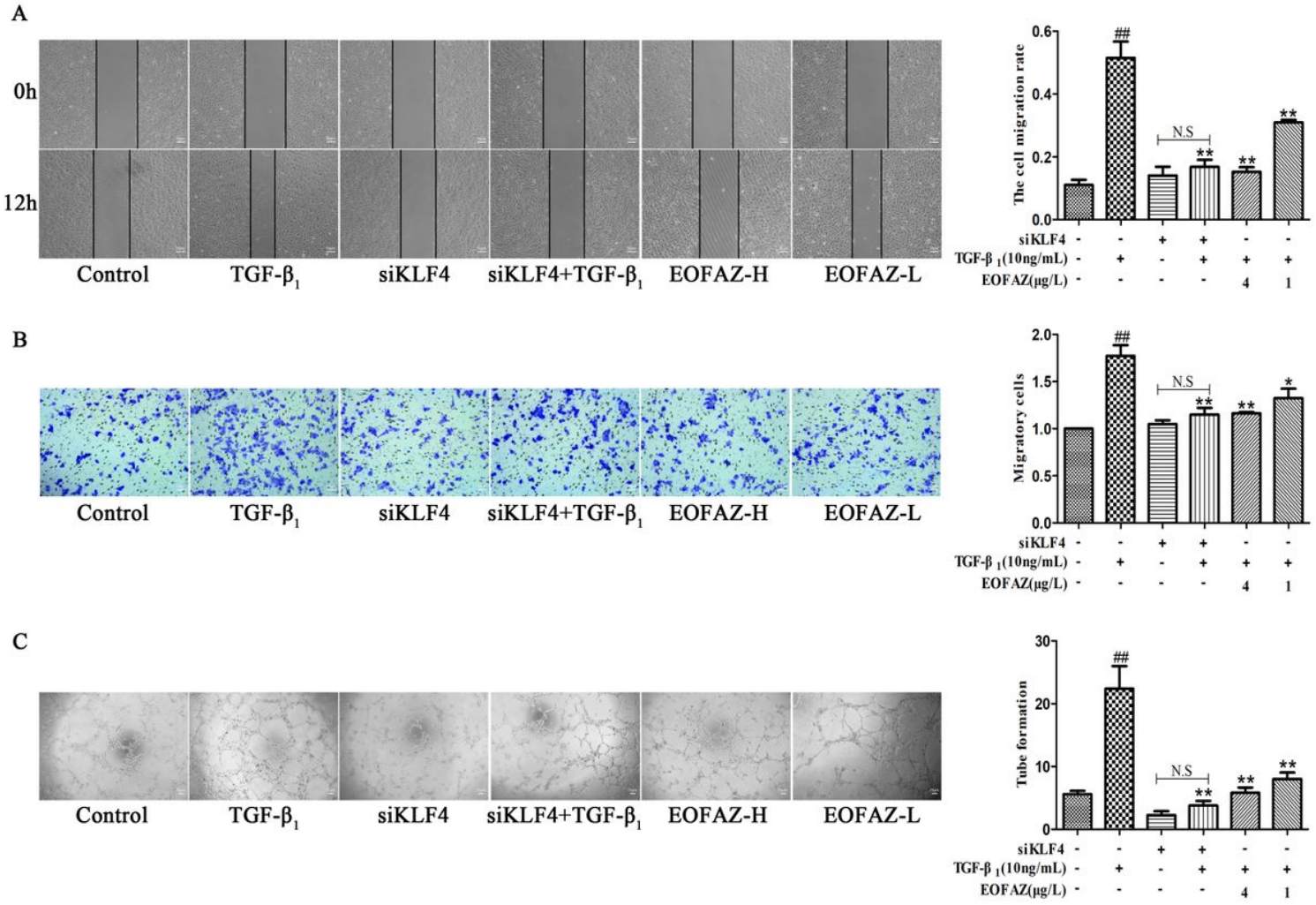

Figure 4

Effects of EOFAZ and KLF4 silencing on HUVECs migration induced by TGF-β1. (A)Wound-healing assay was used to assess the migration of cells transfected with siRNA and added with EOFAZ. (B)Transwell assay was used to assess the migration of cells transfected with siRNA and added with EOFAZ. (C)Vascular ring formation test was used to assess the migration of cells transfected with siRNA and added with EOFAZ. All experiments were repeated at least three independent times and data were presented as mean ± SD. N.S, not significant; ##p < 0.01 control v.s TGF-β1; *p < 0.05, **p < 0.01 siKLF4+ TGF-β1, EOFAZ-H and EOFAZ-L v.s TGF-β1.

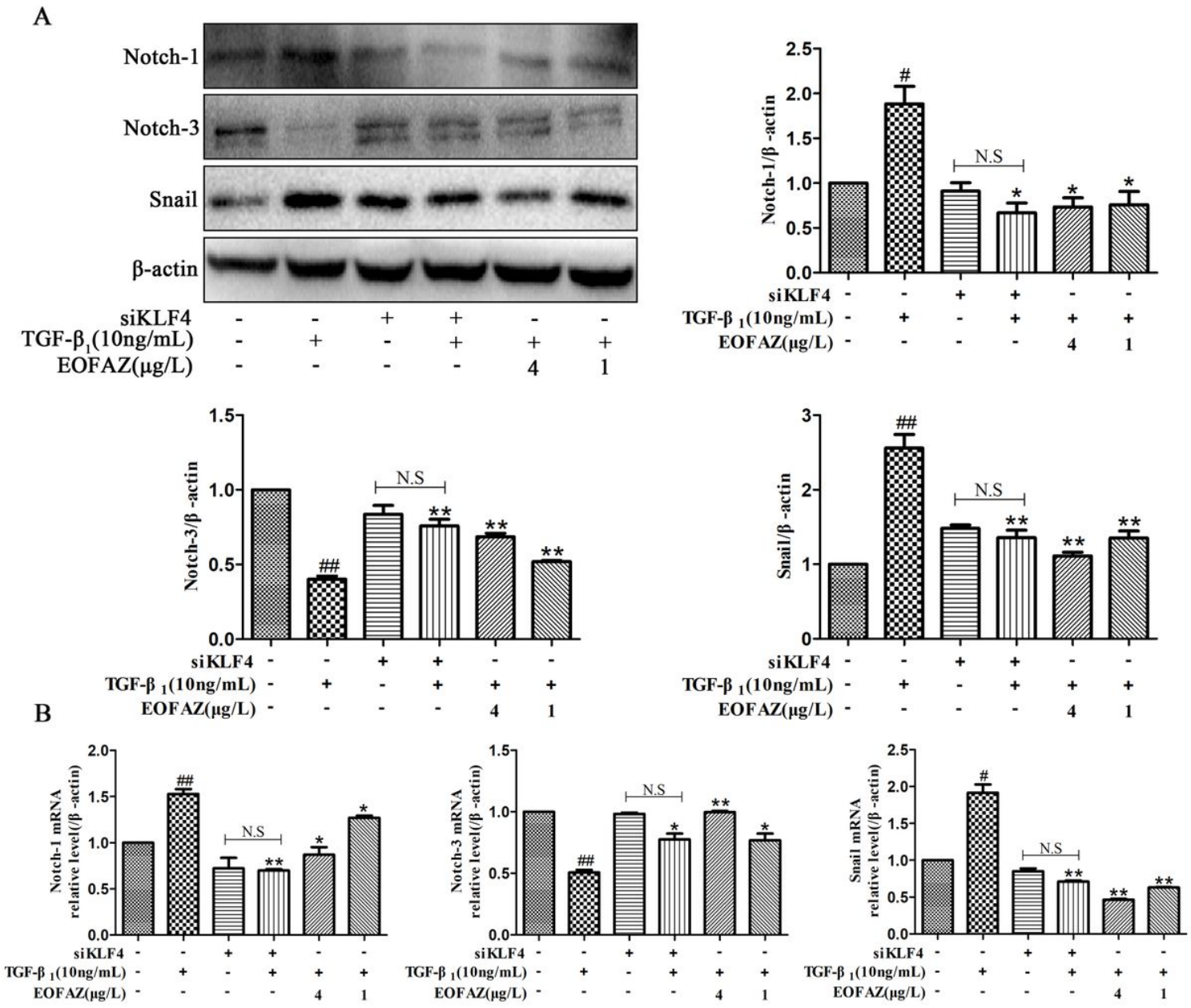

Figure 5

Effects of EOFAZ pretreatment and KLF4 silencing on Notch/Snail signal axis. HUVECs was pretreated with EOFAZ(4 μg·L-1,1 μg·L-1) for 2 h, and then treated with TGF-β1 for 72 h. (A)Protein levels of Notch-1, Notch-3 and Snail were detected by Western blot analysis. Representative western blots are shown in Figure A. (B)Quantitative real-time PCR analysis of Notch-1, Notch-3 and Snail mRNA levels in HUVECs. All experiments were repeated at least three independent times. Densitometry was performed on western blot bands, data were normalized with relevant loading controls and then presented as mean ± SD. N.S, not significant; #p < 0.05, ##p < 0.01 control v.s TGF-β1; *p < 0.05, **p < 0.01 siKLF4+ TGF-β1, EOFAZ-H and EOFAZ-L v.s TGF-β1.

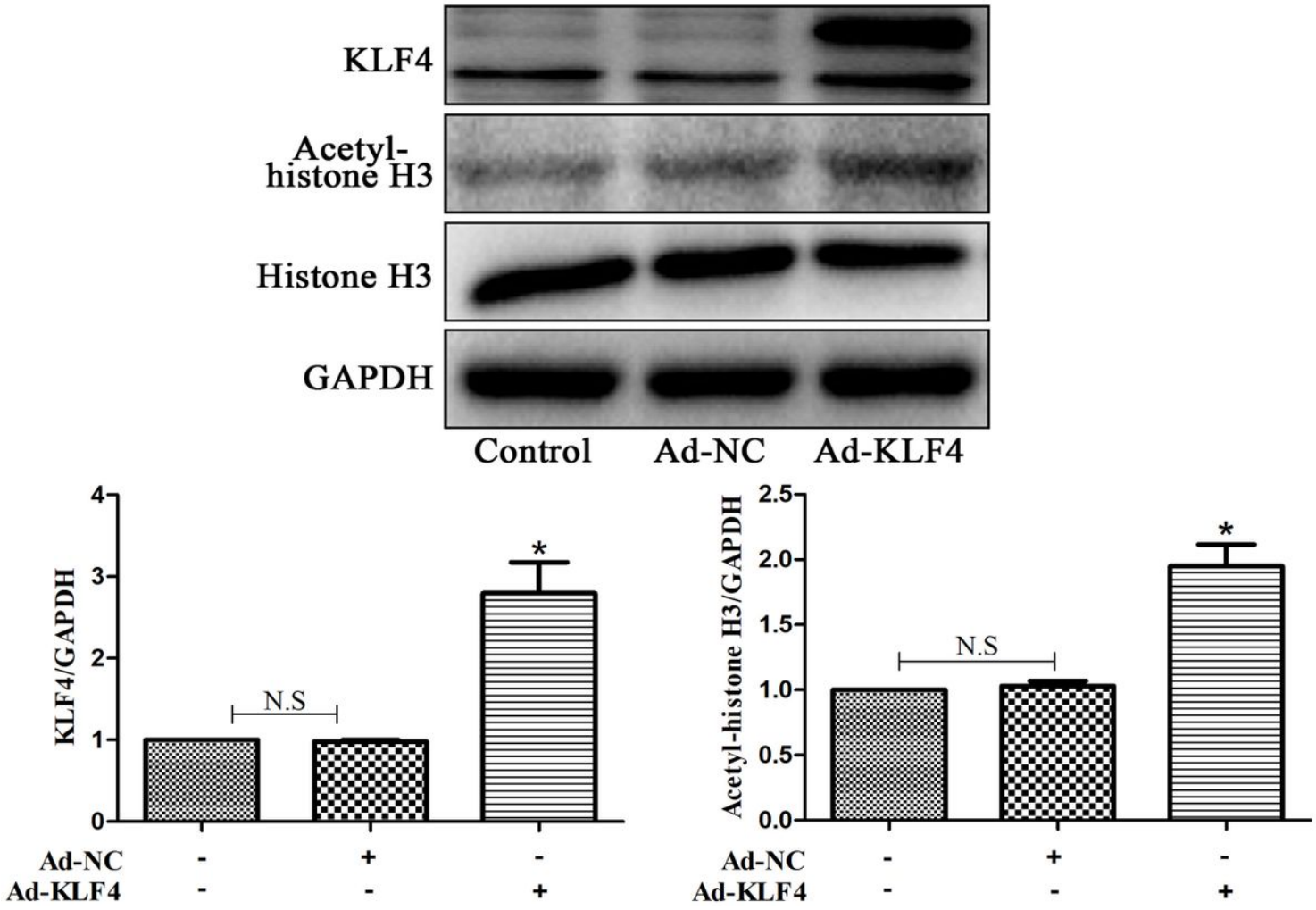

Figure 6

The role of KLF4 on histone H3 acetylation in EndMT. Western Blot analysis of KLF4 protein in HUVECs transfected with adenovirus. Protein levels of KLF4, Acetyl-histone H3 and histone H3 were detected by Western blot analysis. All experiments were repeated at least three independent times. Representative western blots are shown in Figure 6. Densitometry was performed on western blot bands, data were normalized with relevant loading controls and then presented as mean ± SD. N.S, not significant; *p < 0.05, Ad-KLF4 v.s Ad-NC.

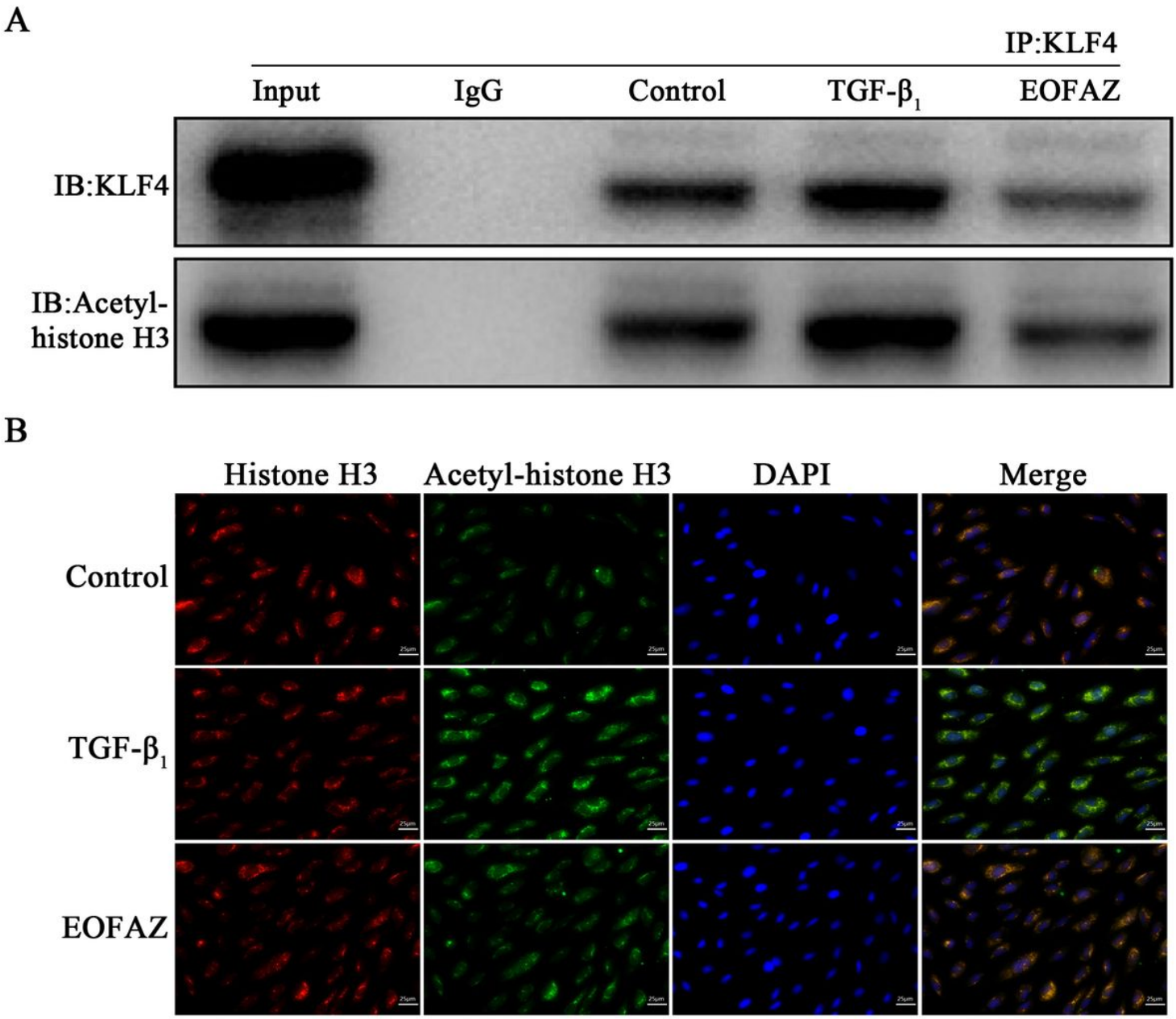

Figure 7

Effect of EOFAZ on histone H3 acetylation induced by TGF-β1. HUVECs was pretreated with EOFAZ(4 μg·L-1,1 μg·L-1)for 2 h, and then treated with TGF-β1 for 72 h. (A)HUVECs were immunoprecipitated with anti-KLF4 antibody and subjected to Western blot analysis of KLF4 and Acetyl-histone H3. (B)The expression of Histone H3 and Acetyl-histone H3 were analyzed by immunofluorescence staining. Red fluorescence labeled Histone H3(fluorescent secondary antibody Cy3), green fluorescence labeled Acetyl-histone H3 (fluorescent secondary antibody 488), and the nucleus was stained blue with DAPI. Yellow fluorescence labeled the co-localization of Histone H3 and Acetyl-histone H3.

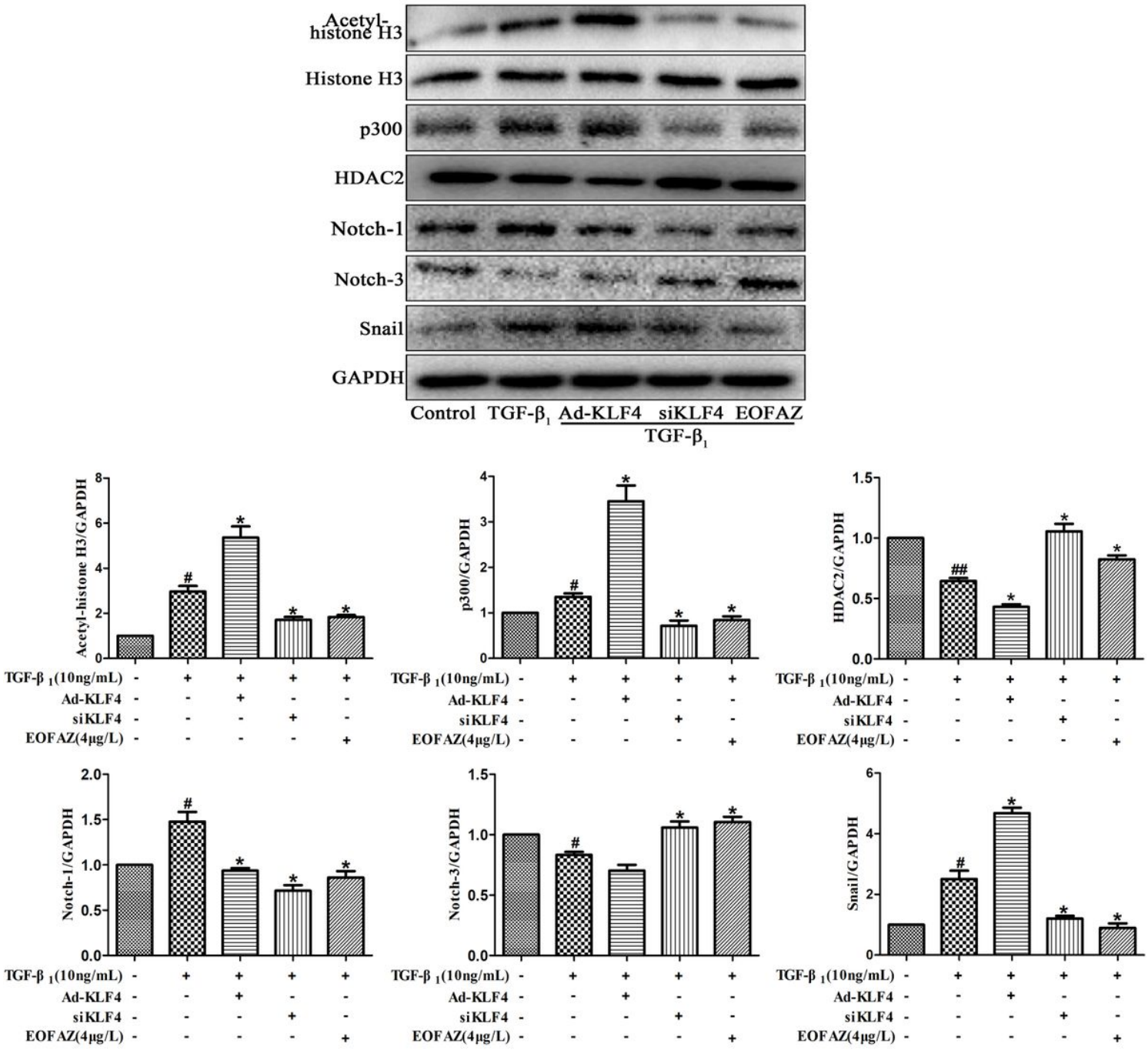

Figure 8

Effects of EOFAZ pretreatment and KLF4 on histone H3 acetylation and Notch/Snail signal axis. HUVECs was pretreated with EOFAZ(4 μg·L-1,1 μg·L-1)for 2 h, and then treated with TGF-β1 for 72 h. KLF4 silencing and overexpressing were subjected to Western blot analysis of acetyl-histone H3, histone H3, p300, HDAC2, Notch-1, Notch-3 and Snail in HUVECs. Representative western blots are shown in Figure 8. All experiments were repeated at least three independent times. Densitometry was performed on western blot bands, data were normalized with relevant loading controls and then presented as mean ± SD. #p < 0.05, ##p < 0.01 control v.s TGF-β1; *p < 0.05 Ad-KLF4+ TGF-β1, siKLF4+ TGF-β1, EOFAZ+ TGF-β1 v.s TGF-β1.

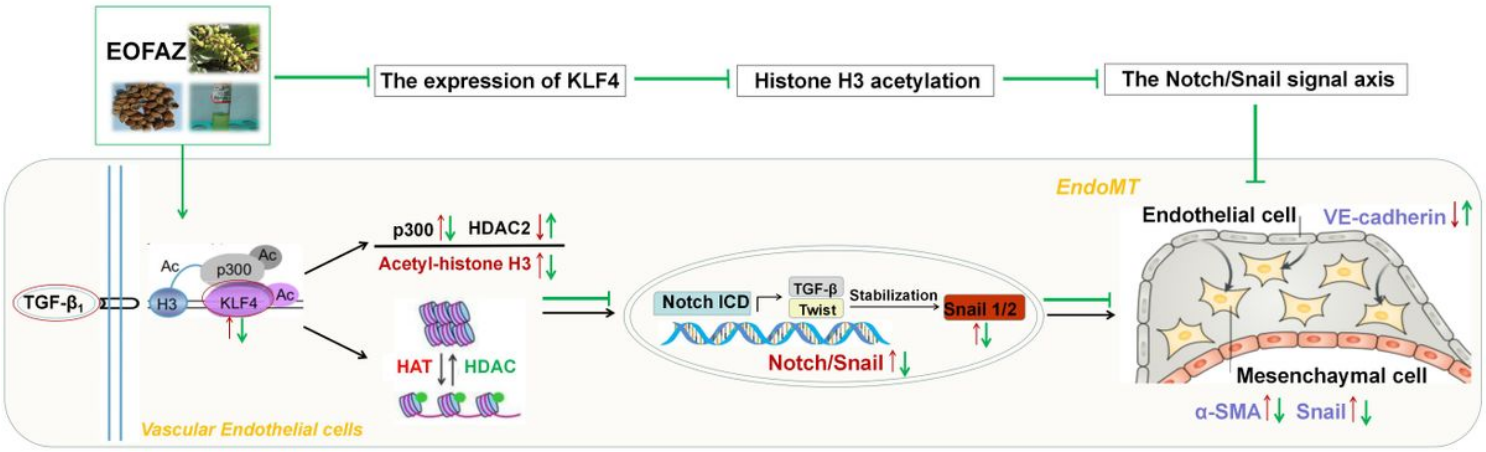

Figure 9

EOFAZ inhibits the EndMT process induced by TGF-β1 in HUVECs. TGF-β1 down-regulates the expression of endothelial marker VE-cadherin and upregulates the expression of mesenchymal markers α-SMA and Snail. In addition, TGF-β1 stimulation increased the expression of KLF4, a key regulator of endothelial cell biology. In this study, it is found that EOFAZ decreases the migration ability of HUVECs by up-regulating the expression of VE-cadherin and down-regulating the expression of α-SMA and Snail, thus inhibiting EndMT induced by TGF-β1. In addition, mechanism studies have shown that KLF4 can be used as an upstream to regulate the level of Notch/Snail signal axis. EOFAZ can reduce the expression of KLF4 and its recruitment for p300, meanwhile prevent the binding of KLF4 to Acetylated-histone H3, thereby reducing the level of histone H3 acetylation induced by TGF-β1. As the looseness of chromatin is changed, it may affect the transduction of Notch/Snail signal axis, thereby weakening the stimulation of HUVECs by TGF-β1 and inhibiting the process of EndMT. HUVECs, human umbilical vein endothelial cells; EOFAZ, essential oil from Alpinia zerumbet; TGF-β1, transforming growth factor-β1; KLF4, Krüpple like factor 4; HAT, Histoneacetylase; HDAC, Histonedeacetylase; Notch ICD, Notch intracellular domain; VE-cadherin, vascular endothelial-cadherin; α-SMA, α-smooth muscle actin; EndMT, endothelial-to-mesenchymal transition.

Supplementary Files

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