Original Paper

MicroRNA-142-3p Induces Atherosclerosis-Associated Endothelial Cell Apoptosis by Directly Targeting Rictor

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Key Words

Microrna-142-3p • HAECs • Apoptosis • Rictor • Atherosclerosis

Abstract

Background/Aims: Atherosclerosis, a multifactorial chronic disease, is the main cause of death and impairment in the world. Endothelial cells (ECs) apoptosis plays a crucial role in the onset and development of atherosclerosis, whereas the underlying molecular mechanisms are unclear. MicroRNA-142-3p (miR-142-3p) is a well-defined tumor suppressor in several types of cancer, while the role of miR-142-3p in ECs apoptosis and the development of atherosclerosis has yet to be elucidated. Therefore, the present study aimed to investigate the role of miR-142-3p in ECs apoptosis during atherosclerosis and the underlying mechanism. **Methods:** Human aortic endothelial cells (HAECs) were treated with oxidized low-density lipoprotein (ox-LDL). The expression level of miR-142-3p was detected using qRT-PCR. Apoptosis was determined via flow cytometry and Caspase-3 activity assay. Prediction of the binding between miR-142-3p and 3'-UTR of Rictor mRNA was performed by bioinformatics analyses and confirmed by a dual luciferase reporter assay. The effects of miR-142-3p on endothelial apoptosis and atherosclerosis were further analyzed in an *in vivo* model using ApoE^{-/-} mice fed with high-fat diet (HFD). **Results:** MiR-142-3p expression was substantially up-regulated during the ox-LDL-elicited apoptosis in HAECs. Forced expression of miR-142-3p exacerbated apoptosis in ECs whereas inhibition of miR-142-3p could partly alleviate apoptotic cell death mediated by ox-LDL. Further analysis identified Rictor as a direct target of miR-142-3p, and Rictor knockdown abolished the anti-apoptotic effect of miR-142-3p inhibitor. Moreover, the Akt/endothelial nitric oxide synthase (eNOS) signaling pathway was found to mediate the beneficial effect of miR-142-3p inhibitor on endothelial apoptosis. Finally, systemic treatment with miR-142-3p antagomir attenuated endothelial apoptosis and retarded the progression of atherosclerosis in the aorta of ApoE^{-/-} mice. **Conclusions:** Down-regulation of miR-142-3p inhibited ECs apoptosis and atherosclerotic development by up-regulating the expression of Rictor and activating the Akt/eNOS signaling pathway. This indicates that miR-142-3p may be a potential target for the prevention and treatment of atherosclerosis.

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Introduction

Atherosclerosis, a chronic multi-factorial vascular disease, has become a primary cause of death worldwide [1]. Atherosclerotic lesions, which primarily occur in large and medium-sized arteries, can be present throughout life and cause stroke or infarction due to ischemia in the brain, heart or extremities, and accumulating evidence has shown that injury and apoptosis in the vascular endothelium are critical events in the development and pathogenesis of atherosclerosis [2]. In certain pathological conditions, endothelial cells (ECs) apoptosis can increase the adhesion of platelets, facilitate infiltration of inflammatory cells and lipids into the intima and promote smooth muscle cells (SMCs) migration and proliferation, leading to atherogenesis [3]. Oxidized low-density lipoprotein cholesterol (ox-LDL) is a well-established risk factor for atherothrombosis and exerts a plethora of effects to promote ECs apoptosis, plaque progression, and inflammatory interactions between monocytes and the underlying vessel wall [4]. Understanding the molecular mechanisms of how ox-LDL incites ECs apoptosis may uncover novel approaches to limit atherosclerosis.

MicroRNAs (miRNAs) are small non-coding RNAs that post-transcriptionally control protein translation through pairing with the 3'-untranslated region (3'-UTR) of the mRNA from their target genes [5]. MiRNAs have been found to play a significant role in vascular biology and atherosclerosis [6]. The activation of ECs promotes leukocyte recruitment from the blood into extravascular tissues and thus contributes to the pathogenesis of atherosclerosis. As one of the most highly expressed miRNAs in ECs, miR-126-3p interferes with this process by inhibiting the expression of VCAM-1 and impeding *in vitro* leukocyte adhesion to ECs [7]. Following their adhesion to the endothelium, inflammatory cells—mainly monocytes—can migrate into the intima, differentiate into macrophages and enhance the local inflammatory response. The expression of miR-155 increases progressively in lesional macrophages during atherogenesis and promotes inflammatory activation by targeting BCL6 [8]. Macrophages take up excessive modified lipoproteins via scavenger receptors, leading to the accumulation of intracellular cholesterol and triglycerides in the form of lipid droplets and the formation of “foam cells”. *In vitro* studies have investigated the role of miRNAs in the regulation of ox-LDL uptake, showing that miR-125a-5p reduces the expression of the scavenger receptors CD68 and lectin-like ox-LDL receptor (LOX)-1 [9]. The dedifferentiation of VSMCs from a “contractile” to a “synthetic” phenotype, their proliferation and their migration from the media to the intima are crucial steps in the progression of atherosclerosis. Knowledge concerning the contribution of miRNAs to the regulation of the phenotypic switch of VSMCs has steadily grown in recent years. In particular, miR-143/-145 cluster thwarts proliferation and the acquisition of a synthetic phenotype [10]. Atherosclerotic plaque destabilization and rupture is the result of biologically active processes intricately regulated by miRNAs. Treatment with LNA anti-miR-24 *in vivo* is associated with a faster progression of atherosclerosis toward an unstable phenotype, characterized by decreased collagen content and increased macrophage infiltration [11]. Additionally, mounting evidence suggests that miRNAs orchestrate lipid metabolism and affect the development and progression of atherosclerosis. For example, antagonism of miR-33 in experimental animal models generally results in increased cholesterol efflux to ApoA1, thus increasing serum high-density lipoprotein (HDL) and decreasing cholesterol levels in peripheral tissues. The overall result is increased plaque stability, improved blood lipid profile, and inhibition of atherosclerosis progression [12]. However, whether miRNAs also participate in regulating atherosclerosis-associated ECs apoptosis remains largely unexplored.

MiRNA-142-3p (miR-142-3p), a miRNA that is well characterized in embryonic development and homeostasis, has been demonstrated to be an epigenetic regulator of cardiovascular diseases [13]. MiR-142-3p inhibits hypoxia/reoxygenation-induced apoptosis and fibrosis of cardiomyocytes, partly at least, by the direct inhibition of HMGB1 expression [14]. Exosomal miR-142-3p is increased during cardiac allograft rejection and compromises endothelial barrier function via down-regulation of RAB11FIP2 [15]. Chen et al. revealed that miR-142-3p is an important regulator of early cardiomyocyte differentiation [16].

Microarray analysis revealed that miR-142-3p is dramatically increased in human umbilical vein endothelial cells (HUVECs) treated with ox-LDL, which is generally considered as an inducer of ECs apoptosis [17]. The present study was designed to investigate the role of miR-142-3p in ECs apoptosis in the setting of atherosclerosis and the underlying mechanisms.

Materials and Methods

Cell culture and transfection

Human aortic endothelial cells (HAECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in Endothelial Cell Medium (ECM, ScienCell, Carlsbad, CA) supplemented with endothelial cell growth factors (ScienCell, Carlsbad, CA), 5% FBS (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured in 6-well plates and exposed to ox-LDL (100 µg/ml) for 0, 6, 12 and 24 h.

Mimic and inhibitor oligonucleotides of miR-142-3p were synthesized by Ribo Bio (Guangzhou, China). MiRNA mimic negative control (NC mimic) and miRNA inhibitor negative control (NC inhibitor) (Ribo Bio, Guangzhou, China) are random sequence miRNA mimic molecule and inhibitor molecule that have been extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function. They are designed to be used as negative controls for experiments using miRNA mimics and inhibitors. The transfection was carried out using the lipid carrier lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Oligonucleotides (50 nM) prepared with lipid carrier were subjected to the transfection in a 2 ml of Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) for each well of the standard 6-well plates. The HAECs were incubated with the oligonucleotides/lipofectamine complex for 24 h and then the medium was replaced by fresh medium with or without ox-LDL (100 µg/ml, 24 h). In order to explore the involvement of Akt/endothelial nitric oxide synthase (eNOS) pathway in miR-142-3p suppression induced protection, HAECs were first pretreated with normal saline, LY294002 (20 µM) or L-NAME (100 µM) (Beyotime, Jiangsu, China) for 2 h, and then were transfected with NC inhibitor or miR-142-3p inhibitor and further exposed to ox-LDL (100 µg/ml) for an additional 24 h. After ox-LDL treatment, the cells were used for apoptosis measurement or protein/RNA extraction.

Rictor-specific siRNA and control scrambled siRNA (NC siRNA) were chemically synthesized from Ribo Bio (Guangzhou, China). HAECs were seeded in six-well plates at a concentration of 3×10^5 per well and grown overnight, and then the cells were transfected with Rictor siRNA at a final concentration of 50 nM using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Rictor-specific siRNA sequence was 5'-UGUAUCUGGACUAUGUCUGTT-3' and 5'-CAGACAUGUCCAGAUACATT-3'. For rescue experiment, HAECs were co-transfected with miR-142-3p inhibitor (50 nM) and si-Rictor or NC siRNA. After that, HAECs were exposed to ox-LDL (100 µg/ml) for an additional 24 h. Then cells were used for apoptosis measurement or protein extraction.

Animal models

ApoE^{-/-} mice on a C57BL/6 background were obtained from Beijing University of Medicine Laboratory. All experimental procedures were approved by the institutional animal care. Male 8-week-old ApoE^{-/-} mice were randomly divided into four groups (10 mice per group): miR-142-3p agomir negative control (AG-NC), miR-142-3p agomir (AG), miR-142-3p antagomir negative control (AN-NC) and miR-142-3p antagomir (AN). All mice from different groups received tail vein injections of miRNA agomir/antagomir or their respective controls (80 mg/kg/d) for 10 days after starting the high fat/high cholesterol western diet (15% fat wt/wt, 0.25% cholesterol wt/wt). After 8 weeks in the western diet, animals were sacrificed. The aortas were carefully excised, washed in cold PBS and freed of connective tissue. Four aortas randomly selected from each group were opened longitudinally and fixed in 4% formalin for 24 h. Atherosclerotic lesions were identified as discrete and raised white areas stained with Sudan IV. The stained aorta was scanned with a digital camera and the total morphometric lesion area was evaluated with the NIH ImageJ software. Data were expressed as the percentage of atherosclerotic area relative to the whole aortic area. The residual aortas were divided into two parts. The thoraco aortas were embedded in paraffin. Serial sections of 4 µm were stained with hematoxylin/eosin (HE) for morphologic analysis. For the analysis of endothelial apoptosis, terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed according

to the specifications for *in situ* apoptosis detection kit. Micrographs were obtained using a Leica microscope (Leica DMI3000 B). To determine the apoptosis rate, 10 random fields from each section were quantified in a blinded manner. Three sections per group were used. The abdominal aortas were stored in liquid nitrogen. The expression of miR-142-3p was determined with qRT-PCR and the protein levels of Rictor and Akt were detected by western blot. All animal procedures were approved by the Sun Yat-sen University Animal Care and Use Committee.

Ox-LDL preparation

Human ox-LDL was isolated from fresh human plasma by sequential ultracentrifugation, as described previously [18].

Measurement of apoptosis by Caspase-3 activity assay and flow cytometry

The Caspase-3 activity was determined using colorimetric assay kits (Beyotime, Jiangsu, China) according to the instruction. Briefly, cells were collected and lysed using lysate buffer. After that, 40 µl of the cell lysate (cytosolic extracts, 100 µg) was mixed with 50 µl of reaction buffer and 10 µl of Ac-DEVD-pNA substrates (Beyotime, Jiangsu, China), and incubated at 37°C for 2 h. After incubation, the chromophores were quantified spectrophotometrically at a wavelength of 405 nm.

Cells were separated and washed three times with PBS (4°C pH 7.4) and centrifuged at 1000 rpm for 5 min. The binding buffer was diluted with pure water at a ratio of 1:4 and added 250 µl to the cells. Cell suspension was stained using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Jiangsu, China) and the mixture was incubated for 15 min protecting from light at room temperature. The apoptosis was analyzed by flow cytometry and the results were analyzed with software LYSISII.

Cell viability assay

The cell viability of HAECs was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. After treatment, 5 mg/ml MTT (MBI, USA) was added to each well. Subsequently, the cultures were incubated for an additional 4 h at 37 °C. The supernatant was aspirated, and formazan crystals were dissolved in 150 µl per well of dimethyl sulfoxide. The absorbance at 540 nm was measured with a microplate reader (Bio-tek, USA). Cell viability was calculated as follows: cell viability (%) = optical density (OD) treatment/OD control (0 h) ×100%.

Cell proliferation assays

The cell counting kit (CCK-8, Beyotime, Jiangsu, China) assay was used to determine cell proliferation. The HAECs (1×10⁴/ml) were seeded in a 96 well plate at 100 µl/well and incubated overnight. HAECs were transfected with miR-142-3p mimic/inhibitor and then cultured with ox-LDL (100 µg/ml) for 24 h. 10 µl CCK8 solution was added to each well, according to the manufacturer's instructions. After 2 h incubation at 37°C, the optical density value was measured at a wavelength of 450 nm. The percentage inhibition of cell proliferation was determined by comparing the absorbance of treated with controls as follows: Inhibition (%) = [1-(A of the experimental sample/A of the control)] ×100%].

MiRNA quantitative real-time PCR (qRT-PCR)

Total RNA from cells was isolated using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. To quantify the expression of miR-142-3p, we synthesized cDNA from 10 ng of RNA sample using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Quantitative PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems, CA, USA) specific for miR-142-3p in a 7900HT RT-PCR machine (Applied Biosystems, CA, USA) according to the manufacturer's protocol. To normalize the expression level of miRNA, U6 RNA was used as an endogenous control.

Western blot and mRNA qRT-PCR analysis

HAECs and aortas were lysed and the protein extracts were denatured. The solubilized proteins (20 µg) were subjected to electrophoresis, blotted to polyvinylidene difluoride membrane, and reacted with specific antibodies. Primary antibodies against Rictor (sc-81538), p-Akt (Ser473) (sc-514032), Akt (sc-81434), p-eNOS (Ser 1177) (sc-81510), eNOS (sc-376751), GAPDH (sc-32233) and β-actin (sc-130065) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the secondary antibody was from KPL

(Gaithersburg, MD, USA). For mRNA analysis, cDNA was synthesized from 1 µg of total RNA sample isolated through TRIzol reagent (Invitrogen, CA, USA) with RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Lithuania) according to the standard protocol. 2 µl cDNA was amplified by real-time polymerase chain reaction using the SYBR® Premix Ex Taq™ II kits (TaKaRa, Dalian, China) with gene specific primers on a Applied Biosystems 7900HT RT-PCR system, according to the manufacturer's instructions.

Luciferase assay

Human Rictor 3'-UTR fragments containing potential binding sites for miR-142-3p were PCR-amplified using human Rictor cDNA as the template. The PCR product was gel purified and subcloned into the SpeI/HindIII site of pMIR-REPORT Luciferase vector (Ambion). We also generated a pMIR-Rictor-3'UTR mutant with three bases in the putative binding site. For luciferase assay, HAECs were co-transfected with 100 ng of pMIR-Rictor-3'UTR or pMIR-Rictor-3'UTR mutant plasmid, and 50 nM of miR-142-3p mimic or NC mimic combined with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48h incubation, the cells were washed and lysed. The relative luciferase activity was assayed using the Promega Dual-Luciferase reporter assay system. All the data were the average of at least three independent transfections.

Statistical analysis

Data are presented as mean±S.D. and evaluated by analysis of variance (ANOVA) or student *t*-test when appropriate. Significance was established at a level of *p* < 0.05.

Results

MiR-142-3p is up-regulated in apoptotic HAECs induced by ox-LDL

After treatment of HAECs with 100 µg/ml ox-LDL for 0 to 24 h, Caspase-3 activity was increased in response to ox-LDL in a time-dependent manner. Flow cytometry analysis using Annexin V-FITC and propidium iodide (PI) double staining demonstrated that the percentage of apoptotic ECs was increased following ox-LDL treatment (Fig. 1A, 1B and 1C). The exposure of HAECs to ox-LDL at 100 µg/ml for 24 h resulted in a significant reduction of cell viability (Fig. 1D). With the increasing apoptotic rate, miR-142-3p was gradually increased in HAECs after ox-LDL incubation, and a more than threefold increase in the miR-142-3p amount was observed at 24 h (Fig. 1E).

Rictor is one of the target genes for miR-142-3p in HAECs

Function of miRNA relies on the inhibition of the target mRNA; therefore, we used the online bioinformatics prediction tools (TargetScan, miRanda and MIRDDB) to identify the putative target genes of miR-142-3p. Among the consensus targets selected by three prediction programs, Rictor was of interest. Rictor knockdown attenuates cell cycle progression and enhances apoptosis in gastric cancer cells [19]. Moreover, miR-142-3p could suppress Rictor expression in YT cells [20]. MiR-142-3p was predicted to bind to the positions of 541-547 in Rictor 3'-UTR (Fig. 2C), and the binding energy between MiR-142-3p and Rictor was relatively low. Thus, Rictor may be a direct target of miR-142-3p in ECs. To investigate the effects of miR-142-3p on Rictor protein and mRNA expressions, miR-142-3p mimic or miR-142-3p inhibitor was transfected into HAECs. As shown in Fig. 2A, ox-LDL treatment decreased Rictor protein expression, which was inversely correlated with miR-142-3p level. MiR-142-3p mimic dramatically reduced the protein level of Rictor and miR-142-3p inhibitor increased the expression of Rictor protein. Although transfection of miR-142-3p mimic or miR-142-3p inhibitor changed Rictor protein levels, transfection did not change Rictor mRNA expression (Fig. 2B). Therefore, miR-142-3p might directly regulate Rictor expression at the post-transcriptional level in HAECs.

To examine whether miR-142-3p directly binds the 3'UTR of Rictor mRNA, we constructed the luciferase reporter plasmid pMIR-ELK1-3'UTR containing the putative miR-142-3p binding site of Rictor 3'UTR downstream of the luciferase open reading frame (Fig. 2C). When miR-142-3p mimic was co-transfected with pMIR-Rictor-3'UTR, the expression of

Fig. 1. Apoptotic/necrotic effects and expression of miR-142-3p in HAECs exposed to ox-LDL for 0 h, 6 h, 12 h and 24 h. (A) Caspase-3 activity analysis of HAECs apoptosis. (B) Flow cytometric analysis of apoptosis and necrosis. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for AV binding (AV⁻/PI⁻). The lower right quadrants represent the apoptotic cells; AV positive and PI negative (AV⁺/PI⁻). The upper right quadrants contain the necrotic cells, positive for AV binding and for PI uptake (AV⁺/PI⁺). FL1-H: the fluorescence height of AV; FL2-H: the fluorescence height of PI. (C) Comparison of the apoptotic and necrotic death rate of HAECs. (D) The viability of HAECs was measured using MTT assay. (E) Relative expression level of miR-142-3p in HAECs was analyzed using qRT-PCR. Each bar represents the means±S.D. (n=3), * p<0.05 versus 0 h group, ** p<0.01 versus 0 h group.

Fig. 2. Experimental validation of Rictor as a target gene of miR-142-3p. (A) HAECs were transfected with NC mimic, miR-142-3p mimic, NC inhibitor or miR-142-3p inhibitor and further exposed to ox-LDL (100 µg/ml) for an additional 24 h. Rictor protein was measured by western blot, and the GAPDH expression was used for protein level normalization. (B) Rictor mRNA was measured by qRT-PCR. (C) The putative target site of Rictor mRNA 3'UTR determined by computational predictions. The target sequence was cloned into pMIR-REPORT vector (pMIR-Rictor-3'UTR). pMIR-Rictor-3'UTR mutant was also generated with three bases mutation (indicated by italic characters) in the target site. (D) HAECs were transfected with pMIR-Rictor-3'UTR or pMIR-Rictor-3'UTR mutant together with miR-142-3p mimic or NC mimic, and luciferase activity was measured and normalized to β-galactosidase expression levels. MiR-142-3p mimic reduced expression of luciferase containing a wild-type miR-142-3p binding site but not a mutant binding site. Values are means±S.D. (n=3). * p<0.05 compared with NC mimic/inhibitor group, ** p<0.01 compared with NC mimic/inhibitor group, # p<0.05 compared with NC mimic/inhibitor + ox-LDL group.

firefly luciferase was significantly decreased compared to the NC mimic co-transfected group (Fig. 2D). We also generated a pMIR-Rictor-3'UTR mutant with three bases mutation in the seed region of the putative binding sequence to investigate whether the down-regulation of firefly luciferase can be attributed to the mutation (Fig. 2C). Bases mutation blocked the inhibition of firefly luciferase induced by miR-142-3p mimic (Fig. 2D). These results strongly suggest a direct inhibitory effect of miR-142-3p on Rictor expression.

Down-regulation of miR-142-3p suppresses ECs apoptosis

The effect of miR-142-3p on ECs apoptosis was determined using Caspase-3 activity assay (Fig. 3A) and flow cytometry (Fig. 3B and 3C). The two methods showed that apoptotic rate of ECs was up-regulated in the presence of ox-LDL. Overexpression of miR-142-3p significantly enhanced apoptosis, whereas transfection with miR-142-3p inhibitor markedly diminished the ability of ox-LDL to induce apoptosis. Moreover, ox-LDL insult significantly inhibited HAECs survival and proliferation in our experimental condition. Overexpression of miR-142-3p after ox-LDL stimulation led to reduced survival and proliferation rate in HAECs, while knockdown of miR-142-3p elevated survival and proliferation of HAECs (Fig. 3D and Table 1).

Fig. 3. Influence of miR-142-3p on apoptosis and survival of HAECs induced by ox-LDL. HAECs were subjected to ox-LDL treatment after miR-142-3p mimic or inhibitor transfection. (A) The activity of Caspase-3 was determined by luminescent substrate assays. (B) Quantitative measurement of survival, apoptosis and necrosis by flow cytometry after AV/PI double stain. The lower left quadrants of each panels show the viable cells, which exclude PI and are negative for AV binding (AV-/PI-). The lower right quadrants represent the apoptotic cells; AV positive and PI negative (AV+/PI-). The upper right quadrants contain the necrotic cells, positive for AV binding and for PI uptake (AV+/PI+). FL1-H: the fluorescence height of AV; FL2-H: the fluorescence height of PI. (C) Comparison of the apoptotic and necrotic death rate of HAECs. (D) Comparison of the survival rate of HAECs. Values are means±S.D. (n=3). * p<0.05 compared with NC mimic/inhibitor group, ** p<0.01 compared with NC mimic/inhibitor group, # p<0.05 compared with NC mimic/inhibitor+ox-LDL group, ## p<0.01 compared with NC mimic/inhibitor+ox-LDL group.

MiR-142-3p mediates endothelial apoptosis through targeting Rictor

To further clarify the role of Rictor in mediating the pro-apoptotic action of miR-142-3p in HAECs, we performed a rescue experiment. As shown in Fig. 4A, transfection of miR-142-3p inhibitor up-regulated Rictor protein expression, whereas co-transfection with si-Rictor led to a significant decrease in the protein level of Rictor. Caspase-3 activity assay and flow cytometry analysis showed that miR-142-3p inhibitor attenuated endothelial apoptosis elicited by ox-LDL, but repression of Rictor protein expression by siRNA abolished the beneficial effect of miR-142-3p inhibitor (Fig. 4B, 4C and 4D).

Involvement of Akt/eNOS signaling pathway in the miR-142-3p-mediated apoptosis of HAECs treated with ox-LDL

Rictor is an important component of mammalian target of rapamycin complex 2 (mTORC2) and the activation of the mTORC2 depends on the presence of the Rictor protein [21]. As the Akt/eNOS signaling pathway has been reported to be activated by mTORC2 and involved in modulating ECs apoptosis, we further elucidated whether Akt/eNOS signaling pathway was involved in the action of miR-142-3p. As shown in Fig. 5A and 5B, ox-LDL reduced the phosphorylation levels of Akt (Ser473) and eNOS (Ser1177) in HAECs after 24 h of incubation, but this tendency was partially reversed by knockdown of miR-142-3p. However, miR-142-3p mimic down-regulated the p-Akt and p-eNOS protein expression in ox-LDL-treated HAECs. While the transfection of miR-142-3p inhibitor increased the phosphorylation of Akt and eNOS, the selective Akt antagonist

Table 1. Anti-proliferation effect of miR-142-3p in HAECs. A₄₅₀, absorption at 450 nm. ^a Percentage of decrease in proliferation rate compared with NC mimic/inhibitor. Values are means±S.D. (n=3). **p<0.01 compared with NC mimic/inhibitor, #p<0.05 compared with NC mimic/inhibitor +ox-LDL group

	A ₄₅₀	Inhibition ratio ^a
NC mimic	0.97±0.03	-
NC mimic+ox	0.71±0.05	24.5%**
miR-142-3p mimic+ox	0.55±0.02	43.3%#
NC inhibitor	0.94±0.06	-
NC inhibitor +ox	0.69±0.03	26.6%**
miR-142-3p inhibitor+ox	0.83±0.04	11.7%#

Fig. 4. MiR-142-3p modulated HAECs apoptosis through Rictor. (A) Rictor protein decreased by transfection of si-Rictor in HAECs. Rictor protein level was measured by Western blotting. Down-regulation of Rictor by si-Rictor blocked the ability of miR-142-3p inhibitor to suppress apoptosis in ox-LDL treated HAECs. The apoptosis was measured by Caspase-3 activity assay (B) and flow cytometry after AV/PI double stain (C and D). Values are means±S.D. (n=3), * p<0.05, ** p<0.01.

Fig. 5. Down-regulation of miR-142-3p reduced endothelial apoptosis via Akt/eNOS signaling pathway. MiR-142-3p regulated the phosphorylation of Akt (A) and eNOS (B). HAECs were exposed to ox-LDL (100 μ g/ml, 24 h) after miR-142-3p mimic or inhibitor transfection. Total lysate from each sample was subjected to western blot analysis using specific antibodies to Akt at Ser-473 and eNOS at Ser-1177 (top panel). The intensity of protein bands was quantified and was shown as relative expression level after normalized by β -actin (bottom panel). HAECs were pretreated with normal saline, LY294002 (20 μ m) or L-NAME (100 μ m) for 2 h, and then were transfected with NC inhibitor or miR-142-3p inhibitor and further exposed to ox-LDL (100 μ g/ml) for an additional 24 h. The expression levels of Akt, pS473-Akt, eNOS and pS1177-eNOS were measured by western blot analysis (C). The apoptosis was detected by Caspase-3 activity assay (D) and flow cytometry after AV/PI double stain (E and F). Values are means $\pm$ S.D. (n=3), *p<0.05, **p<0.01.

LY294002 markedly prevented the miR-142-3p inhibitor-induced phosphorylation of Akt and eNOS. The eNOS inhibitor L-NAME only reduced eNOS phosphorylation, with no effect on p-Akt expression after ox-LDL and miR-142-3p inhibitor treatment (Fig. 5C). Furthermore, inhibition of miR-142-3p markedly ameliorated ECs apoptosis. When the Akt/eNOS signaling pathway was blocked by LY294002 or L-NAME, miR-142-3p inhibitor failed to protect cells against ox-LDL-induced apoptosis (Fig. 5D, 5E and 5F). Altogether, these data reveal that miR-142-3p inhibitor exerts its anti-apoptotic effect through activating the Akt/eNOS signaling pathway.

MiR-142-3p promotes endothelial apoptosis and development of aortic atherosclerosis in ApoE^{-/-} mice

To determine whether endothelial apoptosis was regulated by miR-142-3p *in vivo*, ApoE^{-/-} mice were fed with a high-fat diet (HFD) and injected intravenously with miR-142-3p agomir (AG) or antagomir (AN) or control miRNA oligonucleotides (AG-NC/AN-NC). First we measured the expression of miR-142-3p in the aorta of mice. The expression of miR-142-3p in the aorta was increased in AG treated mice. On the other hand, the expression level of miR-142-3p was decreased by more than 50% in AN treated mice compared with those of mice receiving AN-NC (Fig. 6A). These data suggest that injection of miR-142-3p agomir and antagomir can significantly alter miR-142-3p level in the aorta of mice. TUNEL staining was then performed to assess endothelial apoptosis in the aorta of ApoE^{-/-} mice. Results showed that AG significantly increased the percentage of TUNEL-positive cells compared with AG-NC. Administration of AN significantly reduced the percentage of TUNEL-positive cells (Fig. 6B). Moreover, we investigated the role of miR-142-3p in the development of atherosclerosis in ApoE^{-/-} mice fed with HFD. The atherosclerotic lesion area was examined by an *en face* analysis of the total aorta and the cross-sections of the thoraco aorta. The *en*

Fig. 6. Effects of miR-142-3p on endothelial apoptosis and atherosclerotic lesion area in ApoE^{-/-} mice. Male 8-week-old ApoE^{-/-} mice were given a tail vein injection with miR-142-3p agomir negative control (AG-NC), miR-142-3p agomir (AG), miR-142-3p antagomir negative control (AN-NC) or miR-142-3p antagomir (AN) (80 mg/kg/d) for 10 days and remained in high fat/high cholesterol Western diet until after 8 weeks. (A) Relative expression level of miR-142-3p in the aorta was analyzed using qRT-PCR. Values are means±S.D. (n=6). (B) Apoptotic cells in the aortic endothelium were detected by TUNEL staining. Representative images of TUNEL assay in AG-NC, AG, AN-NC and AN groups (left). Nuclei of normal cells are blue, and nuclei of apoptosis cells are brown. Black arrows represent the positive staining of TUNEL. Quantitative plot of TUNEL positive cells in AG-NC, AG, AN-NC and AN groups (right). Apoptosis rate was determined by the ratio of apoptotic nuclei to total nuclei. Values are means±S.D. Bar=80 μm, (n=6). (C) Representative images and the quantification of atherosclerotic lesion area in the *en face* analysis of the aorta with Sudan IV staining in ApoE^{-/-} mice. Values are means±S.D. Bar=3 mm, (n=4). (D) Representative microscopic images and quantification of atherosclerotic plaque area in cross-sections of thoraco aorta with hematoxylin-eosin staining in ApoE^{-/-} mice. Values are means±S.D. Bar=500 μm, (n=6). (E) The expression levels of Rictor, Akt and pS473-Akt in the aorta of ApoE^{-/-} mice were determined using western blot. Values are means±S.D. (n=6), *p<0.05 compared with AG-NC group, #p<0.05 compared with AN-NC group.

face analysis of the total aorta showed that atherosclerotic lesions in the aorta of ApoE^{-/-} mice were significantly increased in AG group compared with the AG-NC group. Conversely, AN treated mice had a statistically significant reduction in atherosclerotic plaque formation in comparison to AN-NC (Fig. 6C). We then examined atherosclerotic plaque development by thoraco aorta HE stained lesions, and found that the plaque area in AG-treated mice was greater and more severe than that in mice treated with AG-NC. On the other hand, plaques in AN-treated mice were markedly reduced compared with plaques in the AN-NC mice (Fig. 6D). Finally, we investigated the effects of miR-142-3p on Rictor expression and phosphorylation of Akt *in vivo*. The isolated aorta from each group was homogenized for the determination of Rictor expression and Akt phosphorylation by western blot (Fig. 6E). The expression of Rictor in the aorta in ApoE^{-/-} mice was significantly reduced in AG group, but increased in AN group, compared with those in mice injected with the negative control. The level of Akt phosphorylation on serine 473 was significantly lower in AG group compared with control mice, and AN treatment resulted in an increase of aortic Akt phosphorylation. Together, these results indicate that miR-142-3p promotes endothelial apoptosis *in vivo* and the development of atherosclerosis probably through suppression of Rictor expression and Akt phosphorylation.

Discussion

Atherosclerosis is an intricate disease of medium and large-sized arteries. Importantly, the first step in the pathogenesis of atherosclerosis appears to be vascular injury, and

endothelial apoptosis plays a vital role in endothelial dysfunction and atherosclerosis lesion formation [22]. Since ox-LDL is a major risk factor for the onset and development of atherosclerosis [23], the present study used ox-LDL-treated HAECs as an experimental model to identify the specific miRNAs associated with endothelial apoptosis in atherosclerosis.

During the past few years, miRNAs have emerged as key regulators for endothelial biology and dysfunction [6]. Deregulation of miRNA expression has been proved to be associated with endothelial angiogenesis, injury, inflammation and senescence. The mechanistic studies revealed that miR-93-5p can promote angiogenic process through inhibiting EPLIN expression in ECs [24]. It has been demonstrated that miR-126 overexpression activates PI3K/Akt/mTOR pathway, thereby restoring the autophagic flux and alleviating ox-LDL-induced ECs injury [25]. MiR-20b is showed to target RBL1 in the modulation of premature senescence in ECs induced by chronic exposure to TNF- α [26]. Furthermore, aberrantly expressed miRNAs have recently been reported to play a role in the regulation of ECs survival/death in the setting of atherosclerosis. For example, up-regulation of miR-876 induces ECs apoptosis by suppressing anti-apoptotic protein Bcl-xl in the development of atherosclerosis [27]. MiR-351, up-regulated in the serum and ECs of the atherosclerotic mice, can attenuate the survival of ECs and suppress angiogenesis through targeting STAT3 [28]. Thus, the identification of miRNAs and their targets may cast new light on the therapeutic strategies to endothelial dysfunction and atherosclerosis. The present study demonstrated for the first time that miR-142-3p exerted an effect on modulating endothelial apoptosis. Specifically, overexpression of miR-142-3p significantly enhanced endothelial apoptosis whereas inhibition of miR-142-3p could partly alleviate apoptotic cell death *in vitro* and *in vivo*. The study of the mechanism underlying this effect revealed that miR-142-3p promoted ECs apoptosis by suppressing the downstream target Rictor and the Akt/eNOS signaling pathway. Overall, our study provided evidence to suggest that miR-142-3p may be a novel target for the prevention and treatment of atherosclerosis.

MiR-142-3p, localized on chromosome 17q22, is reported to be down-regulated and plays a pro-apoptotic role in various tumors [13]. For example, it has been revealed that overexpression of miR-142-3p improves drug sensitivity of acute myelogenous leukemia (AML) cells by inhibiting cell viability and promoting apoptosis [29]. Zhang et al. indicated that miR-142-3p inhibits non-small cell lung carcinoma (NSCLC) cell proliferation and induces cell apoptosis via targeting HMGB1 [30]. In addition, it has been shown that miR-142-3p is a key regulator of cardiomyocytes apoptosis and fibrosis induced by hypoxia/reoxygenation [14], therefore serving as a candidates for myocardial injury therapeutic strategy. A recent study done by Bao et al. demonstrated that platelet-derived miR-142-3p is delivered into ECs via platelet-derived microparticles (PMPs) and induces ECs apoptosis in hypertension through modulating the expression of target molecule BCL2L1 [31]. Consistent with these findings, we found that miR-142-3p was an essential mediator for ECs apoptosis both *in vitro* and *in vivo*. MiR-142-3p was up-regulated in the apoptotic HAECs elicited by ox-LDL. Over-expression of miR-142-3p promoted ECs apoptosis, whereas depletion of miR-142-3p suppressed ox-LDL-elicited apoptosis in HAECs. Previous research has shown that ox-LDL may induce apoptosis, necrosis or proliferation of cultured ECs depending on the concentration and exposure time [32]. Ox-LDL at concentration lower than 10 μ g/ml could promote proliferation, at concentration higher than 50 μ g/ml could induce apoptosis and at higher than 250 μ g/ml could induce necrosis in HUVECs [18]. Under our experimental conditions, treatment with 100 μ g/ml ox-LDL for 24 h significantly inhibited the survival and proliferation in HAECs, which could be exacerbated by miR-142-3p mimic and alleviated by its inhibitor. Incubation of HAECs with 100 μ g/ml ox-LDL for 0–24 h did not significantly increase the necrotic percentage. Transfection of miR-142-3p inhibitor slightly decreased necrosis in HAECs, but the difference had no statistical significance. To further explore the influence of miR-142-3p on endothelial apoptosis *in vivo*, male ApoE KO mice were applied and transduced with AG or AN through tail vein injection. As a result, there was a marked increase in endothelial apoptosis in mice treated with AG, as visible by TUNEL staining. In contrast, knockdown of miR-142-3p by AN significantly attenuated endothelial apoptosis.

Additionally, treatment with AG caused a significant increase in lesion size in aortic wall. Conversely, the atherogenesis was abrogated by miR-142-3p suppression. These results demonstrated the pro-atherosclerotic effect of miR-142-3p *in vivo*.

The mammalian target of rapamycin (mTOR) pathway is a key regulator of cell growth and proliferation, and increasing evidence suggests that its dysregulation is associated with human diseases, including cancer, diabetes and cardiovascular disease. Recent work identified two structurally and functionally distinct mTOR-containing multiprotein complexes. The first complex, mTORC1, harbors raptor specifically activates protein synthesis via S6 kinase (S6K). The second complex, mTORC2, is associated with Rictor and phosphorylates Akt on Ser473. mTORC2 phosphorylates and activates Akt/protein kinase B, which promotes signaling pathways that ensure cell survival and induce cell proliferation [33]. Rictor is an important component of mTORC2 and the activation of the mTORC2 depends on the presence of the Rictor protein. Rictor is necessary for Akt phosphorylation on Ser473 and that a reduction in Rictor expression inhibits an Akt effector [34]. Upon activation Akt mediates pro-survival and anti-apoptosis in part via phosphorylating downstream target proteins including eNOS, BAD, Caspase-9, Fox1, Fox3a, YAP and MDM2 [35]. Among these target proteins, one of the most important one is eNOS. It is widely known that dysfunction of the eNOS activity leads to the reduction of NO bioavailability, which contributes to atherosclerosis [36]. The Rictor/Akt/eNOS pathway has been shown to play a pivotal role in regulating ECs apoptosis and dysfunction. Knockdown of Rictor inhibited activation of Akt and reduced ECs proliferation, migration, neovascularization [37, 38] and promoted endothelial apoptosis [37, 39], inflammation [39] and senescence [40]. To the contrary, fibroblast growth factor (FGF) [38], vascular endothelial growth factor (VEGF) [41] and 14, 15-epoxyeicosatrienoic acid [40] could provoke the expression of Rictor and phosphorylation of Akt, suppressing apoptosis and senescence of ECs. Ox-LDL induced apoptosis and oxidative stress in HAECs through inhibiting Akt/eNOS signaling pathway [42]. In the current study, we found that overexpression of miR-142-3p decreased Rictor protein expression and Akt phosphorylation, whereas knockdown of miR-142-3p enhanced the expression of Rictor and activation of Akt both *in vitro* and *in vivo*. Further luciferase reporter assay analysis revealed that Rictor gene is one of the direct targets of miR-142-3p in ECs. With Rictor down-regulation, miR-142-3p inhibitor lost its ability to suppress apoptosis. Ox-LDL inhibited Akt/eNOS activity and endothelial apoptosis correspondingly increased. While miR-142-3p inhibitor enhanced activity of Akt and eNOS, application of LY294002 and L-NAME reduced the phosphorylation of Akt and eNOS as well as abolished the anti-apoptotic effect of miR-142-3p inhibitor. These results suggest that down-regulation of miR-142-3p can ameliorate ECs apoptosis by activating the Rictor/Akt/eNOS signaling pathway.

Our study has several limitations that warrant further investigation. A major limitation of this study is that we have not examined the immune-related side-effects. Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by innate and adaptive immune system involvement. A key component of atherosclerotic plaque inflammation is the persistence of different immune cell types including monocytes, macrophages, dendritic cells, T cells, mast cells, *etc* [43]. Over the years, miR-142-3p has gained considerable attention for its quintessential role in regulating the function of immune cells [44]. The study done by Lagrange et al. identified the contribution of miR-142-3p to CSF1-induced differentiation of human monocytes into macrophages and demonstrated that this miRNA is part of a molecular circuitry involved the transcription factor Egr2 [45]. MiR-142-3p plays a critical role in modulating age-associated dysfunction of macrophages and LPS-induced dendritic cells responses and mortality via targeting IL-6 [46, 47]. The expression of miR-142-3p is down-regulated in CD4⁺ T cells from patients with arteriosclerosis obliterans. The down-regulation of miR-142-3p could increase the migration of CD4⁺ T cells to the vascular walls by regulation of actin cytoskeleton via its target genes, RAC1 and ROCK2 [48]. In the current study, injection of miR-142-3p agomir and antagomir *in vivo* may also influence miR-142-3p expression and function of these immune cells, which may affect the development and progression of atherosclerosis. Secondly, it has been demonstrated that miR-142-3p is

induced upon TGF β signaling in VSMCs, acting as a critical regulator of the TGF β -mediated contractile phenotype of VSMCs by targeting DOCK6. The phenotypic switching of VSMCs has long been considered of fundamental importance to atherosclerosis [49]. Thus, VSMCs may also be involved in the effect of miR-142-3p on development of atherosclerosis. Moreover, thousands of potential target genes of miR-142-3p were predicted by database, and the participation of other target genes such as IL-6, RAC1 and DOCK6 in the process requires further study. Finally, because we did not recruit a sufficient number of patients with atherosclerosis, the precise effect of miR-142-3p on atherosclerosis remains to be further validated in individuals.

In conclusion, we provide compelling evidence that down-regulation of miR-142-3p could alleviate ECs apoptosis and development of atherosclerosis via up-regulating Rictor expression and activating Akt/eNOS signaling pathway. This study provides a new target for the treatment of endothelial dysfunction and atherosclerosis at the gene level.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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