Effect of microRNA-144-5p on the proliferation, invasion and migration of human umbilical vein endothelial cells by targeting SMAD1

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Abstract. Atherosclerosis is a multifactorial chronic disease that is a major cause of death and injury worldwide. Apoptosis of endothelial cells (ECs) serves an important role in the occurrence and development of atherosclerosis. MicroRNAs (miRNAs) serve a key role in atherosclerosis though regulating the function of ECs. At present, the role of miRNA-144-5p (miR-144-5p) in atherosclerosis is unclear. The aim of this study was to investigate the effect of miR-144-5p on atherosclerosis in oxidized low-density lipoprotein (ox-LDL)-stimulated human umbilical vein endothelial cells (HUVECs). Results from the present study demonstrated that miR-144-5p overexpression could inhibit proliferation and induce apoptosis in HUVECs. To further study the biological function of miR-144-5p, the effects of modulating miR-144-5p expression on the invasion and migration of HUVECs were also examined. The results demonstrated that miR-144-5p upregulation suppressed HUVEC migration and invasion. TargetScan and dual luciferase reporter assay results demonstrated that SMAD1 was a direct target gene of miR-144-5p. miR-144-5p upregulation inhibited the expression of phosphorylated-SMAD1/5/8 in the SMAD pathway. In conclusion, the data indicated that miR-144-5p serves an important role in the development of atherosclerosis through regulating the function of HUVECs by targeting SMAD1.

Introduction

Atherosclerotic cardiovascular disease and its clinical complications, such as myocardial infarction and ischemic stroke, are the world’s first and third causes of death, respectively, causing 247.9 deaths/100,000 persons in 2013, representing 84.5% of cardiovascular deaths and 28.2% of all-cause mortality (1). Atherosclerosis can lead to lipid accumulation, extracellular matrix protein deposition, and calcification in the intima and media of the arteries causing arterial stiffness and reducing arterial elasticity (2). Oxidized low-density lipoprotein (ox-LDL) is able to induce apoptosis of endothelial cells (ECs) and is considered as the major risk factors for atherosclerosis (3). In response to oxidized low-density lipoproteins (oxLDL), endothelial cells express a range of chemokines and adhesion molecules that contribute to leukocyte recruitment, adherence, and migration into the subendothelium (4), the first stage in the development of atherosclerosis (5). Recently, increasing evidence has indicated that microRNAs (miRNAs) serve an important role in atherosclerosis development and progress (6).

miRNAs, widely found in plants and animals, including humans, are a group of non-coding, single-stranded RNA molecules that participate in sequence-specific post-transcriptional regulation of gene expression (7-9). miRNAs have emerged as crucial players in many biological processes, and changes in their expression or function are associated with numerous human diseases (10). It has been reported that miRNAs regulate several cellular and molecular biological processes related to the development of atherosclerosis ranging from interacting with risk factors to initiating the development, promoting the progression and causing the rupture of atherosclerotic plaques (11). miRNA (miR)-144-5p has been studied in several diseases, including cancer (12-14), chronic periodontitis (15) and depressive disorders (16). However, the specific function and mechanism of action of miR-144-5p in atherosclerosis remain unclear.

SMAD proteins are intracellular mediators of the transforming growth factor-β family. Smad1/5/8 play an important role in angiogenesis. Phosphorylation and activation of the transcription factors SMAD1/5/8 results in the promotion of angiogenesis. The SMAD1/5/8 signaling pathway, which inhibits extracellular matrix deposition, promotes endothelial cell proliferation and migration (17). A previous study has reported that the balance of proliferation/apoptosis of vascular smooth muscle cells in atherosclerosis is modulated by long non-coding RNA-MEG3 via the regulation of the miR-26a/Smad1 axis (18). In addition, activation of CD137 signaling promotes
angiogenesis in atherosclerosis by modulating the endothelial Smad1/5-nuclear factor of activated T cells pathway (19). In a previous study using bioinformatics, it was predicted that SMAD1 was a direct target gene of miR-144-5p (20).

The present study aimed to investigate the role of miR-144-5p in atherosclerosis and to further explore the molecular mechanism of action of miR-144-5p. It was hypothesized that miR-144-5p alleviates ox-LDL-induced HUVEC proliferation, invasion and migration by targeting SMAD1, and further implicates the potential therapeutic targets to reverse atherosclerosis.

**Materials and methods**

**Cell culture and cell transfection.** HUVECs were acquired from the Shanghai Institute of Life Sciences, Chinese Academy of Sciences. Culture media for HUVECs contained routine medium 199 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (unless otherwise stated) (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.), 1% endothelial cell growth supplement (Sigma-Aldrich; Merck KGaA) and 10 ng/ml epidermal growth factor in a 5% CO₂ humidified atmosphere at 37°C. To mimic atheroprone conditions, HUVECs were exposed to proatherogenic oxLDL (25 µg/ml; Biomedical Technologies S.L.).

HUVECs (5.0x10⁴ cells/well) were transfected with 100 nM miR-144-5p mimic or 100 nM mimic control for 48 h at 37°C using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 48 h of transfection, the transfection efficiency was detected using reverse transcriptase quantitative PCR (RT-qPCR).

**MTT assay.** Cell proliferation was detected by MTT assay. HUVECs (2.0x10⁵ cells/well) were plated into 96-well plates and cultured at 37°C for 12, 24 or 48 h, then 20 µl MTT (5 mg/ml; Sigma-Aldrich, Merck KGaA) was added to medium. Following 4-h incubation, 150 µl DMSO was used to dissolve the formazan crystals. The absorbance was measured at a wavelength of 490 nm using a microplate reader.

**Dual-luciferase reporter assay.** TargetScan (v7.2) bioinformatics software (http://www.targetscan.org/vert_72/) was used to predict target genes of miR-144-5p. The 3'-untranslated region (UTR) of SMAD1 was cloned into the luciferase reporter vector psiCHECK-2 (Promega Corporation) according to the manufacturer's instructions. Briefly, 500 ng of each reporter construct [wild-type (WT) or mutant 3'-UTR of SMAD1 or the psiCHECK-2 vector] and miR-144-5p mimic or mimic control were co-transfected into pre-confluent (60‑70%) HUVECs plated at a density of 1x10⁵ per well. At 48 h after transfection, total RNA from each well was subjected to qPCR using the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instruction. qPCR was performed with the SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. U6 and GAPDH were used as the endogenous controls. The thermocycling conditions were as follows: 95°C for 5 min, followed by 38 cycles of denaturation at 95°C for 15 sec and annealing/elongation at 60 sec for 30 sec. The primers used were as follows: U6, forward 5'-GCTTCGGCAG CACATACTAACAAT-3', reverse 5'-CGCTTTCACGAGTTT GCGTGTGTCAT-3'; GAPDH, forward 5'-CCATTGTGATCATGGA GAAGACTC-3', reverse 5'-GTAGAGCCAGGTATGATGTT TCT-3'; SMAD1, forward 5'-ACAGTCTGTGAAACGATGT GT TTA-3', reverse 5'-TGAGGTTGAACCATTTGGAGTAA GAA-3'. The experiments were repeated in triplicate, and the 2−ΔΔCT method was used to calculate the relative gene expression and normalized to the internal reference gene U6 or GAPDH, respectively (21).

**Western blotting.** HUVECs (5x10⁴ cells/well) were lysed using RIPA Lysis Buffer (Gibco; Thermo Fisher Scientific, Inc.) with protease inhibitor PMSF (Shanghai Biocolor BioScience & Technology Co., Ltd.). BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) was used to measure protein concentrations. A total of 20 µg of protein was subjected to 10% SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% BSA, and then the membranes were probed with primary antibodies against SMAD1 (cat. no. 6944; 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5753; 1:1,000; Cell Signaling Technology, Inc.) and incubated for the appropriate amount of time. After washing three times with PBS to remove any unbound antibodies, the membranes were probed with secondary antibodies conjugated to horseradish peroxidase and incubated for the appropriate amount of time. Following washing, the membranes were developed using an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific, Inc.) and imaged using a GelDoc™ Imaging System (Bio-Rad Laboratories, Inc.).

**Transwell invasion assay.** Transwell invasion assays were performed with using a Transwell chamber (8 µm pore size; Corning, Inc.). Cells (2x10⁴ cells/well) were collected and re-suspended in human endothelial serum-free culture medium (cat. no. 11111044; Gibco; Thermo Fisher Scientific, Inc.) containing 0.5% FBS and plated on the top of polycarbonate Transwell filter pre-coated with Matrigel (BD Biosciences). Complete culture medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS was added to the lower chamber. After incubation for 24 h at 37°C, the non-invasive free cells were carefully removed with cotton swab from the top chamber. The invaded cells on the lower membranes were fixed with 4% paraformaldehyde for 20 min at room temperature and stained by 0.1% crystal violet for 15 min at room temperature. Cells were counted in five random fields for each chamber at a magnification of x100 under Olympus IX71 Inverted Microscope (Olympus Corporation) to evaluate invasive capacity.

**Cell proliferation was detected by MTT assay.** HUVECs (2.0x10⁵ cells/well) were plated into 96-well plates and cultured at 37°C for 12, 24 or 48 h, then 20 µl MTT (5 mg/ml; Sigma-Aldrich, Merck KGaA) was added to medium. Following 4-h incubation, 150 µl DMSO was used to dissolve the formazan crystals. The absorbance was measured at a wavelength of 490 nm using a microplate reader.
Signaling Technology, Inc.), SMAD5 (cat. no. 12534; 1:1,000; Cell Signaling Technology, Inc.), p-SMAD5 (cat. no. ab92698; 1:1,000; Abcam), SMAD8 (cat. no. sc-293413; 1:1,000; Santa Cruz Biotechnology, Inc.), p-SMAD8 (cat. no. sc-12353, 1:1,000; Santa Cruz Biotechnology, Inc.), and GAPDH (cat. no. 5174; 1:1,000; Cell Signaling Technology, Inc.), at 4˚C overnight. The next day, the membrane was washed three times with PBS-Tween-20 (0.05%) buffer and then incubated with horse radish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (cat. no. 7074; 1:1,000; Cell Signaling Technology, Inc.) or mouse IgG κ light chain binding protein -HRP (cat. no. sc-516102; 1:1,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. The residue antibody solution was completely washed off with PBST, and protein bands were visualized using RapidStep™ ECL Reagent (cat. no. 345818; Merck KGaA). GAPDH served as loading control for normalization. Blot bands were semi-quantitively analyzed using ImageJ version 4.0 (National Institutes of Health).

Flow cytometry apoptosis assay. The Annexin V-FITC Apoptosis Detection kit I (BD Biosciences) was used to detect cell apoptosis, according to the manufacturer's protocol. A total of 100,000 suspended cells were washed twice with PBS, collected, centrifuged with 1,000 x g for 5 min at 20˚C, and re-suspended in 100 µl of FITC-binding buffer. Subsequently, ~5 µl ready-to-use Annexin V-FITC (BD Biosciences) and 5 µl propidium iodide (PI) were added. Cells were incubated for 30 min at room temperature in the dark. Annexin V-FITC and PI fluorescence were assessed by BD FACSCalibur flow cytometer (BD Biosciences). The percentages of cells in early apoptosis (Annexin V+/PI−) and late apoptosis (Annexin V+/PI+) were calculated.

Statistical analysis. All data are presented as the mean ± SD from three independent experiments in triplicate. Student's t-test or one-way ANOVA followed by Tukey's post hoc test was used for biostatistical analysis, using SPSS 19.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of miR-144-5p on HUVEC proliferation and apoptosis. Apoptosis of endothelial cells serves an important role in the occurrence and development of atherosclerosis (22). To study the role of miR-144-5p in atherosclerosis, firstly the effects of miR-144-5p on HUVEC proliferation and apoptosis were explored. HUVECs were transfected with mimic control or miR-144-5p mimic for 48 h. RT-qPCR was used to detect the transfection efficiency, and the results demonstrated that compared with the control group, transfection with miR-144-5p mimic significantly increased the level of miR-144-5p in HUVECs (Fig. 1A). Results from the MTT assay demonstrated that miR-144-5p mimic transfection...
reduced cell proliferation of HUVECs (Fig. 1B). In addition, to determine whether miR-144-5p regulated the apoptosis of HUVECs, flow cytometric analyses were performed to detect cell apoptosis. The results indicated that miR-144-5p mimic transfection significantly increased the apoptotic rates of HUVECs compared with the control (Fig. 1C and D).

Effects of miR-144-5p on HUVEC migration and invasion. To further examine the effects of miR-144-5p on the biological function of HUVECs, wound healing and Matrigel assays were performed (Fig. 2). The wound healing assay results demonstrated that miR-144-5p mimic suppressed cell migration compared with control groups (Fig. 2A). Matrigel assay results indicated that miR-144-5p mimic significantly inhibited the invasive ability of HUVECs (Fig. 2B). Taken together, these results showed that miR-144-5p inhibited the invasive and migratory potential of HUVECs.

**SMAD1 is the direct target gene of miR-144-5p.** To investigate the underlying mechanism of miR-144-5p on HUVECs, a putative biological target gene of miR-144-5p was identified and verified; the online bioinformatics tool TargetScan was used to predict target genes. The results identified a miR-144-5p target site in the SMAD1 3’UTR (Fig. 3A). Dual-luciferase reporter
assay results revealed that miR-144-5p mimic reduced the luciferase activity of the WT SMAD1 3’ UTR, but miR-144-5p mimic did not inhibit that of the reporter fused to the mutant SMAD1 3’ UTR (Fig. 3B). Taken together, these results demonstrated that SMAD1 was a direct target gene of miR-144-5p.

miR‑144‑5p inhibited SMAD1/5/8 pathway in HUVECs. To investigate the specific mechanism of action of miR‑144‑5p on HUVECs, the expression levels of proteins within the SMAD1/5/8 signaling pathway were examined. Western blot assay suggested that miR -144-5p mimic markedly decreased the protein level of SMAD1 and p-SMAD1 (Fig. 4A), and significantly reduced the ratio of p-SMAD1/SMAD1 (Fig. 4B). RT-qPCR assay results showed that miR-144-5p mimic decreased the relative mRNA expression of SMAD1 in HUVECs (Fig. 4C). Moreover, western blotting indicated that miR‑144‑5p mimic significantly decreased p-SMAD5/8 protein expression in HUVECs (Fig. 4D and E).

Discussion

In this present study, the primary aim was to explore the effect of miR-144-5p on HUVECs in vitro, to determine the role of miR-144-5p in atherosclerosis. It was demonstrated that miR-144-5p mimic transfection could suppress proliferation and induce apoptosis in HUVECs. It was also found that miR-144-5p mimic suppressed HUVEC migration and invasion. Furthermore, it was found that SMAD1 was a direct target of miR-144-5p, and miR-144-5p mimic suppressed the SMAD1/5/8 pathway in HUVECs.

miRNAs are a group of small, non-coding RNAs (9). Recent reports have identified multiple miRNAs that are involved in the pathogenesis of atherosclerosis (23-29). Vascular endothelial cells are crucial barriers in the vascular lumen (30). They maintain the stability of hemodynamics and material exchange, secrete inflammatory cytokines and regulate blood pressure through vasodilation and contraction factors (31). Previous studies have demonstrated that various miRNAs have different roles in the injury of vascular endothelial cells in both normal and tumor tissues. For example, previous research indicated that miRNA-129-1 and miRNA-133 promote vascular endothelial cell proliferation (32). However, the specific function and mechanism of action of miR-144-5p on HUVECs remain unclear. Consistent with these previous results, the present study provided evidence supporting the important role of miR-144-5p in HUVECs; the data demonstrated that miR‑144‑5p mimic suppressed the proliferation, migration and invasion of HUVECs and induced cell apoptosis.

The pathological role of miR-144-5p was further explored by identifying its direct target gene. Through bioinformatics, it was predicted that SMAD1 was a direct target of miR-144-5p. SMAD1 is a member of the SMAD family, and the family members are signal transducers and transcriptional modulators that mediate multiple signaling pathways (20). SMAD1 mediates bone morphogenetic proteins (BMP) signaling, and BMP stimulation increases SMAD1 phosphorylation,
allowing it to form complexes with GAL4 to act as a functional transcription regulator (33). The BMP-SMAD1/5/8 signaling pathway participates in multiple biological processes including cell growth, apoptosis, morphogenesis, differentiation and immune modulation. A previous study also reported that SMAD1 is a target gene of miR-144 (20). Ren et al reported that miR-144 upregulation could decrease the activity of mTOR signaling pathways and suppress cell proliferation in osteosarcoma cells (34). In the present study, it was found that miR-144-5p was associated with the SMAD signaling pathway. miR-144-5p mimic inhibited the expression of β-SMAD1/5/8. Consequently, these results further confirm that miR-144-5p serves a crucial role in HUVECs.

In conclusion, miR-144-5p may modulate HUVECs proliferation, apoptosis, invasion and migration through affecting the SMAD signaling pathway by altering the expression of SMAD1, and thus may participate in the onset and development of atherosclerosis. Therefore, the data from this present study may provide a new theoretical basis and strategy for the diagnosis and treatment of atherosclerosis. However, this study is only a preliminary investigation in to the role of miR-144-5p in atherosclerosis. Further studies are needed to better understand the role of miR-144-5p in atherosclerosis. For example, it would be interesting to investigate whether SMAD1 upregulation could reverse the effect of miR-144-5p on HUVECs. How the SMAD1/5/8 pathway is involved in the effect of miR-144-5p in HUVECs should also be further explored. Furthermore, the effect of downregulating miR-144-5p in HUVECs should be investigated. Finally, the relationship between the expression of miR-144-5p and SMAD1, in the context of the clinical features of atherosclerosis needs to be 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.

Competing interests
The authors declare that they have no competing interests.

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