

Effect of miR-144-5p on the proliferation, migration, invasion and apoptosis of human umbilical vein endothelial cells by targeting RICTOR and its related mechanisms

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Abstract. The purpose of the present study was to investigate the effect of microRNA (miR)-144-5p on human umbilical vein endothelial cells (HUVECs) to explore the role of miR-144-5p in atherosclerosis. miR-144-5p expression was upregulated in HUVECs using miR-144-5p mimics. The relative expression level of miR-144-5p in HUVECs was detected using reverse transcription-quantitative PCR (RT-qPCR). Cell proliferation was detected by performing an MTT assay. Apoptosis was determined via flow cytometry. Cell migration ability was detected by a wound-healing assay. Cell invasion was determined by a transwell assay. The protein levels of phosphorylated (p)-PI3K, p-Akt and endothelial nitric oxide synthase (eNOS) were detected using western blot analysis. The binding sites between miR-144-5p and 3'-untranslated region of rapamycin-insensitive companion of mTOR (RICTOR) mRNA were predicted by TargetScan and confirmed by a dual luciferase reporter assay. The present study showed that miR-144-5p mimics significantly inhibited cell proliferation and induced apoptosis in HUVECs. In addition, miR-144-5p mimics could suppress migration and invasion of HUVECs. Further analysis identified that RICTOR was a direct target gene of miR-144-5p. Moreover, miR-144-5p upregulation decreased the protein level of p-PI3K, p-Akt and eNOS. In conclusion, miR-144-5p regulated HUVEC proliferation, migration, invasion, and apoptosis through affecting the PI3K-Akt-eNOS signaling pathway by altering the expression of RICTOR. These results indicated that miR-144-5p may be a potential target for the prevention and treatment of atherosclerosis.

Introduction

Atherosclerosis is a chronic inflammatory and vascular disease, which frequently causes heart disease and stroke (1). Atherosclerosis is involved in the accumulation of lipid-engorged macrophages in the subendothelial layer of arterial vessels (2,3). Endothelial dysfunction is one of the main contributors to atherosclerosis (4) and results in a phenotypic alteration that increases the expression of adhesion molecules released by endothelial cells (ECs). Apoptosis of endothelial cells plays an important role in the occurrence and development of atherosclerosis (5).

MicroRNAs (miRNAs/miRs) are a group of small, single-stranded, non-coding RNA molecules which influence the synthesis of proteins via their interactions with target mRNAs (6,7). It has been reported that miRNAs could modulate a number of biological processes, including tumorigenesis, development, invasion, metastasis and other biological characteristics (8,9). Accumulating evidence suggests that miRNAs serve as diagnostic and prognostic biomarkers of numerous types of cancer (10,11). Some miRNAs have been reported to be involved in atherosclerosis through regulating vascular cells (12). Previous research indicated that miRNAs were closely related to the pathogenesis of atherosclerosis (13,14).

Previous studies reported that several miRNAs, such as miR-34a, miR-217, and miR-146a, could regulate the proliferation and differentiation of ECs and may also stimulate cellular senescence, which can act as a trigger for endothelial dysfunction (15-17). miR-144 is a conservative miRNA and tumor suppressor gene that plays a role in tumor suppression in a variety of cancers, such as bladder cancer (18), thyroid cancer (19), gastric cancer (20), colorectal cancer (21), ovarian cancer (22) and nasopharyngeal carcinoma (NPC) (23). Moreover, previous studies reported that miR-144 upregulation may inhibit the proliferation, invasion and metastasis of cancer cells (21,24). However, the specific function and mechanism of miR-144-5p in atherosclerosis remain unclear. Apoptosis of endothelial cells plays an important role in the occurrence and development of atherosclerosis. Therefore, the present study aimed to investigate the effects of miR-144-5p

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on human umbilical vein endothelial cells (HUVECs) to explore the role of miR-144-5p in atherosclerosis.

Materials and methods

Cell culture. HUVECs were obtained from Shanghai Institute of Life Sciences, Chinese Academy of Sciences. HUVECs were cultured in medium 199 supplemented with 10% FBS, 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.), endothelial cell growth supplement (Sigma-Aldrich; Merck KGaA) and epidermal growth factor (10 ng/ml, Roche Diagnostics) in a humidified atmosphere at 37°C with 5% CO₂.

Cell transfection. Once cells reached 70-80% confluency, miR-144-5p mimics (Guangzhou RiboBio Co., Ltd.) or the negative control of miR-144-5p mimics (mimics-NC; Guangzhou RiboBio Co., Ltd.) were transfected into HUVECs using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocols. The transfection efficiency was detected by reverse transcription-quantitative PCR (RT-qPCR), as described below.

Flow cytometry analysis. After transfection, HUVECs were washed three times with PBS and centrifuged at 1,000 x g for 5 min at 20°C. Harvested cells were stained with 200 µl annexin V/FITC binding buffer (Beyotime Institute of Biotechnology) and then incubated with 5 µl propidium iodide (PI; Beyotime Institute of Biotechnology) for 25 min at room temperature without light. Cell apoptosis was analyzed by flow cytometry (BD FACSCalibur[™]; Becton, Dickinson and Company) and data were analyzed using FlowJo software (version 7.6.1; FlowJo LLC). Each experiment was performed in triplicate.

Cell invasion assay. Cell invasion ability was detected using a Transwell chamber (pore size, 8 µm; Costar; Corning Inc.) pre-coated with Matrigel (1 mg/ml; BD Biosciences) at 4°C overnight. HUVECs (2x10⁴ cells/well) were briefly plated into the upper chambers using human endothelial serum-free medium (cat. no. 11111044; Gibco; Thermo Fisher Scientific, Inc.) containing 0.5% FBS. Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS was added to the lower chamber as a chemoattractant, stimulating cells to move to the lower chamber. At 24 h after incubation, the invasive cells on the lower chamber were fixed with 4% paraformaldehyde at room temperature for 30 min. Subsequently, the membrane was stained for 30 min with 0.1% crystal violet at room temperature. Cells located on the top of membrane were wiped with a cotton swab. The Transwell chamber was then washed with 95% ethanol. A total of 10 fields of view were selected randomly to count the number of cells under a light microscope (magnification, x200; Nikon Corporation). Each group was repeated in triplicate and all experiments were repeated three times to obtain average values.

Cell migration assay. A wound-healing assay was performed to detect cell migration ability. HUVECs (3x10⁵ cells/well) were seeded in six-well plates. Cells were grown until 90% confluency before each well was scraped with a 10 µl pipette

tip to create a linear region devoid of cells. Subsequently, the cells were washed with PBS three times. Human endothelial serum-free culture medium (cat. no. 11111044; Gibco; Thermo Fisher Scientific, Inc.) was then added to cells and incubated at 37°C with 5% CO₂. Wound healing was monitored using a light microscope (magnification, x100; Nikon Corporation) at 0 and 24 h after scraping. Percentage of wound (%) = (Wound width at 24 h/Wound width at 0 h) x100.

MTT assay. HUVECs were inoculated in a 96-well plate at a density of 1x10⁴ cells/well at 37°C with 5% CO₂ for 12, 24 and 48 h. Subsequently, MTT (20 µl; 5 mg/ml) was added to each well, and cells were incubated for 4 h. A total of 150 µl DMSO was added to dissolve formazan crystals and gently shaken for 10 min. The optical density of each sample was determined with a microplate reader (Multiskan[™] FC; Thermo Fisher Scientific, Inc.) by measuring the absorbance at 490 nm. The experiments were repeated at least three times.

Western blot analysis. Total protein was extracted from HUVECs using radioimmunoprecipitation assay buffer containing 1 mM PMSF. Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.) was then used to quantify protein concentration following which the protein samples were separated by 10% SDS-PAGE (20 µg/lane) and transferred onto PVDF membranes. Subsequently, the membrane was blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies against phosphorylated (p)-AKT (cat. no. ab81283; 1:1,000; Abcam), AKT (cat. no. ab179463, 1:10,000, Abcam), p-PI3K (cat. no. ab182651, 1:1,000, Abcam), PI3K (cat. no. ab86714, 1:1,000, Abcam), eNOS (cat. no. ab66127, 1:1,000, Abcam) or GAPDH (cat. no. ab181602, 1:10,000, Abcam) at 4°C overnight. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody Goat Anti-Rabbit IgG H&L (ab205718; 1:2,000; Abcam), Goat Anti-Mouse IgG H&L (ab205719; 1:2,000; Abcam) and for 2 h at room temperature. The GAPDH was used as internal reference. Afterwards, the protein bands were detected and visualized using an enhanced chemiluminescence method (RapidStep[™] ECL Reagent; Merck KGaA). The densities of bands were semi-quantitatively analyzed using ImageJ (version 4.0; National Institutes of Health).

RT-qPCR assay. Total RNA was extracted from HUVECs using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocols. Total RNA concentration was detected by NanoDrop[™] 2000 (Thermo Fisher Scientific, Inc.) and RNA samples were subsequently stored at -80°C. cDNA synthesis was performed using the RevertAid[™] First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The temperature protocol was as follows: 42°C for 30 min and 85°C for 5 min. RT-qPCR was performed using a QuantiFast SYBR Green PCR kit (Qiagen GmbH) in accordance with the manufacturer's protocols. The following primers were used: U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'; miR-144-5p forward, 5'-GCGCGAATTCGA GATCTTAACAGACCCTAGCTC-3' and reverse, 5'-GCG CGGATCCGTGCCCTGGCAGTCAGTAGG-3'. Relative expression levels were calculated using the 2^{-ΔΔC_q} method (25)

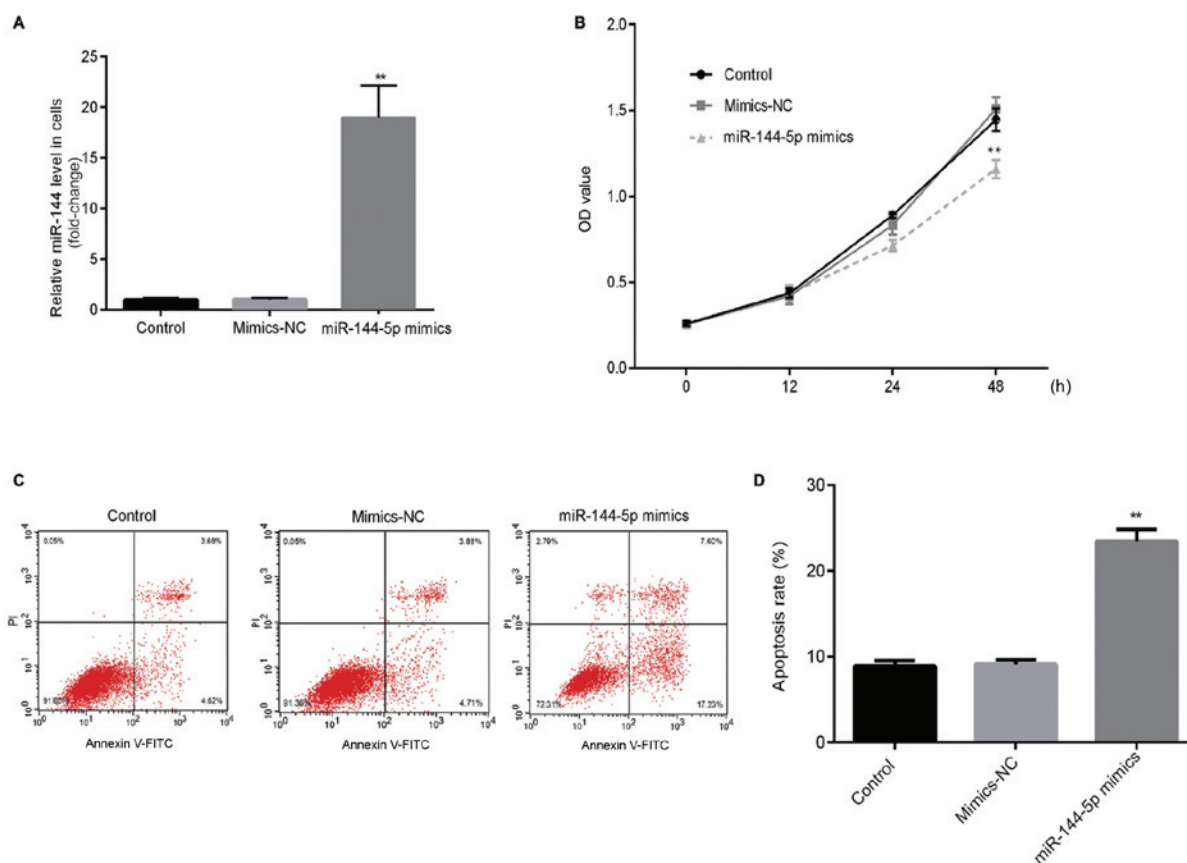


Figure 1. miR-144-5p suppresses cell proliferation and promotes apoptosis. (A) A reverse transcription-quantitative PCR assay detected the relative expression of miR-144-5p in HUVECs transfected with miR-144-5p mimic or mimic-NC for 48 h. (B) An MTT assay was performed to determine the proliferation in HUVECs transfected with miR-144-5p mimics or mimics-NC for 48 h. (C) A flow cytometry assay was performed to determine apoptosis in HUVECs transfected with miR-144-5p mimics or mimics-NC for 48 h. (D) Apoptotic rate of transfected cells compared with non-transfected cells. ** $P < 0.01$ vs. mimic-NC. NC, negative control; miR, microRNA.

after normalization with reference to U6 expression. All experiments were performed in triplicate.

Dual-luciferase reporter assay. TargetScan 7.2 (http://www.targetscan.org/vert_72/) was used to predict the potential targets of miR-144-5p. The results showed the binding sites between miR-144-5p and the RICTOR 3'-untranslated region (UTR). Subsequently, a dual-luciferase reporter assay was performed to confirm the predicted binding sites. According to the bioinformatics analysis results, a fragment of the RICTOR 3'-UTR containing the wild type (WT) or mutant (5'-AGAAGAGGUUAGGAACACCCCGA-3') seed regions of miR-144-5p was chemically synthesized *in vitro* and cloned into *Bam*HI and *Asc*I sites of the pMIR-RB-REPORT™ vector (Guangzhou RiboBio Co., Ltd.). HUVECs were then co-transfected with miR-144-5p mimics or mimics-NC and WT-RICTOR or Mutant-RICTOR with Lipofectamine 2000 for 48 h. After a 48-h incubation, relative luciferase activity was calculated by normalizing to *Renilla* luciferase activity using the Dual Luciferase Reporter Assay System (Promega Corporation) following the manufacturer's protocol.

Statistical analysis. All data were presented as the mean \pm standard deviation. All experiments were performed at least three times. SPSS 19.0 software (IBM Corp.) was used to perform data analysis. Comparisons between groups were

analyzed using Student's t-test and one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of miR-144-5p on cell proliferation and apoptosis of HUVECs. An RT-qPCR assay was first performed to detect the transfection efficiency of miR-144-5p. RT-qPCR results showed that miR-144-5p mimics significantly increased the relative expression of miR-144-5p in HUVECs compared with the mimic-NC (Fig. 1A). To investigate the effects of miR-144-5p on HUVEC proliferation and apoptosis, MTT and flow cytometry assays were performed. MTT assay results demonstrated that miR-144-5p mimics significantly reduced cell proliferation compared to the mimic-NC group (Fig. 1B). Flow cytometry results indicated that miR-144-5p mimics significantly promoted cell apoptosis of HUVECs compared with the mimic-NC group (Fig. 1C and D).

Effect of miR-144-5p on cell migration and invasion of HUVECs. In order to investigate the migration and invasion of cells in different groups, wound healing and transwell assays were performed. Results from the wound healing assay revealed that miR-144-5p mimics significantly inhibited the cell migration ability of HUVECs compared with

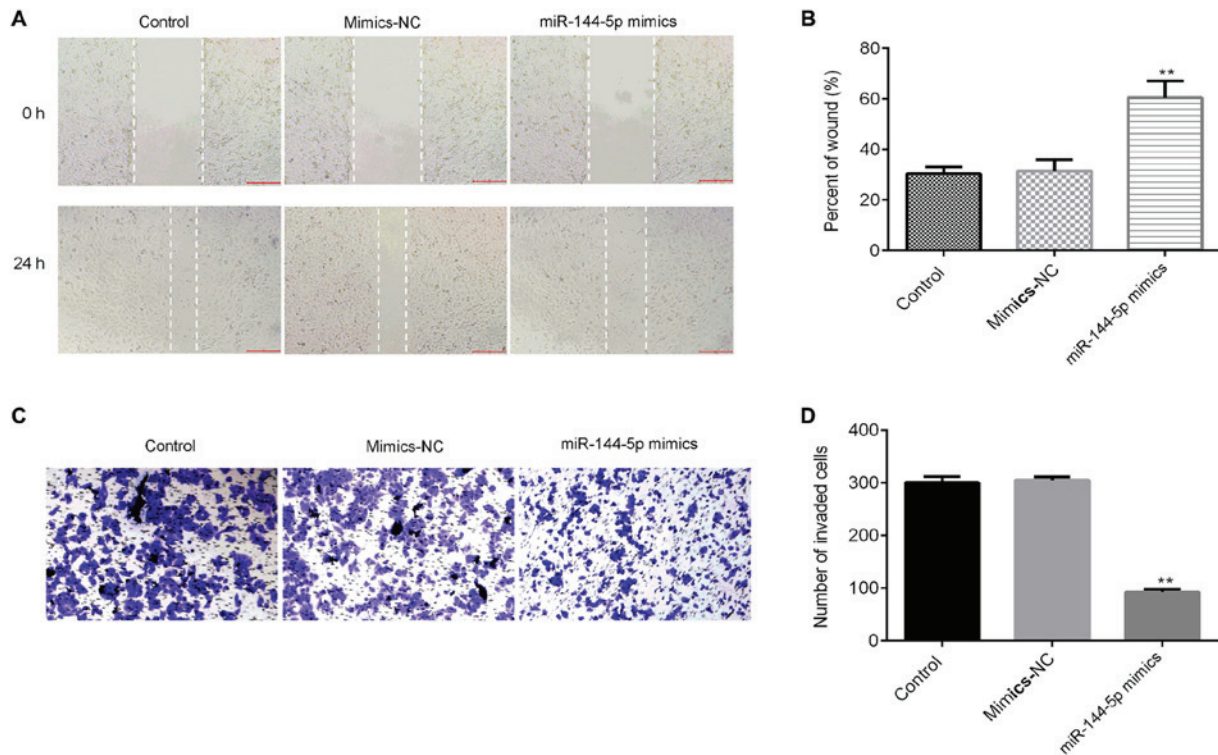


Figure 2. miR-144-5p mimics suppress cell migration and invasion of HUVECs. (A) A wound healing assay was performed to determine cell migration in HUVECs transfected with miR-144 mimic or mimic-NC for 24 h. Scale bars, 50 μ m. (B) Percentage of wound data are presented as the mean \pm SD from three independent experiments. (C) A Transwell assay was performed to determine cell invasion in HUVECs transfected with miR-144-5p mimics or mimic-NC for 24 h (magnification, x200). (D) The number of invaded cells was calculated and presented as the mean \pm SD from three independent experiments. **P<0.01 vs. mimic-NC. NC, negative control; miR, microRNA.

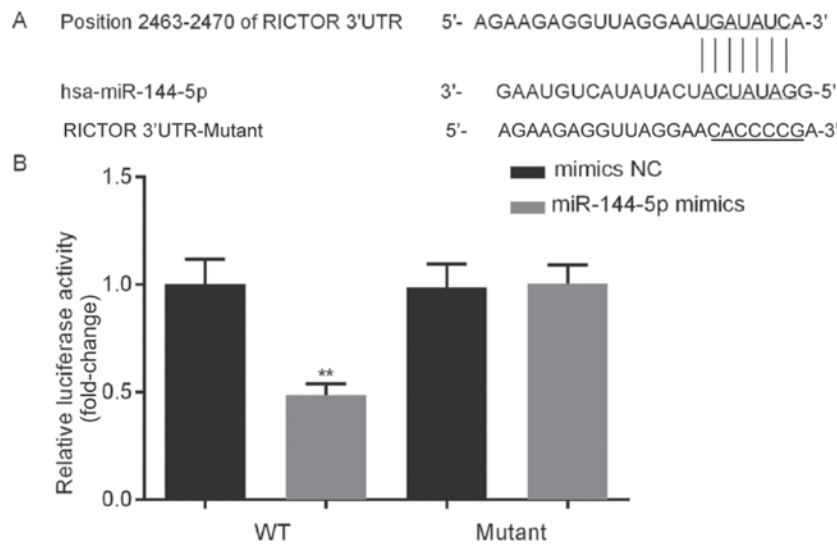


Figure 3. RICTOR is a direct target gene of miR-144-5p. (A) Binding sites between miR-144-5p and RICTOR. (B) A dual-luciferase reporter assay was used to confirm the binding sites between miR-144-5p and RICTOR in HUVECs. **P<0.01 vs. mimic-NC. RICTOR, rapamycin-insensitive companion of mTOR; UTR, untranslated region; NC, negative control; miR, microRNA; WT, wild-type.

the mimic-NC group (Fig. 2A and B). The transwell assay results showed that miR-144-5p mimics significantly reduced the invasive capacity and decreased the number of invasive HUVECs (Fig. 2C and D).

RICTOR is a direct target gene of miR-144-5p. To investigate the potential role of miR-144-5p in the growth of HUVECs,

bioinformatics analysis was performed to predict the potential targets of miR-144-5p. The results identified binding sites between the 3'-UTR of RICTOR and miR-144-5p (Fig. 3A). A dual-luciferase reporter system was used to determine if RICTOR was a direct target of miR-144-5p. The results indicated that compared with the relative luciferase activity of cells co-transfected with WT RICTOR 3'-UTR and mimics-NC,

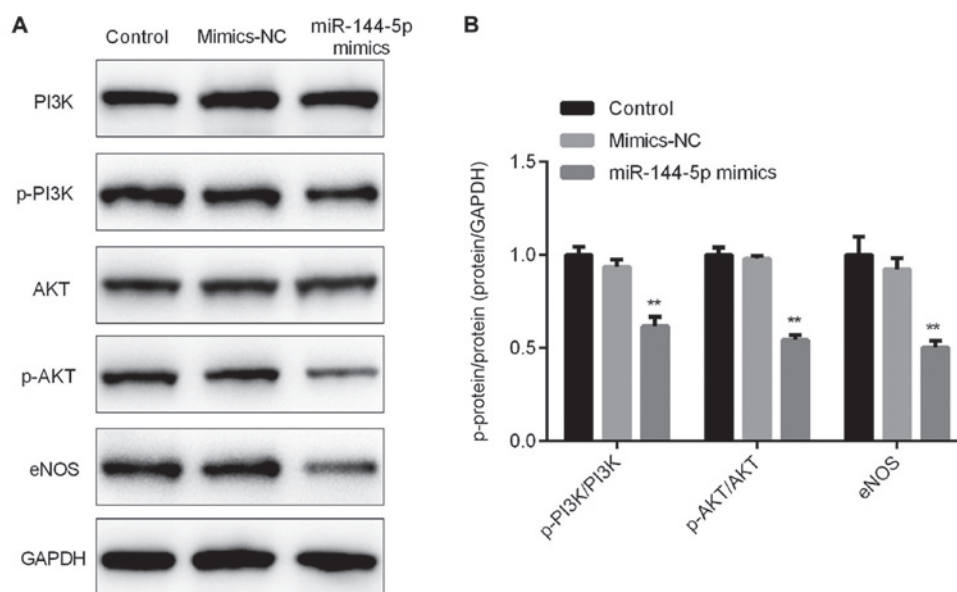


Figure 4. miR-144-5p mimics inhibit the protein levels of p-PI3K, p-Akt and eNOS. (A) Western blot assay detected the protein levels of p-PI3K, PI3K, p-Akt, Akt and eNOS, and GAPDH was used as an internal control. (B) Ratios of p-PI3K/PI3K, p-Akt/Akt, and eNOS/GAPDH were calculated. ** $P < 0.01$ vs. mimic-NC eNOS, endothelial nitric oxide synthase; NC, negative control; miR, microRNA.

the relative luciferase activity of cells co-transfected with WT RICTOR 3'-UTR and miR-144-5p mimics significantly decreased (Fig. 3B). Furthermore, when comparing the relative luciferase activity of cells co-transfected with mutant RICTOR 3'-UTR and mimics-NC, the relative luciferase activity of cells co-transfected with mutant RICTOR 3'-UTR and miR-144-5p mimics showed no significant changes (Fig. 3B). These data indicated that RICTOR was a direct target gene of miR-144-5p.

miR-144-5p is associated with PI3K-Akt-endothelial nitric oxide synthase (eNOS) pathway in HUVECs. In order to further explore the specific mechanism of the effect of miR-144-5p on HUVECs, the expression levels of related proteins in the PI3K-Akt-eNOS pathway were detected. Results from the western blot analysis indicated that miR-144-5p mimics decreased the protein levels of phosphorylated (p)-PI3K, p-Akt and eNOS in HUVECs (Fig. 4A and B).

Discussion

Atherosclerosis is a relatively complex disease affecting medium and large-sized arteries. Vascular injury plays an important role in the pathogenesis of atherosclerosis, and endothelial apoptosis plays a crucial role in endothelial dysfunction and atherosclerosis lesion formation (26). Currently, atherosclerosis is being extensively studied. Therefore, the current study aimed to explore the role of miR-144-5p in atherosclerosis development through investigating the effects of miR-144-5p on endothelial cells. miR-144-5p mimics inhibited HUVEC proliferation and promoted cell apoptosis. In addition, miR-144-5p mimics could decrease cell migration and invasion of HUVECs. Moreover, RICTOR was a direct target of miR-144-5p. Finally, miR-144-5p mimics could repress PI3K-Akt-eNOS signaling pathway in HUVECs.

Previously, miRNAs have been confirmed to be involved in numerous important physiological processes (27-30).

miRNAs are vital regulators for endothelial biology and dysfunction. Deregulation of miRNA expression is involved in endothelial angiogenesis, injury, inflammation and senescence (31-33).

miR-144-5p has been studied in various cancers such as renal cell carcinoma (34) and miR-144-5p has also been studied in chronic periodontitis (35). A previous study demonstrated that circulating miR-144-5p was associated with depressive disorders (36). However, the role and mechanism of miR-144-5p in atherosclerosis remains unclear. Apoptosis of endothelial cells plays an important role in the occurrence and development of atherosclerosis (26). The present study investigated the effect of miR-144-5p on HUVECs to explore the role of miR-144-5p in atherosclerosis.

The effect of miR-144-5p upregulation on the biological behaviors of HUVECs was determined by performing MTT, migration and invasion assays, and flow cytometry. The results demonstrated that miR-144-5p mimics significantly inhibited HUVEC proliferation, migration and invasion, and induced cell apoptosis.

Subsequently, to explore the underlying mechanism of miR-144-5p on HUVECs, bioinformatics analysis was used to predict the potential targets of miR-144-5p. RICTOR was a direct target of miR-144-5p in HUVECs. RICTOR is a crucial component of mTOR complex 2 (mTORC2), and the activation of mTORC2 depends on the presence of the inactivating Ser473 phosphorylation on Akt (37). Furthermore, eNOS activity dysfunction leads to a decrease in NO bioavailability, which results in atherosclerosis (38). A previous study revealed that the RICTOR/Akt/eNOS pathway played an important role in regulating apoptosis and dysfunction in EC cells (39). Thus, the present study explored whether the RICTOR/Akt/eNOS pathway was affected by miR-144-5p in HUVECs. The results indicated that miR-144-5p mimics decreased the protein levels of p-PI3K, p-Akt and eNOS in

HUVECs. However, p-eNOS levels were not detected in the current study, which is a limitation.

In conclusion, miR-144-5p inhibited the proliferation, invasion and migration of HUVECs, and induced apoptosis through regulating the PI3K-Akt-eNOS signaling pathway, at least partly by modulating RICTOR expression. Thus, miR-144-5p might participate in the occurrence and development of atherosclerosis through regulating the apoptosis of ECs. However, the current study only included a preliminary analysis of miR-144-5p in atherosclerosis. To completely elucidate the role of miR-144-5p in atherosclerosis, further in-depth research is needed. For example, the protective effects of RICTOR, PI3K, Akt, and eNOS on HUVECs should be investigated. The role of miR-144-5p in atherosclerosis should be studied *in vivo*. In addition, the expression of miR-144-5p in patients with atherosclerosis and its relationship with the clinicopathological features of patients can be further explored.

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Availability of data and materials

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

Authors' contributions

ZL designed the study and revised the manuscript. WF and JZ wrote the manuscript and collected the data. YS and RZ searched the literature and interpreted the data. HZ collected the data. 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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