

miR-454-3p prevents ox-LDL-induced apoptosis in HAECs by targeting TRPC3

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Abstract. Endothelial-cell (EC) apoptosis serves a vital role in the pathogenesis of atherosclerosis. Accumulating evidence has implicated microRNA (miRNA/miR) dysregulation in EC apoptosis. Although the role of miR-454-3p in carcinogenesis has been well documented, its role and underlying mechanism in EC apoptosis remain unclear. In the present study, the results revealed that miR-454-3p expression was substantially downregulated in human aortic endothelial cells (HAECs) following oxidized low-density lipoprotein (ox-LDL) treatment. miR-454-3p suppression significantly attenuated the viability of HAECs, while miR-454-3p overexpression repressed ox-LDL-induced HAEC apoptosis. Bioinformatics analysis and luciferase reporter assays revealed that transient receptor potential canonical 3 (TRPC3), a key regulator of atherosclerosis development, was the direct target of miR-454-3p. Furthermore, TRPC3 overexpression abolished the anti-apoptotic effect of miR-454-3p on HAECs. These results revealed a novel role of miR-454-3p in ox-LDL-induced apoptosis in HAECs.

Introduction

Atherosclerosis, a chronic vascular disorder of the arterial wall, has become a predominant cause of a variety of

cardiovascular (CV) disorders, including ischemic stroke, myocardial infarction, heart attack and aortic aneurysm (1). Despite the advances in novel therapeutic strategies against atherosclerosis, this disorder remains the leading cause of death and disability worldwide (2). Endothelial-cell (EC) apoptosis serves a critical role in the pathogenesis of atherosclerosis via multiple mechanisms: i) Disrupting normal function of the endothelium to dysregulate lipid homeostasis, immunity and inflammation (3); ii) breaking the integrity and barrier functions of the endothelium to facilitate lipid deposition, leading to atherogenesis (4); and iii) destabilizing plaque to predispose patients to arterial thrombosis (5). Therefore, elucidating the molecular mechanism underlying EC apoptosis is required to further understand the disorder.

MicroRNAs (miRNAs/miRs) are small, non-coding RNA molecules which are 17-22 nucleotides in length that negatively regulate the expression of their target genes by promoting mRNA degradation or by repressing their translation (6). miRNAs participate in various cellular functions, including proliferation, differentiation, senescence and apoptosis (7). Accumulating evidence has demonstrated that abnormally expressed miRNAs are implicated in the initiation and progression of atherosclerosis (8,9). Various miRNAs, including miR-34a, miR-122, miR-210 and miR-876, have been reported to be involved in the pathogenesis of atherosclerosis by controlling EC apoptosis (10-13).

Human aortic endothelial cells (HAECs) are implicated in the pathogenesis of atherosclerosis (4). The dysfunction of HAECs can be affected by oxidized low-density lipoprotein (ox-LDL), which are a critical risk factor in atherosclerosis (12). Therefore, in the present study, ox-LDL-induced HAECs were used as atherosclerosis cell models to explore the molecular mechanism underlying atherosclerosis. Although the role of miR-454-3p in tumorigenesis is well documented (14-17), its involvement in the regulation of HAEC apoptosis remains to be elucidated. The present study investigated the role of miR-454-3p in cell apoptosis in atherosclerosis.

Materials and methods

Antibodies and reagents. TRPC3 antibodies (cat. no. ab241343; 1:1,000) were purchased from Abcam. Cleaved caspase-3

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Abbreviations: miRNAs, microRNAs; HAECs, human aortic endothelial cells; TRPC3, transient receptor potential canonical 3; ox-LDL, oxidized low-density lipoprotein; 3'-UTR, 3'-untranslated region

Key words: microRNA-454-3p, transient receptor potential canonical 3, oxidized low-density lipoprotein, human aortic endothelial cells, apoptosis

antibodies (cat. no. 9661; 1:1,000) and tubulin antibodies (cat. no. 2128; 1:2,000) were purchased from Cell Signaling Technology, Inc. Horseradish peroxidase (HRP) conjugated secondary antibody (anti-rabbit IgG, HRP-linked Antibody; cat. no. 7074; 1:5,000) was purchased from Cell Signaling Technology, Inc. miR-454-3p mimics and antagomiR-454-3p were obtained from Guangzhou RiboBio. HAECs were treated with 0, 25, 50 or 100 $\mu\text{g}/\text{ml}$ of ox-LDL to elucidate the effects of ox-LDL, which were provided by Beijing Xiasheng BioTechnology Co., Ltd.

Cell culture. HAECs were obtained from ScienCell Research Laboratories, Inc. (cat. no. 6100) and cultured in Endothelial Cell Growth Medium (Sigma-Aldrich; Merck KGaA) supplemented with Endothelial Cell Growth Supplement (BD Biosciences), 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.). HAECs were cultured in a humidified atmosphere with 5% CO_2 at 37°C. To investigate the potential role of miR-45-3p in atherosclerotic progression, HAECs were exposed to ox-LDL at various concentrations (0, 25, 50 or 100 $\mu\text{g}/\text{l}$) for 24 h or to 50 $\mu\text{g}/\text{ml}$ ox-LDL at various durations (0, 12, 24 or 48 h).

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was isolated from HAECs using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. miR-454-3p was quantified by synthesizing complementary (c)DNA using a TaqMan Advanced miRNA cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega Corporation) according to the manufacturer's protocol to detect TRPC3 expression. RT-qPCR was performed using a GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.) and a PrimeScript miRNA RT-PCR kit or Takara SYBR RT-PCR kit (both, Takara Biotechnology Co., Ltd.), according to the manufacturer's protocol. For the measurement of miR-454-3p, the thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min; followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 25 sec and elongation at 72°C for 40 sec. For the measurement of TRPC3, Initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 1 min, and a final elongation at 72°C for 2 min. Relative gene expression was calculated via the $2^{-\Delta\Delta C_q}$ method (18) and normalized to endogenous controls U6 or GAPDH. The following primer pairs were used for the qPCR: miR-454-3p forward, 5'-GCGCGTAGTGCAATATTGCTTA-3' and reverse, 5'-AGTGCAGGGTCCGAGGTATT-3'; U6 forward, 5'-CGCTTCGCGCAGCACATATACTAA-3' and reverse, 5'-TATGGAACGCTTCACGAATTTGC-3'; TRPC3 forward, 5'-AGCAGCTCTTGACGATCTGG-3' and reverse, 5'-GCACAACGAGACACTTGATAGC-3' and GAPDH forward, 5'-AATCCCATCACCATCTTC-3' and reverse, 5'-AGGCTGTTGTCATACTTC-3'.

Transient transfection of miR-454-3p mimics or inhibitors. miR-454-3p mimics (50 nM) or antagomiR-454-3p

(100 nM) were transiently transfected into HAECs using Lipofectamine RNAiMAX Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. These sequences are listed as follows: miR-454-3p mimic, 5'-UAGUGCAAUAUUGCUUAUAGGGU-3'; mimic control miR-NC, 5'-UCACAACCUCUAGAAAGAGUAGA-3'. antagomiR-454-3p, 5'-ACCCUAUAAGCAAUAUUGCACUA-3'; negative control antagomiR-NC, 5'-UUGUACUACACAAAAGUACUG-3'. Cells were used for further experiments 24 h post-transfection. For the luciferase reporter assay, cells were collected for the detection 48 h post-transfection.

Transfection with siRNA and plasmids. siRNA (50 nM) or plasmids was transfected were transiently transfected into HAECs using Lipofectamine RNAiMAX Transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. siRNA targeting TRPC3 (si-TRPC3) and siRNA negative control (si-NC) were synthesized by GenePharma Co. Ltd. (Shanghai, China). The sequences are listed as follows: si-TRPC3: 5'-CCCAGTTTACATGGACTGAAA-3'; si-NC (5'-CAACAAGATGAAGAGCACCAA-3'). The full length cDNA sequences of TRPC3 were cloned into pcDNA3.1 vector (Invitrogen) to construct 3'-UTR-deleted TRPC3 plasmid.

Cell viability assay. Cell viability of HAECs was examined using a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories) assay according to the manufacturer's protocol. Briefly, cells were seeded into 96-well plate at a density of 2,500 cells/well. Prior to analysis, CCK-8 reagent was added to each well and HAECs were cultured at 37°C for 4 h. Absorbance at 450 nm was detected using a microplate reader (Molecular Devices, LLC). Experiments were conducted in triplicate.

Apoptosis analysis. Cell apoptosis was detected using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer's protocol. Harvested cells were washed twice with PBS and then stained with 1 $\mu\text{g}/\text{ml}$ Annexin V-FITC for 15 min in the dark at room temperature. Following washing with PBS, cells were stained with 1 $\mu\text{g}/\text{ml}$ propidium iodide (PI) for 10 min in the dark at room temperature. Cells were then subjected to flow cytometric (FCM) analysis using a FACSCanto FCM flow cytometer (BD Biosciences). A total of 1×10^4 cells were collected using the forward-scatter/side-scatter scatterplot method to exclude mutually adherent cells and cell debris (19). Data analysis was conducted using FlowJo software (version 7.6.5; FlowJo LLC).

Lactate dehydrogenase (LDH) release assay. Cell culture medium was collected and LDH activity was measured using a commercial LDH Activity Assay kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol. Briefly, 25 μl cell supernatant was mixed with 25 μl substrate in the kit and the solution was incubated at 37°C for 15 min. A total of 25 μl 2,4-dinitrophenylhydrazine in the kit was then added into the samples. Following incubation at 37°C for 15 min in the dark, 250 μl 0.4 mol/l NaOH solution was added and the mixture was further incubated at room temperature for 5 min. Absorbance was recorded at a wavelength of 450 nm using a microplate reader.

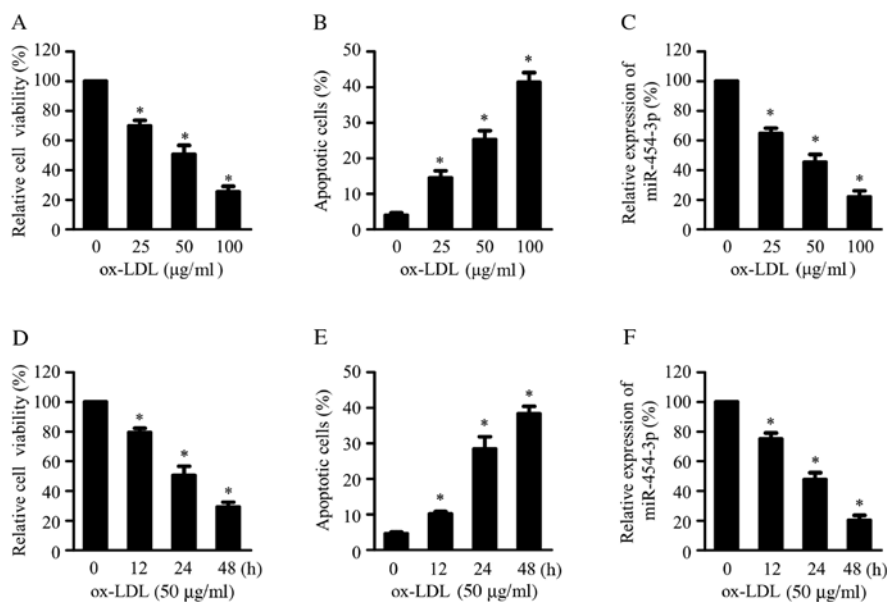


Figure 1. miR-454-3p is downregulated in ox-LDL-treated HAECs. HAECs treated with 0, 25, 50 or 100 $\mu\text{g/ml}$ ox-LDL for 24 h, followed by (A) CCK-8 and (B) cell apoptosis analyses. (C) RT-qPCR analysis of miR-454-3p expression in HAECs treated with 0, 25, 50 or 100 $\mu\text{g/ml}$ ox-LDL for 24 h. HAECs treated with 50 $\mu\text{g/ml}$ ox-LDL for 0, 12, 24 or 48 h, followed by (D) CCK-8 and (E) cell apoptosis analyses. (F) Following treatment with 50 $\mu\text{g/ml}$ ox-LDL of HAECs for 0, 12, 24 or 48 h, miR-454-3p expression was measured by RT-qPCR. The expression of miR-454-3p was normalized to U6 small nuclear RNA. * $P < 0.05$, compared with the group treated with 50 $\mu\text{g/ml}$ ox-LDL for 0 h. miR, microRNA; ox-LDL, oxidized low-density lipoprotein; HAEC, human aortic endothelial cells; CCK-8, Cell Counting Kit-8; RT-qPCR, reverse transcription-quantitative PCR. Experiments were conducted in triplicate and data are presented as mean \pm standard deviation (SD).

Western blotting. Collected cells were lysed with RIPA buffer (Pierce; Thermo Fisher Scientific, Inc.) supplemented with protease inhibitors (Roche Diagnostics). The protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The detailed protocol is described previously (20). Following centrifugation at 12,000 \times g, 4°C for 10 min, the supernatant was harvested and quantified using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (50 $\mu\text{g/lane}$) were then separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk at room temperature for 1 h, the membranes were probed with primary antibodies overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Bands were visualized using an Enhanced Chemiluminescence Western Blotting Analysis kit (Invitrogen; Thermo Fisher Scientific, Inc.). Tubulin was used as a loading control.

Caspase-3 activity assay. Caspase-3 activity was determined using a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Cells were harvested and lysed for 15 min at 4°C using the cell lysis buffer supplied by the kit. Suspensions were then centrifuged at 12,000 \times g for 10 min at 4°C. Supernatants were collected and 10 μl caspase-3 substrate Ac-LEHD-pNA (from kit) and 10 μl supernatant was added to 80 μl reaction buffer (from kit). Following incubation at 37°C for 2 h, absorbance was measured at a wavelength of 405 nm using a microplate reader.

Bioinformatics analysis and Luciferase reporter assay. The bioinformatics tool TargetScan (http://www.targetscan.org/vert_72/) was utilized to predict potential target genes

of miR-454-3p. HAECs were seeded into 24-well plates at a density of 7.5×10^4 cells/per well. After 24 h, cells were co-transfected with pGL3 luciferase reporter vectors (Promega Corporation) containing wild-type (WT) or mutated 3'-untranslated region (3'-UTR) sequences of TRPC3 alongside miR-454-3p mimics or antagomiR-454-3p using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). pRL-TK vectors (Promega Corporation) were co-transfected as an internal control of transfection efficiency. At 48 h post-transfection, cells were harvested and subjected to luciferase activity analysis using a Dual-Luciferase Reporter Assay system (Promega Corporation), according to the manufacturer's protocol. Relative reporter gene activity was measured by normalizing to *Renilla* luciferase activity.

Statistical analysis. All data are presented as the mean \pm standard deviation (SD). Statistical analyses were conducted using a two-tailed Student's t-test or one-way ANOVA followed by Dunnett's test with SPSS software (version 17.0; SPSS, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ox-LDL-treatment downregulates miR-454-3p in HAECs.

Ox-LDL is a key atherosclerotic risk factor that induces EC apoptosis and contributes to the initiation and progression of atherosclerosis (12). HAECs were exposed to ox-LDL at various concentrations (0, 25, 50 or 100 $\mu\text{g/l}$) for 24 h or to 50 $\mu\text{g/ml}$ ox-LDL at various durations (0, 12, 24 or 48 h) to investigate the potential role of miR-45-3p in atherosclerotic progression. Ox-LDL treatment reduced cell viability and increased cell apoptosis in a concentration- (Fig. 1A-B) and

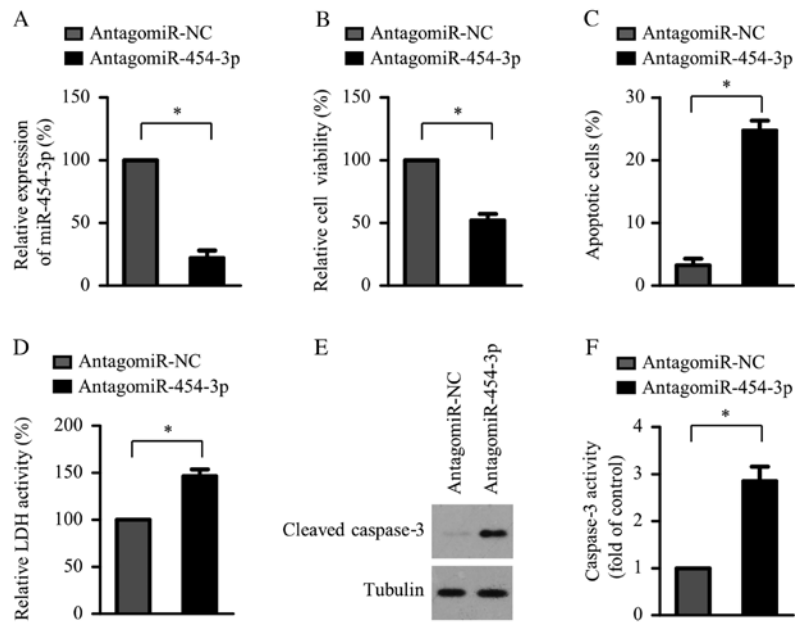


Figure 2. miR-454-3p inhibition induces HAEC apoptosis. (A) Relative miR-454-3p levels in HAECs following 24-h transfection with antagomiR-NC or antagomiR-454-3p. Transfected HAECs were treated with 50 μ g/ml ox-LDL for 24 h, followed by (B) Cell Counting Kit-8, (C) cell apoptosis and (D) LDH release analyses. (E) Western blotting of cleaved-caspase-3 protein expression and (F) caspase-3 activity measurement in transfected HAECs treated with ox-LDL. Data are presented as mean \pm standard deviation (SD) and experiments were performed in triplicate. * P <0.05. miR, microRNA; HAEC, human aortic endothelial cells; NC, negative control; ox-LDL, oxidized low-density lipoprotein; LDH, lactate dehydrogenase.

time-dependent (Fig. 1D-E) manner, as assessed by CCK-8 and apoptosis analyses. Furthermore, RT-qPCR results demonstrated that ox-LDL treatment reduced miR-454-3p expression in a dose- (Fig. 1C) and time-dependent manner (Fig. 1F).

miR-454-3p inhibition induces HAEC apoptosis. The effect of miR-454-3p suppression on cell viability and apoptosis of HAECs was determined. miR-454-3p expression significantly decreased in the miR-454-3p inhibitor transfection group compared with the negative control group (Fig. 2A). CCK-8 assay results demonstrated that miR-454-3p suppression significantly reduced the viability of HAECs compared with the negative control group (Fig. 2B). Consistent with cell viability inhibition, compared with negative control groups, HAEC apoptosis was significantly increased by miR-454-3p suppression (Fig. 2C) and the LDH assay confirmed that this suppression significantly induced HAEC cell death (Fig. 2D). Furthermore, compared with negative control groups, miR-454-3p suppression significantly increased cleaved caspase-3 protein expression and caspase-3 activities in HAECs (Fig. 2E and F), confirming increased cell apoptosis.

miR-454-3p attenuates ox-LDL-induced apoptosis in HAECs. Based on the previous results of ox-LDL treatment on miR-454-3p and the suppressive role of miR-454-3p on HAEC apoptosis, it was determined whether miR-454-3p overexpression could rescue ox-LDL-induced apoptosis of HAECs. HAECs were transfected with miR-454-3p or miR-negative control (miR-NC), followed by treatment with 50 μ g/ml ox-LDL for 24 h. RT-qPCR results demonstrated successful miR-454-3p overexpression in HAECs transfected with miR-454-3p compared with miR-NC-transfected cells (Fig. 3A). CCK-8 assay revealed that miR-454-3p overexpression reversed the

inhibitory effect of ox-LDL on the viability of HAECs (Fig. 3B). Additionally, Annexin V-FITC/PI double-staining analysis and LDH release assay revealed that ox-LDL-induced apoptosis of HAECs was significantly attenuated by miR-454-3p overexpression compared with miR-NC-transfected cells (Fig. 3C-D). Consistently, ox-LDL-induced increase in cleaved caspase-3 protein expression and activity were significantly reduced by miR-454-3p overexpression (Fig. 3E and F).

TRPC3 is a direct target of miR-454-3p in HAECs. To elucidate the molecular mechanism by which miR-454-3p regulated HAEC apoptosis, the bioinformatics tool TargetScan (David Bartel Lab; Whitehead Institute for Biomedical Research; Massachusetts Institute of Technology) was utilized to predict potential target genes of miR-454-3p. Among the potential target genes (Table SI), TRPC3 has been reported to be a key gene contributing to the pathogenesis and development of atherosclerosis (21,22). The present study then explored whether TRPC3 might be used as a potential target gene of miR-454-3p. The possible binding site of miR-454-3p in TRPC3 3'-UTR is shown in Fig. 4A. To determine whether miR-454-3p directly targeted TRPC3, a Dual-Luciferase Reporter Assay system containing WT or mutated miR-454-3p binding sites in the 3'-UTR was used. miR-454-3p overexpression significantly inhibited WT 3'-UTR luciferase activity compared with miR-NC-transfected cells (Fig. 4B); however, there was no significant effect on mutant 3'-UTR between miR-454-3p overexpression and miR-NC group. Consistently, miR-454-3p suppression significantly increased WT 3'-UTR luciferase activity compared with the negative control group but showed no significant effect on the mutant 3'-UTR of TRPC3 (Fig. 4C). RT-qPCR and Western blotting analyses revealed that miR-454-3p transfection decreased TRPC3 mRNA and protein expression (Fig. 4D and E).

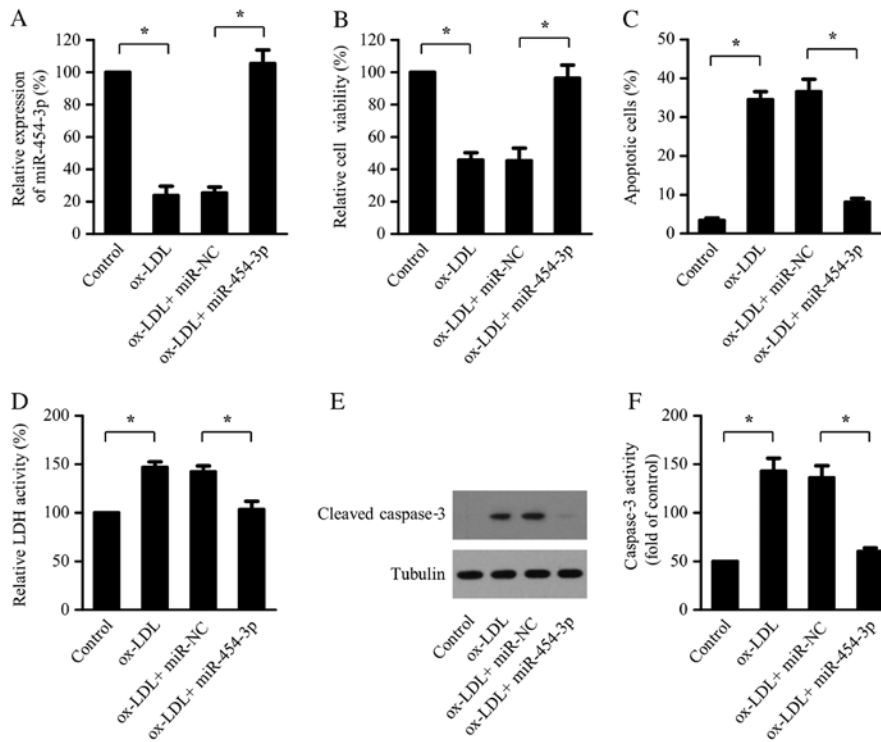


Figure 3. miR-454-3p attenuates ox-LDL-induced apoptosis in HAECs. miR-NC or miR-454-3p was transfected into HAECs and at 24 h post-transfection (A) miR-454-3p expression was determined by reverse transcription-quantitative PCR. Transfected HAECs were treated with 50 μ g/ml ox-LDL for 24 h, followed by (B) Cell Counting Kit-8, (C) cell apoptosis and (D) LDH release analyses. (E) Western blotting of cleaved-caspase-3 protein expression and (F) caspase-3 activity measurement in transfected HAECs treated with ox-LDL. Data are presented as the mean \pm standard deviation (SD) and experiments were performed in triplicate. *P<0.05. miR, microRNA; ox-LDL, oxidized low-density lipoprotein; NC, negative control; HAECs, HAEC, human aortic endothelial cells; LDH, lactate dehydrogenase.

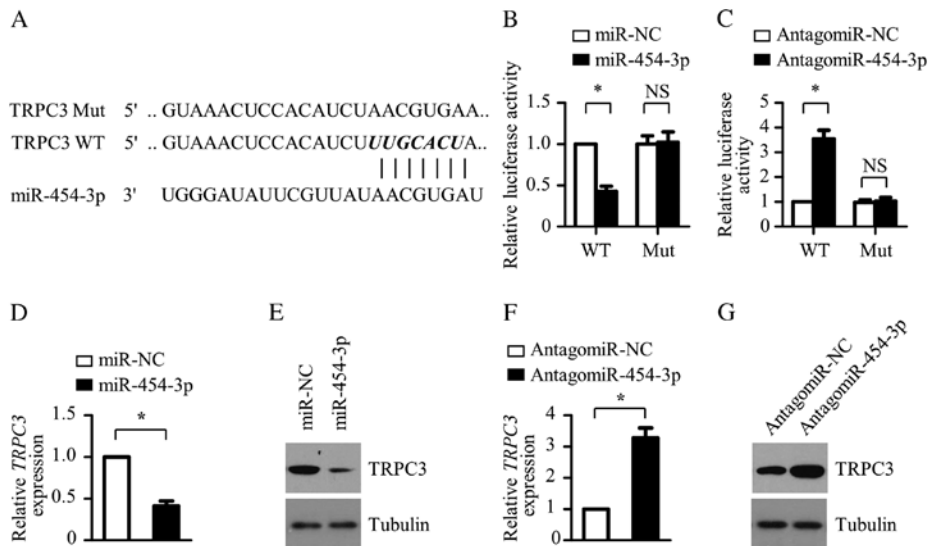


Figure 4. TRPC3 is a direct target of miR-454-3p in HAECs. (A) Target sites of miR-454-3p in TRPC3 3'-UTR mRNA. WT or mut TRPC3 3'-UTR luciferase reporter vectors alongside miR-NC or miR-454-3p were co-transfected into HAECs. After 48 h, luciferase activities were determined in HAECs transfected with (B) miR-NC or miR-454-3p or with (C) antagomiR-NC or antagomiR-454-3p. Reverse transcription-quantitative PCR analysis of TRPC3 mRNA levels in HAECs transfected with (D) miR-NC or miR-454-3p or with (F) antagomiR-NC or antagomiR-454-3p. Expression of TRPC3 mRNA was normalized to GAPDH. Western blotting results of TRPC3 protein levels in HAECs transfected with (E) miR-NC or miR-454-3p or with (G) antagomiR-NC or antagomiR-454-3p. Tubulin was used as a loading control. Data are presented as the mean \pm standard deviation (SD) and experiments were performed in triplicate. *P<0.05. TRPC3, transient receptor potential canonical 3; miR, microRNA; HAEC, HAEC, human aortic endothelial cells; UTR, untranslated region; WT, wild-type; mut, mutant; NC, normal control; NS, not significant.

Furthermore, miR-454-3p inhibition increased TRPC3 mRNA and protein expression (Fig. 4F and G). In summary, these results revealed that TRPC3 was a direct target of miR-454-3p.

miR-454-3p inhibits EC apoptosis via inhibition of TRPC3 expression. To address the role of TRPC3 in mediating the anti-apoptotic effect of miR-454-3p in HAECs, TRPC3

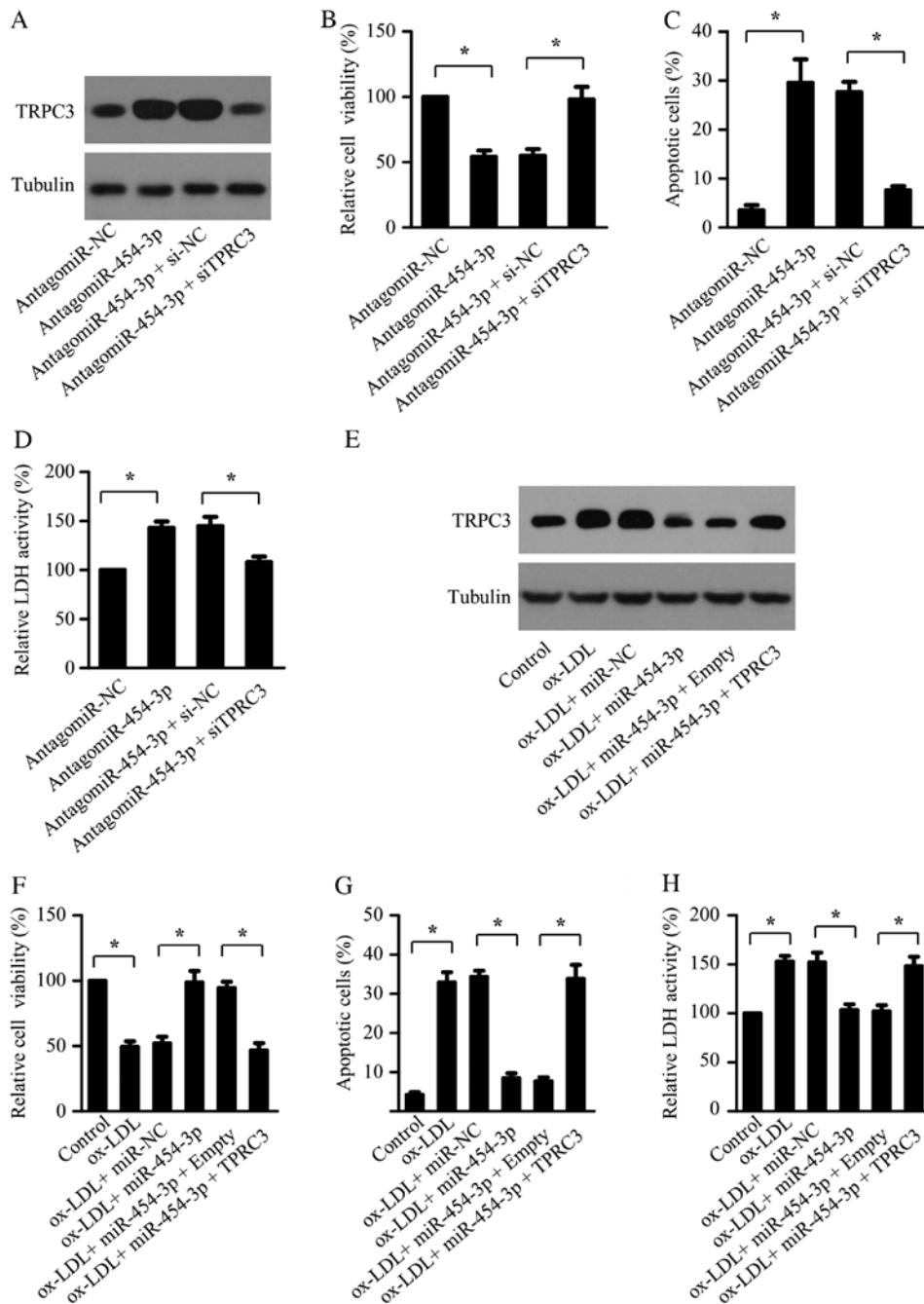


Figure 5. miR-454-3p inhibits endothelial apoptosis via inhibition of TRPC3 expression. Western blotting of TRPC3 expression in HAECs transfected with (A) antagomiR-454-3p or antagomiR-454-3p + siTRPC3. Tubulin was used as a loading control. HAECs were transfected with antagomiR-454-3p or antagomiR-454-3p + siTRPC3, followed by (B) CCK-8, (C) cell apoptosis and (D) LDH release analyses. HAECs transfected with miR-454-3p or miR-454-3p + TRPC3 were treated with 50 μ g/ml ox-LDL for 24 h, followed by (E) western blotting of TRPC3 expression, (F) CCK-8, (G) cell apoptosis and (H) LDH release analyses. Data are presented as the mean \pm standard deviation (SD) and experiments were performed in triplicate. * P <0.05. miR, microRNA; TRPC3, transient receptor potential canonical 3; HAEC, human aortic endothelial cells; si, small interfering RNA; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; NC, negative control; ox-LDL, oxidized low-density lipoprotein.

knockdowns were used to determine whether they reversed HAEC apoptosis mediated by miR-454-3p suppression. As shown in Fig. 5A, the expression of TRPC3 significantly decreased in the siTRPC3 transfection group compared with the si-NC group. Compared with their respective negative control groups, antagomiR-454-3p transfection suppressed the viability of HAECs, whereas TRPC3 silencing reversed this repression of cell viability (Fig. 5B). Accordingly, the increased apoptosis caused by miR-454-3p inhibition was

reversed by TRPC3 silencing (Fig. 5C-D), as assessed by Annexin V-FITC/PI double-staining analysis and an LDH release assay. To further validate the role of TRPC3 in mediating the anti-apoptotic action of miR-454-3p in HAECs, a rescue experiment was performed by introducing a 3'-UTR-deleted TRPC3 plasmid into HAECs transfected with miR-454-3p. Western blotting indicated that miR-454-3p decreased TRPC3 protein levels, which were partially restored by co-transfection with a TRPC3 plasmid (Fig. 5E). The CCK-8 assay

demonstrated that cell viability was significantly decreased in ox-LDL-treated HAECs compared with control cells, while miR-454-3p transfection restored cell viability (Fig. 5F). However, TRPC3 overexpression significantly decreased the beneficial effect of miR-454-3p. Additionally, ox-LDL treatment significantly increased HAEC apoptosis compared with controls (Fig. 5G-H). This effect was significantly attenuated following miR-454-3p overexpression. However, TRPC3 overexpression inhibited the ability of miR-454-3p to suppress ox-LDL-induced apoptosis. Thus, these results indicated that miR-454-3p inhibited EC apoptosis by targeting TRPC3.

Discussion

EC apoptosis manifests in the early stages of atherosclerosis and contributes to the pathogenesis of the disorder (4). Accumulating evidence has demonstrated that aberrantly-expressed miRNAs serve critical roles in EC apoptosis by targeting crucial factors or key pathways regulating cell apoptosis (8). For instance, the lethal-7g gene was demonstrated to suppress EC apoptosis by directly targeting caspase-3 (23). miR-429 suppressed atherosclerosis-associated EC apoptosis by inhibiting B-cell lymphoma 2 (24). Li *et al* (12) reported that miR-210 upregulation induced EC apoptosis by suppressing pyruvate dehydrogenase lipoamide kinase isozyme 1 during atherosclerosis development. It was reported that downregulation of miR-142-3p suppressed EC apoptosis and atherosclerotic progression by increasing the expression of rapamycin-insensitive companion of mTOR and activating protein kinase B signaling (25). Therefore, identifying novel miRNAs that participate in EC apoptosis will expand the understanding of the molecular mechanism underlying the pathogenesis of atherosclerosis.

miR-454-3p, which has been reported to be abnormally expressed in various types of cancers, may have an oncogenic role. miR-454-3p promoted breast cancer metastasis by suppressing the regulation of nuclear pre-mRNA domain containing 1A and by activating Wingless/Integrated signaling (26). Furthermore, miR-454-3p may act as a tumor suppressor; a previous study demonstrated miR-454-3p downregulation in glioblastoma and that it exerted tumor-suppressive functions by targeting nuclear factor of activated T cells, cytoplasmic 1 (17). By inhibiting signal transducer and activator of transcription 3 and autophagy-related protein 12, miR-454-3p negatively regulated the growth of chondrosarcoma (16). Although its role in carcinogenesis has been well documented, its roles and underlying mechanisms in EC apoptosis remain to be elucidated.

In the present study, ox-LDL-induced HAECs were used as an *in vitro* cell model of atherosclerosis and the potential role of miR-454-3p in ox-LDL-induced EC apoptosis was investigated. The results demonstrated that ox-LDL suppressed miR-454-3p expression in a dose- and time-dependent manner. This suppression significantly attenuated EC viability, mimicking the apoptosis-inducing effects of ox-LDL treatment. miR-454-3p overexpression almost completely reversed ox-LDL-induced EC apoptosis and relieved ox-LDL-elicited suppression of cell viability, revealing the anti-arteriosclerotic effects of miR-454-3p.

Previous studies demonstrated that plasma miR-454-3p is upregulated in gliomas and may act as a sensitive biomarker for the diagnosis of gliomas (27,28). In future studies, determining the difference between miR-454-3p in plasma in patients with atherosclerosis and in plasma from healthy subjects will be beneficiary in order to investigate whether miR-454-3p acts as a novel potential diagnostic biomarker for atherosclerosis.

Through *in silico* algorithm analyses and experimental verification, the present study demonstrated that TRPC3 was a direct target of miR-454-3p. TRPC3, a member of the TRPC family of calcium-permeable, non-selective cation channels, participates in diverse functions in CV and hematopoietic systems (24). Microarrays have demonstrated significant increases of TRPC3 mRNA levels in plaques obtained from patients with atherosclerosis (29). Consistent with this observation, TRPC3 was reported to exhibit higher expression in the aortic roots of atherosclerotic mice compared with aortic cross-sections from non-atherosclerotic animals (30), indicating an association between TRPC3 overexpression and the presence of atherosclerosis (30). Additionally, TRPC3 deficiency impaired atherosclerotic-lesion formation in a mouse model of atherosclerosis (31). Apoe knockout mice, a unique mouse model of atherosclerosis, with endothelial-specific overexpression of human TRPC3 exhibited increased size and cellularity of advanced atherosclerotic lesions (32), supporting a pro-atherogenic role of endothelial TRPC3. Additionally, a previous study reported that TRPC3 is required for EC apoptosis induced by endoplasmic-reticulum stress (33). Therefore, the present study hypothesized that miR-454-3p may exert its anti-apoptotic effect by suppressing TRPC3. The results demonstrated that TRPC3 silencing completely blocked the increased apoptosis caused by miR-454-3p suppression, while TRPC3 overexpression reversed the inhibitory effect of miR-454-3p in ox-LDL-induced HAEC cell apoptosis. These results indicated that miR-454-3p inhibited EC apoptosis by directly targeting TRPC3 expression.

In conclusion, the results of the present study revealed that miR-454-3p is a mediator for EC apoptosis. In HAECs, miR-454-3p overexpression reversed ox-LDL-induced apoptosis by repressing TRPC3. Additionally, the results demonstrated that the miR-454-3p/TRPC3 pathway was required for the survival of HAECs under normal culture conditions. These findings further elucidated the molecular mechanisms underlying ox-LDL-induced apoptosis in HAECs.

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Availability of data and materials

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

Authors' contributions

LL and YL designed the present study. LL, QY, HL and RM performed the experiments and analyzed data. LL and YL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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