


LncRNA HOXA11-AS promotes vascular endothelial cell injury in atherosclerosis by regulating the miR-515-5p/ROCK1 axis

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Abstract

Aims Long non-coding RNA HOXA11-AS participated in heart disease. In this study, we aim to evaluate the potential roles of HOXA11-AS in atherosclerosis and its underlying mechanisms.

Methods and results The expression levels of HOXA11-AS in ox-LDL-treated HUVECs and arch tissues of high-fat diet-fed ApoE^{-/-} mice ($n = 10$) were assessed by qRT-PCR. The effects of HOXA11-AS knockdown on the development of atherosclerosis were evaluated using *in vitro* and *in vivo* models. Luciferase reporter and RNA immunoprecipitation (RIP) assays verified the potential relationships between HOXA11-AS or ROCK1 and miR-515-5p. The interactive roles between HOXA11-AS and miR-515-5p and between miR-515-5p and ROCK1 were further characterized in ox-LDL-treated HUVECs. Our data showed that HOXA11-AS was significantly up-regulated ($P < 0.001$), whereas miR-515-5p was dramatically down-regulated in AS mice tissues ($P < 0.001$) and ox-LDL-treated HUVECs ($P < 0.01$). Ox-LDL could induce endothelial injuries by inhibiting cell proliferation ($P < 0.001$) and SOD synthesis ($P < 0.001$), promoting apoptosis ($P < 0.01$), ROS ($P < 0.001$), and MDA production ($P < 0.001$), increasing Bax ($P < 0.001$) and cleaved Caspase-3 ($P < 0.001$), and decreasing Bcl-2 ($P < 0.001$) and phosphorylated eNOS ($P < 0.01$). HOXA11-AS knockdown attenuated endothelial injuries via increasing eNOS phosphorylation. Luciferase assay and RIP results confirmed that miR-515-5p is directly bound to HOXA11-AS and ROCK1. HOXA11-AS promoted ox-LDL-induced HUVECs injury by directly inhibiting miR-515-5p from increasing ROCK1 expression and subsequently decreasing the expression and phosphorylation of eNOS. MiR-515-5p mimics could partially reverse the effects of HOXA11-AS knockdown.

Conclusions HOXA11-AS contributed to atherosclerotic injuries by directly regulating the miR-515-5p/ROCK1 axis. This study provided new evidence that HOXA11-AS might be a candidate for atherosclerosis therapy.

Keywords LncRNA HOXA11-AS; Atherosclerosis; miR-515-5p; ROCK1; Vascular endothelial cell dysfunction

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Introduction

Atherosclerosis is a leading blood vessel disease, mainly caused by the inflammatory response, lipid deposition, fibrosis of the arterial wall, and the vulnerable plaques. It can pro-

gressively induce the lesion and narrowing of arteries.¹ Increasing evidence confirmed that the low-density lipoprotein cholesterol (LDL) accumulation in endothelial cells is responsible for the development of atherosclerosis.¹ The depositions of lipoproteins can cause the dysfunction and

structural alteration of endothelial cells.² After being activated by oxidized lipids and ox-LDL, the endothelial cells could further recruit other inflammatory cells to exacerbate the atherosclerotic injuries. Hence, the endothelial cell was a crucial factor for atherosclerosis. Hence, the study of how ox-LDL influenced endothelial cells and their subsequent responses will provide us with new insights into atherosclerosis therapeutics.

lncRNAs are transcribed RNAs with about 200 nucleotides but no protein-coding function.^{3,4} They participate in transcriptional regulation, post-transcriptional regulation and act as endogenous decoys to control the expression of miRNAs.⁴ Further studies showed that lncRNAs could regulate lipid metabolism, inflammatory response, and angiogenesis.³ Until now, several lncRNAs are closely related to vascular diseases and atherosclerosis, such as lncRNA GAS5,² NORAD,⁵ ZEB1-AS1,⁶ and TUG1.⁷ lncRNA HOAX11-AS exhibited a high level in symptomatic atherosclerotic patients.⁸ Recently, studies confirmed that HOAX11-AS was closely related to the expressions of inflammation-related genes TNF- α ,⁸ adipocyte differentiation,⁹ and cardiac fibrosis.¹⁰ Mechanically, lncRNAs could bind to miRNA 3' untranslated regions (3' UTRs) and regulate mRNA transcript during angiogenesis and tumorigenesis.¹¹ By bioinformatics analysis, we found HOAX11-AS contained a binding sequence for miR-515-5p. Therefore, we wondered whether HOAX11-AS promoted atherosclerosis by directly regulating miR-515-5p.

ROCK1 can regulate cell cytoskeleton organization, adhesion, proliferation, and apoptosis.¹² A series of evidence showed ROCK1 as a downstream effector of TGF- β was actively involved in the pathophysiological events in cardiovascular diseases, such as hypertension,¹³ heart fibrosis,¹⁴ heart failure,¹⁵ and atherosclerosis.¹⁶ Endothelial nitric oxide synthase (eNOS) is a crucial modulator of cardiovascular homeostasis, which is responsible for the production of nitric oxide (NO).¹⁷ The phosphorylation of eNOS could directly regulate the eNOS-mediated NO production.¹⁸ Abnormal synthesis of eNOS and phosphorylation of eNOS contributed to cardiovascular diseases, including atherosclerosis.¹⁹ Previous experiments confirmed that ROCK negatively modulates the activation of eNOS via the inactivation of protein kinase B (PKB).¹⁷ Hence, ROCK1 is an essential target for treating cardiovascular diseases, including atherosclerosis.²⁰ Intriguingly, the starBase database predicted that ROCK1 had a complementary binding site of miR-515-5p, which helped us hypothesize that the HOXA11-AS/miR-515-5p/ROCK1 pathway might regulate atherosclerosis via influencing the activation of eNOS.

In the current study, we aimed to check the expressions of HOXA11-AS and miR-515-5p in atherosclerotic mice tissue and ox-LDL-induced HUVEC and further investigate the functions of HOXA11-AS knockdown and miR-515-5p on the progression of atherosclerosis. Mechanistically, we eval-

uated the relationships among HOXA11-AS, miR-515-5p, and ROCK1 and further characterized the potential mechanisms of HOXA11-AS/miR-515-5p/ROCK1 in atherosclerosis. This study will provide us a new insight to treat atherosclerosis.

Methods

Characteristics of atherosclerotic models in ApoE^{-/-} mice

We purchased thirty 6–8 weeks aged ApoE knockout (ApoE^{-/-}) male mice and ten C57BL/6J mice (bodyweight 20–30 g) from Medical Science Experimental Animal Center (Guangdong, China). Mice were maintained in 5 mice per cage with free access to water and food in an SPF grade laboratory environment (22 \pm 2°C and a 12 h day/night cycle). After acclimatization, the ApoE^{-/-} mice were randomly separated into three groups (10 mice per group) and fed a high-fat diet (HFD, Research Diets, New Brunswick, NJ, USA). C57BL/6J mice were fed with a regular chow diet (Lactamin, Stockholm, Sweden). Then, the ApoE^{-/-} mice were injected with sh-NC or sh-HOAX11-AS lentivirus by tail vein (7.5 \times 10⁷ TU per mice). The sh-NC and sh-HOAX11-AS lentivirus were purchased from Shanghai GenePharma (Shanghai, China). We sacrificed the mice after 12 weeks following lentivirus injection and high-fat feeding. After sacrifice, we collected blood to isolate the plasma. Then, plasma lipids total cholesterol (TC), triglyceride (TG), high-density lipoprotein, and LDL were measured by ELISA assays using commercial kits (Nanjing Jiancheng Technology Co. Nanjing, China). The aorta was collected and stored at -80°C for gene expression. The remaining mice were perfused with 4% paraformaldehyde in 1 \times pre-cold PBS for immunostaining assays. The Ethics Committee of The First Affiliated Hospital of Anhui Medical University approved all the above-mentioned experimental procedures.

Cell transfection

Human Umbilical Vein Endothelial Cell (HUVEC, Thermo Fisher Scientific, Shanghai, China) was grown in Medium 200 with low serum supplement (LSGS, cat. no. S-003-10) 37°C in a humidified incubator. We purchased 1 \times 10⁸ TU/mL pGPU6-GFP-Neo-HOAX11-AS (sh-HOAX11-AS) and the empty vector pGPU6-GFP-Neo, miR-515-5p mimics/inhibitor, and the corresponding negative or positive mimics control, pcDNA3.1-ROCK1, and pcDNA3.1 empty vector from Guangzhou RiboBio Co., Ltd, China. For HUVECs transfection, we transfected cells with sh-HOAX11-AS lentivirus, miR-515-5p

mimics/inhibitors, pcDNA3.1-ROCK1, or the corresponding control by Invitrogen Lipofectamine 2000 (Fisher Scientific, MA, USA). The characteristics of sh-HOXA11-AS lentiviral vector were shown in supporting information, *Data S1*. qRT-PCR confirmed the effectiveness of transfection.

MTT assay

CyQUANT™ MTT Cell Proliferation Assay Kit (V13154) was bought from Invitrogen, USA. The 1×10^3 HUVECs cells were plated into each well of 96-well plates. Subsequently, cells were incubated with 0, 20, 40, 60, 80 $\mu\text{g}/\text{mL}$ ox-LDL (Sigma-Aldrich, MO, USA) for 24 h. Then, 5 mg/mL of MTT reagent (Sigma-Aldrich, MO, USA) was added and incubated for a further 4 h at 37°C. After being dissolved by 0.01 M HCl-SDS solution, the dissolved formazan was measured at 570 nm using a Thermomax microplate reader (BioTek EL, Vermont, USA).

Flow cytometry

The HUVECs were collected after treatment and stained with AnnexinV-FITC/propidium iodide (PI) (Sigma-Aldrich, MO, USA). Cells were detected by Cytomics FC500 Flow Cytometer (Beckman Coulter, CA, USA). The ratio of the apoptotic cells to total cells was calculated.

Dual-luciferase reporting assay

We purchased pGL3 vectors containing the wide-type and mutant binding sequences of miR-515-5p in HOXA11-AS and ROCK1 from Guangzhou RiboBio Co., Ltd, China (Guangdong, China). They are named as pGL3-HOXA11-AS wide-type (HOXA11-AS WT), pGL3-HOXA11-AS mutant (HOXA11-AS MUT), pGL3-ROCK1 wide-type (ROCK1 WT), and pGL3-ROCK1 mutant (ROCK1 MUT).

HUVECs were transfected with the indicated luciferase vector single or cotransfected with mimics NC or miR-515-5p mimics using Lipofectamine 2000 (Thermo Fisher Scientific, USA). The luciferase activities were measured 48 h after transfection using a commercial kit (Promega, Madison, USA). The Renilla luciferase acted as an internal control.

RIP

Whole HUVECs extracts were obtained and reacted with magnetic beads conjugated with human anti-Ago2 antibody (MilliporeSigma, MA, USA) or negative control IgG (MilliporeSigma). After the antibody was recovered using protein A/G beads, the bounded RNAs were eluted

by Trizol (Thermo Fisher Scientific, USA). qRT-PCR was performed to measure the levels of HOXA11-AS and miR-515-5p.

Haematoxylin and eosin staining

The aortic arch to the branch of the abdominal aorta was dissected and immersed in precooled 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS at 4°C overnight. After a brief wash with $1 \times$ phosphate-buffered saline, the aortic arch was processed directly for paraffin embedding and microtome sectioned at thickness 5 nm by a microtome (HistoCore BIO-CUT, Leica, USA) according to the previous method.²¹ After serial dewax and rehydration, the sections were stained by haematoxylin solution (Sigma-Aldrich) for 5–10 min. After being rinsed under tap water, the tissues were differentiated using 1% aqueous hydrochloric acid, and the development of staining was checked under a microscope. After rinsing for 5 min in running tap water, the sections were counterstained by eosin alcoholic solution for 0.5–1 min. Finally, the sections were dehydrated by increasing serial concentration of ethanol (70%, 90%, and 100% ethanol, each for 2 min) and cleared in xylene for 2 min. After mounting with neutral resin, the sections were imaged under a microscope with a camera. Two independent pathologies evaluated the lesions in a blinded manner.

Measurement of ROS, MDA, and SOD

The HUVECs were transfected by a sh-NC, sh-HOXA11-AS single, or together with inhibitor NC, miR-515-5p inhibitor. Twenty-four hours later, 80 $\mu\text{g}/\text{mL}$ ox-LDL was added into cells and incubated for a further 24 h. Subsequently, HUVECs were incubated with 10 μM of ROS-sensitive substrate CM-H₂DCFDA (Beyotime Biotechnology, Shanghai, China) for 20 min and measured by flow cytometry. A commercial MDA ELISA kit was used to determine MDA and SOD production (Beyotime Biotechnology, Shanghai, China).

Measurement of blood lipid

After collecting blood from each mouse, the blood triglyceride (TG), total cholesterol (TC), and LDL-C levels in the sera were detected using the corresponding commercial kits (Abcam, CA, USA) according to the protocols.

Quantitative real-time PCR

Total RNA was isolated using a Qiagen RNeasy Mini Kit (Fisher Scientific). A PrimeScript RT Reagent Kit was used to reversely transcribe 1 μg of total RNA into cDNA

(TaKaRa, Dalian, China). The expressions of HOXA11-AS and ROCK1 were detected by the SYBR Green Master Mix kit (Takara). MiR-515-5p expression was detected by TaqMan™ MicroRNA Assay (Thermo Fisher Scientific). β -actin and U6 were the corresponding internal control. qRT-PCR procedures were performed on the Rotor-Gene Q (Qiagen). The $2^{-\Delta\Delta Ct}$ method was used to analyse relative expression. The primer sequences were listed below:

HOXA11-AS, F: 5'-CCAAGTCCGAGTTCATTTC-3', R: 5'-GGCTCTGGGTTGACGATAAA-3'.

miR-515-5p, F: 5'-GCGTTCTCCAAAAGAAAGCAC-3', R: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGAC CAGAAA-3'.

ROCK1, F: 5'-TGCAACTGGAACCAACCA-3', R: 5'-GTTTAGCAGCAATTGCTCA-3'.

β -actin, F: 5'-CCCTGGAGAAGAGCTACGAG-3', R: 5'-CGTACAGGCTTTGCGGATG-3'.

U6: F: 5'-CTCGCTTCGGCAGCAC-3', R: 5'-AACGCTTCACGAATTTGCGT-3'.

Western blot analysis

Total proteins were isolated from HUVECs or mice aortic tissues using RIPA buffer with 1 mM PMSF. After pre-clear by centrifuge, the concentrations were quantified by BCA Assay Kit (Thermo Fisher Scientific, USA). Then, 10 μ g of total protein was separated by 10% SDS-PAGE and was transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After 1 h blocking by 5% BSA in TBST (Sigma-Aldrich, USA), the membranes were incubated with primary antibodies in a cold room on a rotator. The primary antibodies are mouse anti-Bax (ab182733, Abcam, 1:2000), mouse anti-Bcl-2 (ab182858, Abcam, 1:2000), rabbit anti-cleaved caspase-3 (9661, Cell signalling, 1:1000), rabbit anti-ROCK1 (4035, Cell signalling, 1:1000), rabbit anti-eNOS (ab199956, Abcam, 1:2000), rabbit anti-p-eNOS (ab215717, Abcam, 1:2000), and rabbit anti-GAPDH (5174, Cell signalling, 1:1000). After incubation with secondary antibodies, the membranes were developed by SuperSignal™ & Pierce™ ECL (Thermo Fisher Scientific, USA). Image Lab™ 4.1 (Bio-Rad, USA) took and quantified the images.

Statistical analysis

All the results were expressed as means \pm SD and performed by Student's t-test or one-way ANOVA to compare the multiple groups by SPSS. We conducted three separate experiments. * $P < 0.05$, ** $P < 0.01$. $P < 0.05$ was considered as statistical significance.

Results

HOXA11-AS was up-regulated in arteriosclerosis

Our data showed that HOXA11-AS was remarkably up-regulated in atherosclerosis mice aortic plaque tissue compared with the control group (Figure 1A). As shown in Figure 1B, ox-LDL caused a dose-dependent decrease in cell viability. The relative expression of HOXA11-AS was dramatically increased in a dose-dependent manner (Figure 1C). These results indicated that HOXA11-AS was up-regulated in atherosclerosis.

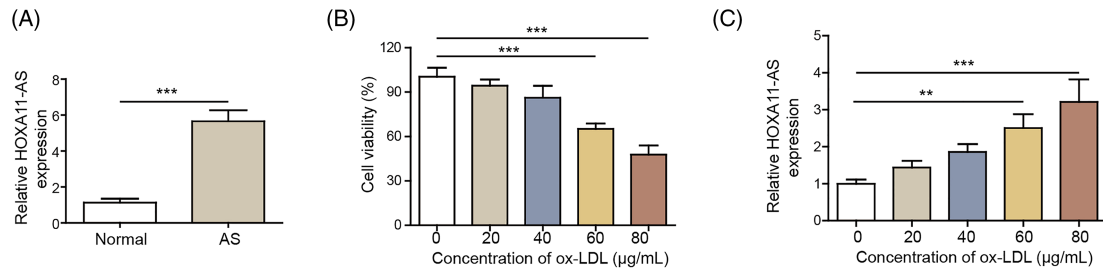
HOXA11-AS silencing diminished ox-LDL induced injury in HUVECs

Our data indicated HOXA11-AS expression was significantly inhibited after the transfection of sh-HOXA11-AS compared with vehicle control and sh-NC (Figure 2A). Meanwhile, knockdown of HOXA11-AS could attenuate the ox-LDL-induced high level of HOXA11-AS (Figure 2B). Further experiments showed HOXA11-AS silencing could remarkably alleviate the ox-LDL-induced cell proliferation inhibition (Figure 2C) and cell apoptosis promotion (Figure 2D). Compared with sh-NC, we found HOXA11-AS knockdown markedly suppressed the ox-LDL-induced production of ROS (Figure 2E) and Malondialdehyde (MDA) (Figure 2F). The further experiment identified that HOXA11-AS knockdown reversed ox-LDL-induced low level of Superoxide dismutase (SOD) (Fig. 2G). HOXA11-AS knockdown could lead to distinct up-regulation of anti-apoptotic Bcl-2 protein and apparent down-regulation of pro-apoptotic Bax and cleaved-caspase-3 proteins (Figure 2H). However, compared with the control, knockdown of HOXA11-AS could antagonize the inhibitory effects of ox-LDL on the phosphorylation of eNOS rather than the total eNOS (Figure 2I). Those results revealed that HOXA11-AS silencing alleviated ox-LDL-induced injuries in HUVECs, possibly through increasing the phosphorylation of eNOS.

Knockdown HOXA11-AS alleviated atherosclerosis in ApoE^{-/-} mice

To silence the HOXA11-AS gene in atherosclerosis, we injected lentivirus sh-HOXA11-AS or sh-NC to ApoE^{-/-} mice. Then, ApoE^{-/-} mice were administered a 10 week high-fat diet to develop the atherosclerosis model. After sacrifice, the relative HOXA11-AS levels in the aortic arch tissue of the control or HFD-fed ApoE^{-/-} mice. When HOXA11-AS was knockdown in AS mice through the injection of sh-HOXA11-AS lentivirus, our result revealed that knockdown of HOXA11-AS could significantly inhibit the expression of HOXA11-AS in AS mice (Figure 3A). The haematoxylin and

Figure 1 HOXA11-AS was up-regulated in arteriosclerosis. (A) The levels of HOXA11-AS were detected by qRT-PCR. (B) Cell viabilities were measured by MTT assay in ox-low-density lipoprotein (LDL)-treated Human Umbilical Vein Endothelial Cell (HUVEC). (C) The levels of HOXA11-AS were detected by qRT-PCR in ox-LDL treated HUVEC.



eosin staining assay showed that atherosclerosis mice's aortic arch tissue exhibited the thickened aortic arch vascular wall, the increased accumulation of foam cells, the ruptured vascular wall, and the atherosclerotic lesion in the aortic arch (*Figure 3B*). In contrast, we did not find the atherosclerotic lesion in the aortic arch of the normal mice (*Figure 3B*). By silencing HOXA11-AS, we found a significant decrease in TC, TG, and LDL-c concentrations (*Figure 3C–3E*) but enhanced the phosphorylation of eNOS rather than the total eNOS in the aortic arch tissues of AS mice (*Figure 3F*). Those data confirmed inhibition of HOXA11-AS protected mice from atherosclerotic injury.

HOXA11-AS inhibited miR-515-5p expression

The bioinformatics analysis indicated miR-515-5p contained a putative binding site for HOXA11-AS (*Figure 4A*). So we wondered whether miR-515-5p was altered in atherosclerosis. Using the normal and atherosclerosis aortic tissues, we checked miR-515-5p expression and found it reduced in the atherosclerosis aortic arch (*Figure 4B*). Then, we measured the miR-515-5p expression in ox-LDL-induced HUVECs. Interestingly, we observed a significant reduction in miR-515-5p expression in a dose-dependent manner (*Figure 4C*). However, in contrast with the effect of sh-NC transfection, HOXA11-AS knockdown reversed miR-515-5p (*Figure 4D*). Next, we performed a luciferase assay to investigate their regulation. After transfection of miR-515-5p mimics, we found the level of miR-515-5p was significantly enhanced, miR-515-5p inhibitor dramatically inhibited miR-515-5p expression (*Figure 4E*). Then, we transfected WT and MUT HOXA11-AS luciferase reporter vector with mimic NC or miR-515-5p mimics. The results indicated that miR-515-5p mimics decreased the luciferase activity compared to the mimics NC group, but no significant change was observed on the luciferase activity in the HOXA11-AS-MUT reporter (*Figure 4F*). Subsequent RIP assay further confirmed their relationship (*Figure 4G*). The results showed that both HOXA11-AS and miR-515-5p were highly enriched by anti-Ago2 com-

pared with anti-IgG, indicating HOXA11-AS had the endogenous interaction with miR-515-5p.

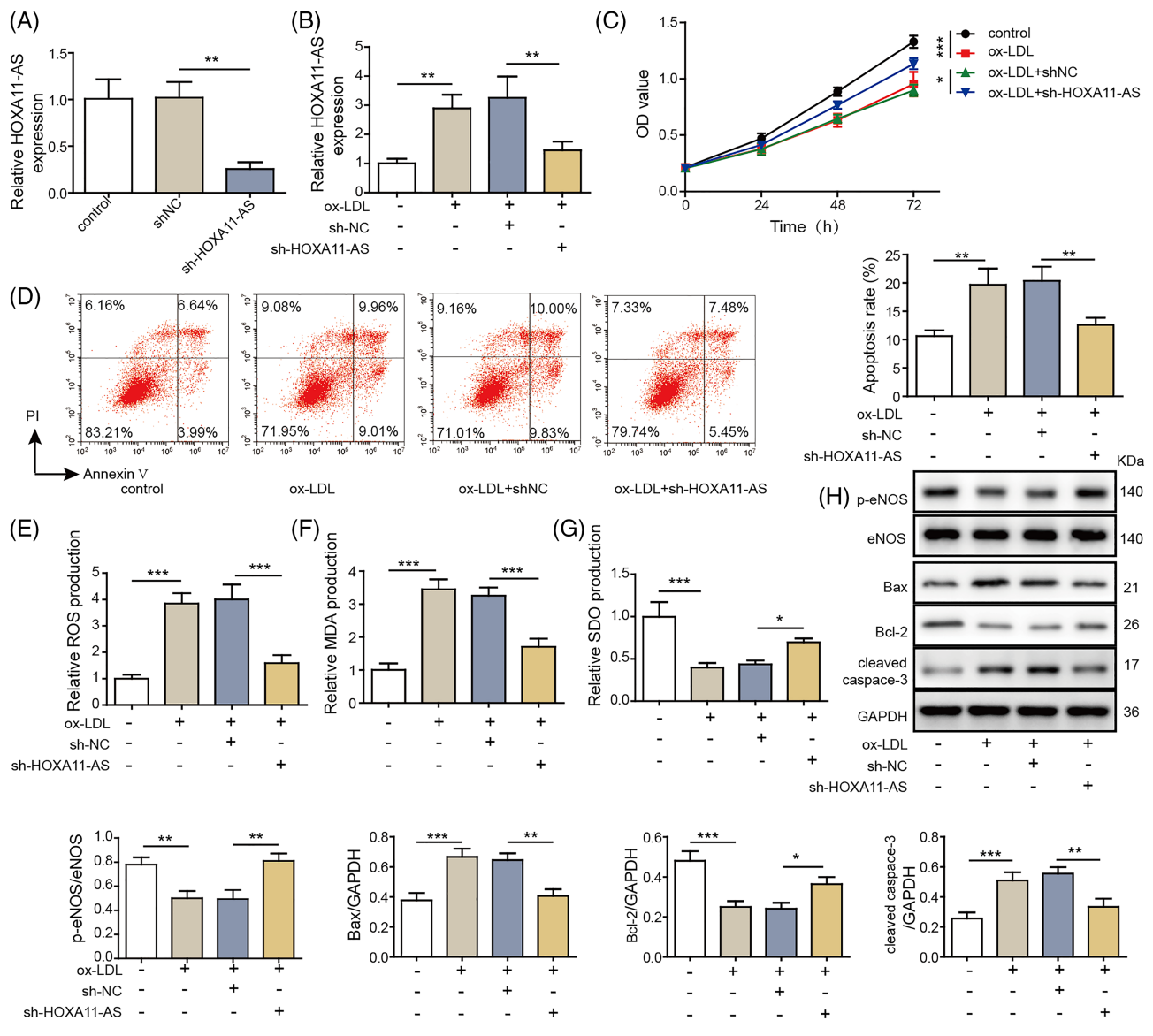
HOXA11-AS exacerbated ox-LDL-induced HUVECs injuries by inhibiting miR-515-5p

Our data revealed that miR-515-5p inhibition by transfection of miR-515-5p inhibitor significantly suppressed the increased cell proliferation (*Figure 5A*) and promoted cell apoptosis (*Figure 5B*). Functionally, silencing of HOXA11-AS remarkably inhibited ROS and Malondialdehyde (MDA) and promoted Superoxide dismutase (SOD) synthesis, but a miR-515-5p inhibitor overturned the effects (*Figure 5C–5E*). Further experiments showed silencing of HOXA11-AS distinctly increased cell viability and inhibited cell apoptosis, as showed by the increased Bcl-2 and the decreased Bax and cleaved caspase-3, whereas miR-515-5p inhibition abolished the effects (*Figure 5F*). Knockdown of HOXA11-AS could induce remarkably increased phosphorylated eNOS in ox-LDL-treated HUVECs, but knockdown of HOXA11-AS could enhance the expression of phosphorylated eNOS. However, inhibition of miR-515-5p could significantly attenuate the increased expression of phosphorylated eNOS by knockdown of HOXA11-AS in ox-LDL-treated HUVECs (*Figure 5F*). These data pointed out that HOXA11-AS regulated atherosclerosis, possibly via controlling the expression of miR-515-5p.

miR-515-5p directly inhibited ROCK1

To elucidate the mechanism of miR-515-5p in HOXA11-AS-mediated atherosclerosis, we screened out that the 3' UTR of ROCK1 contained the binding sequence by StarBase (*Figure 6A*). Western blot assay showed that atherosclerosis enhanced ROCK1 expression in aortic tissues compared with the control group (*Figure 6B*). Further experiments indicated that ROCK1 expressions gradually increased in ox-LDL-induced HUVECs (*Figure 6C*). Additional experiments showed that knockdown of HOXA11-AS could remarkably attenuate

Figure 2 HOXA11-AS silencing diminished ox-low-density lipoprotein (LDL) induced injury in Human Umbilical Vein Endothelial Cells (HUVECs). After sh-NC or sh-HOXA11-AS transfection followed by vehicle treatment or ox-LDL for 24 h, the HOXA11-AS expressions were detected by qRT-PCR (A,B). (C) Cell proliferation was detected by MTT assay. (D) Cell apoptosis was measured by flow cytometry. (E) The ROS was measured by flow cytometry. (F,G) ELISA assays measured malondialdehyde (MDA) and superoxide dismutase (SOD). H. The apoptosis-related proteins and the protein levels of p-eNOS and total eNOS were determined by western blot.



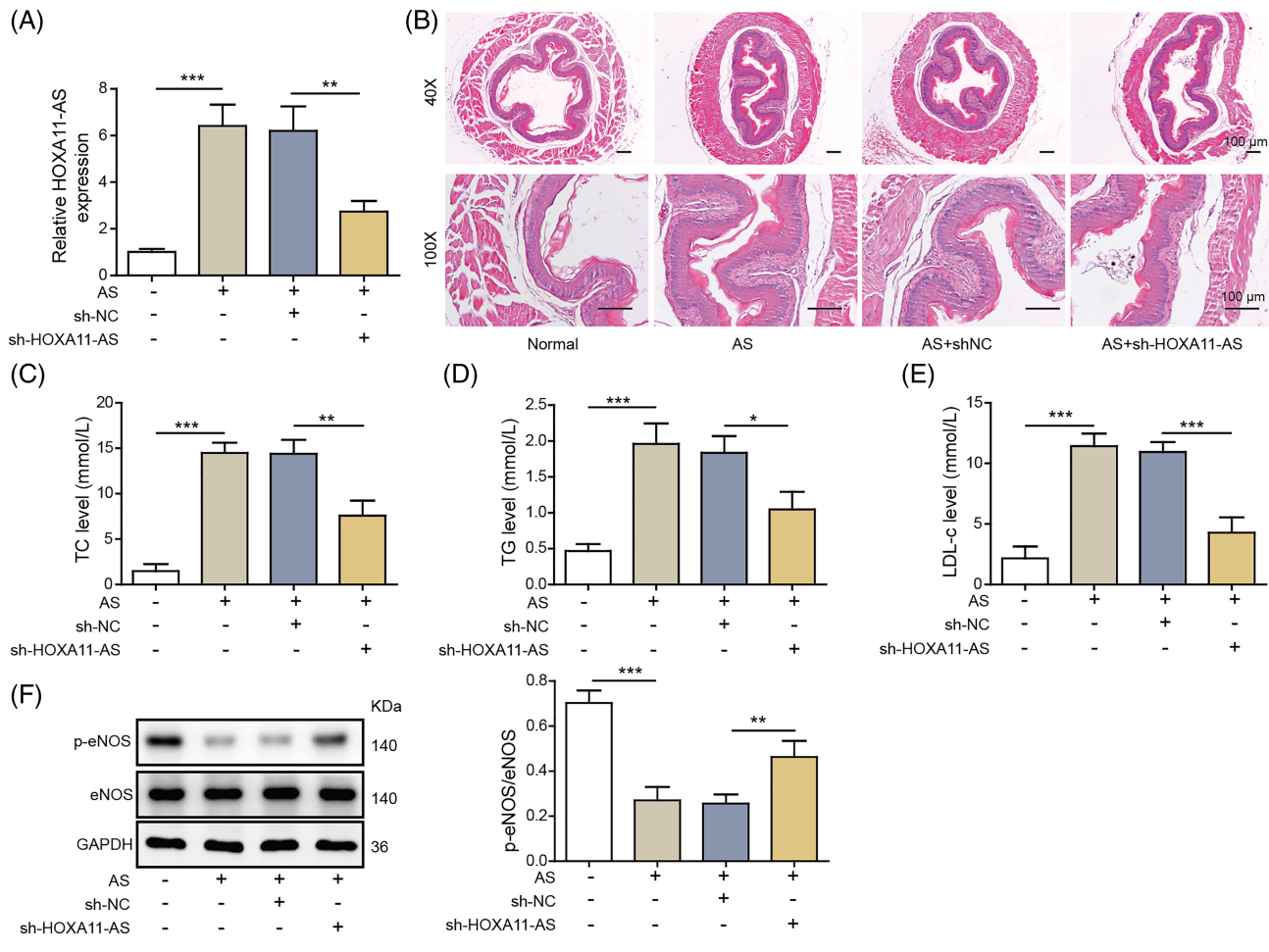
the increased ROCK1 protein levels in AS aortic tissue (Figure 6D) or ox-LDL-induced HUVECs (Figure 6D). miR-515-5p mimics decreased ROCK1 expression, but the miR-515-5p inhibitor positively increased ROCK1 expression (Figure 6F). Additionally, miR-515-5p mimics markedly reduced the luciferase activity of ROCK1-WT reporter, but no significant change was found in mimics NC or miR-515-5p mimics groups (Figure 6G). After transfection of ROCK1 overexpression vector (pcDNA3.1-ROCK1), our qRT-PCR result showed that the pcDNA3.1-ROCK1 transfection highly up-regulated ROCK1

expression rather than vehicle control and pcDNA3.1 empty vector (Figure 6H).

miR-515-5p antagonized ox-LDL-induced injuries in HUVECs by directly suppressing ROCK1

To clarify whether miR-515-5p was via regulating ROCK1 to suppress ox-LDL-induced injuries, we transfected HUVECs with miR-515-5p mimics plus pcDNA3.1-ROCK1, followed by

Figure 3 Knockdown HOXA11-AS alleviated atherosclerosis in ApoE^{-/-} mice. (A) The expression of HOXA11-AS in atherosclerosis aortic tissue was detected by qRT-PCR. (B) The formations of the vulnerable atherosclerotic plaques were evaluated by haematoxylin and eosin staining in atherosclerosis aortic tissues. (C–E) The total cholesterol (TC), triglyceride (TG), and low-density lipoprotein (LDL)-c were measured in the serum by ELISA assays. (F) The protein levels of p-eNOS and total endothelial nitric oxide synthase (eNOS) were assessed by western blot.



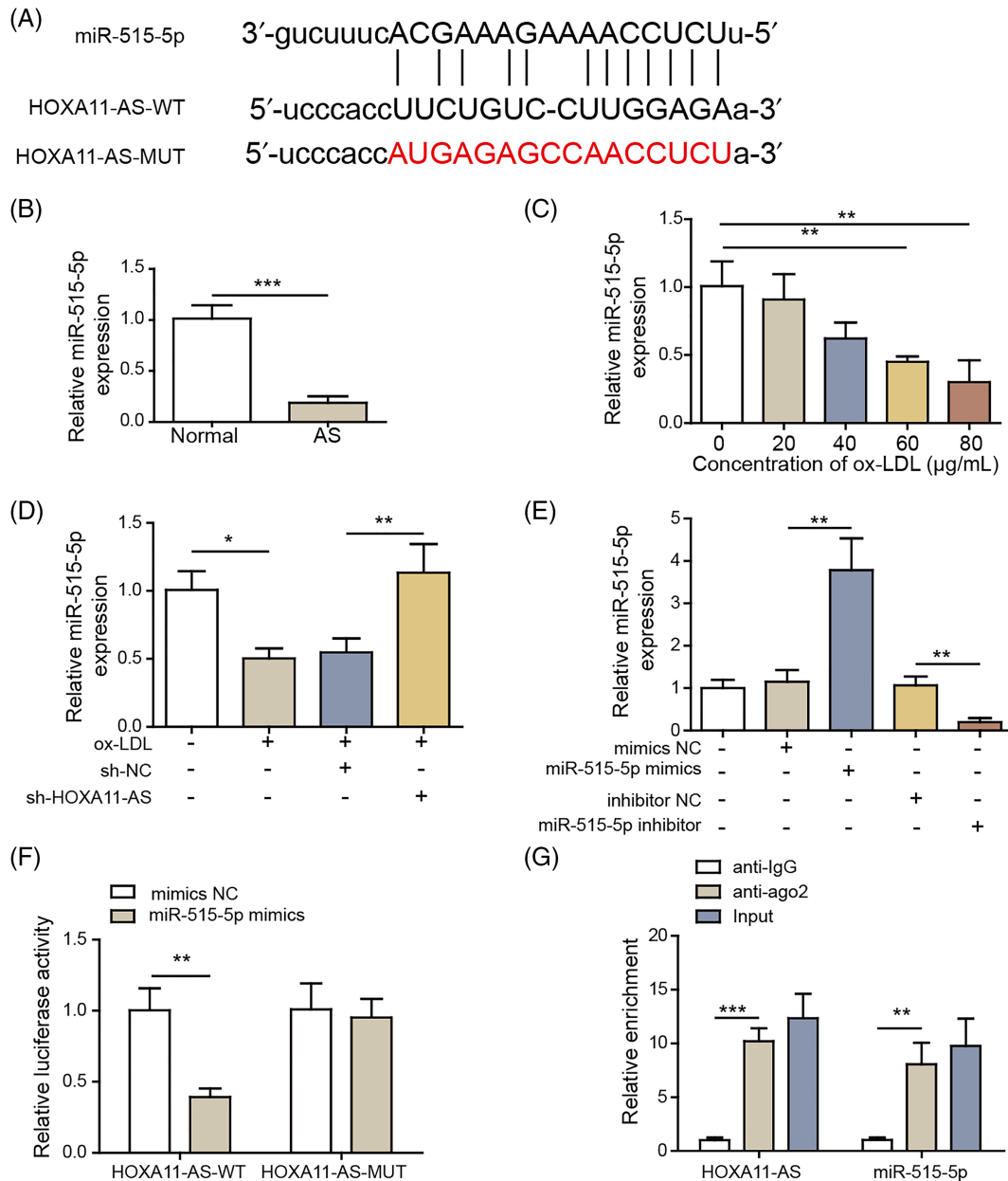
ox-LDL treatments. Our result indicated that ROCK1 overexpression could diminish the increased cell proliferation (Figure 7A) and the decreased cell apoptosis (Figure 7B) induced by miR-515-5p. Furthermore, miR-515-5p overexpression decreased the production of ROS and Malondialdehyde (MDA) and increased Superoxide dismutase (SOD) synthesis. However, ROCK1 overexpression could reverse the effects induced by miR-515-5p mimics (Figure 7C–7E). When cotransfection of miR-515-5p mimics with pcDNA3.1-ROCK1, ROCK1 overexpression increased Bax and cleaved caspase-3 levels and suppressed the Bcl-2 (Figure 7F). However, ROCK1 overexpression could antagonize the effect of miR-515-5p mimics (Figure 7F). Compared with mimics NC, miR-515-5p mimics could distinctly up-regulate the expression of phosphorylated eNOS in ox-LDL-treated HUVECs. But ROCK1 overexpression could significantly inhibit the expression of phosphorylated eNOS. However, when cotransfection of miR-515-5p mimics and pcDNA3.1-ROCK1, we found that

ROCK1 overexpression could slightly antagonize the promoting effects of miR-515-5p mimics. Taken together, miR-515-5p protected ox-LDL-induced injury by directly suppressing ROCK1.

Discussion

Atherosclerosis is a chronic progressive arterial vascular disease and a leading cause of vascular death.²² Many risk factors such as lipid infiltration, injury-response, and endothelial injuries contributed to the pathogenesis of atherosclerosis.²³ Oxidized cholesterols in the diet, particularly ox-LDL, were believed as the primary source of oxidized lipoproteins, which could accelerate the pathogenesis of atherosclerosis.²⁴ After oxidation, ox-LDL stimulates foam cells, induces endothelial dysfunction, plaque formation,

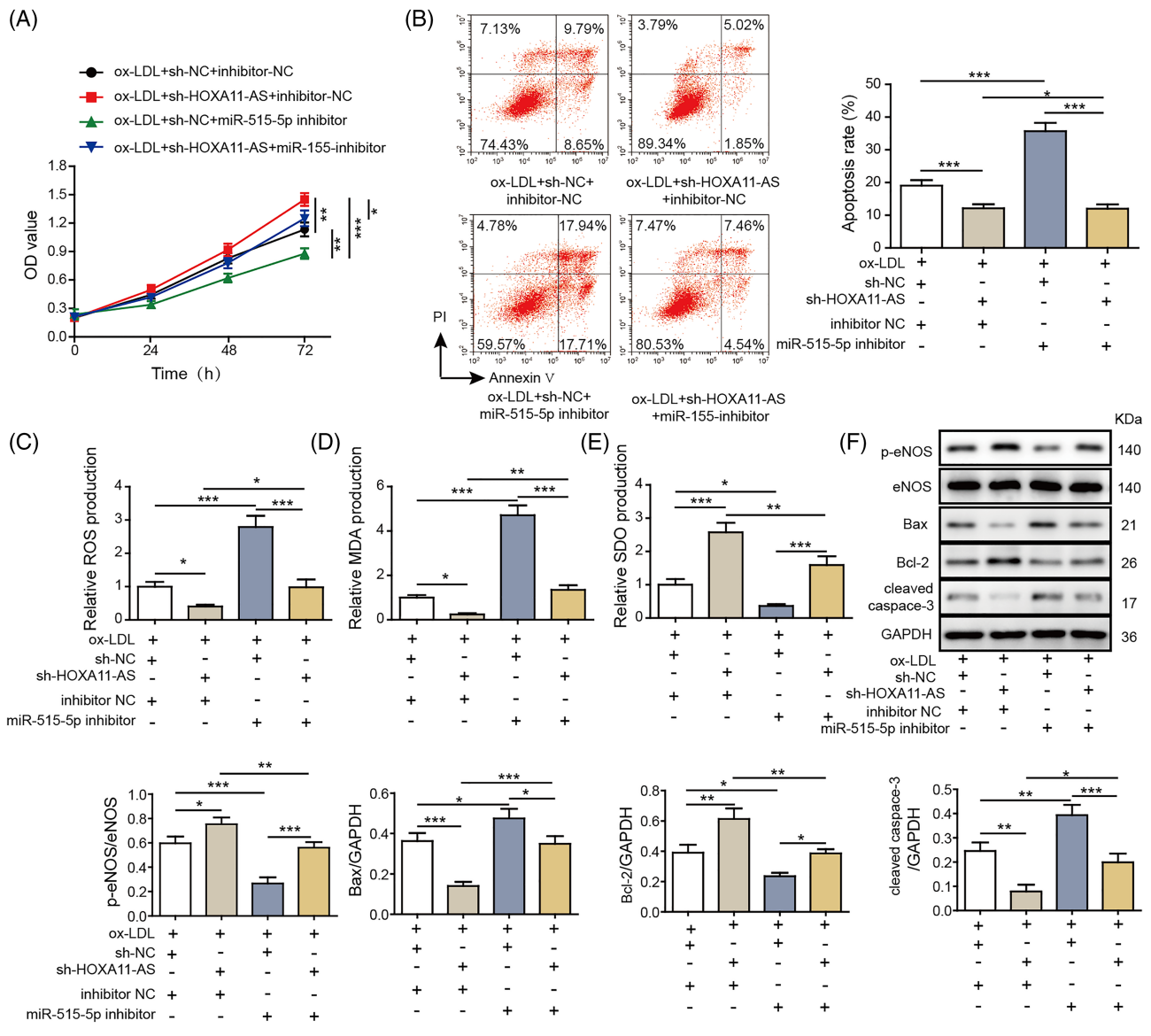
Figure 4 HOXA11-AS inhibited miR-515-5p expression. (A) The potential binding sequence in HOXA11-AS for miR-515-5p was predicted by StarBase. (B) The miR-515-5p expression was detected by qRT-PCR in atherosclerosis aortic tissue. (C) The miR-515-5p expression was detected in ox-low-density lipoprotein (LDL)-treated Human Umbilical Vein Endothelial Cells (HUVECs) by qRT-PCR. (D) The miR-515-5p expression was detected in ox-LDL-treated HUVEC with or without HOXA11-AS knockdown by qRT-PCR. (E) The miR-515-5p expression was detected after transfections of miR-515-5p mimics, miR-515-5p inhibitor or the corresponding control by qRT-PCR. (F) The luciferase activity was analysed after 48 h post-transfection of HOXA11-AS WT or Mut together with mimics NC or miR-515-5p mimics. (G) The direct interaction between HOXA11-AS and miR-515-5p was determined by RIP assays.



and inflammatory response. The experiment showed ox-LDL could induce the production of TNF- α , IL-6, and IL-8 and suppressed cell viability.^{5,25} Additional studies confirmed ox-LDL could regulate several lncRNAs and contribute to the progress of atherosclerosis,^{5,25} but the definite mechanisms are still poorly elucidated. Here, our data exhibited that

HOXA11-AS was overexpressed in both atherosclerosis mice aortic tissue and ox-LDL-stimulated HUVECs. AS mice subjected to the injection of sh-HOXA11-AS lentivirus exhibited significantly decreased expression of HOXA11-AS compared with that of control. Meanwhile, the down-regulation of HOXA11-AS could attenuate ox-LDL-induced injuries, possibly

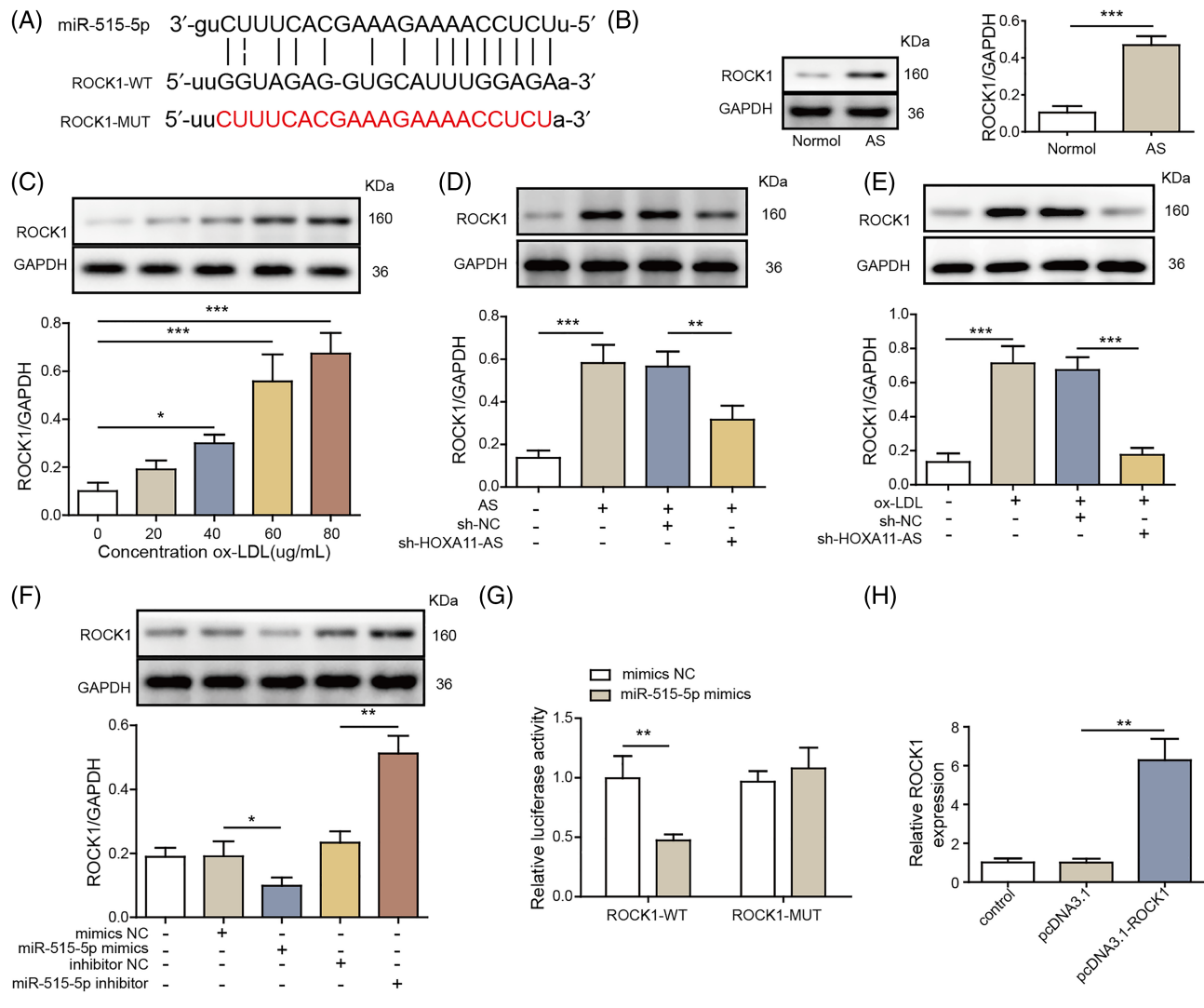
Figure 5 HOXA11-AS exacerbated ox-low-density lipoprotein (LDL)-induced Human Umbilical Vein Endothelial Cells (HUVECs) injuries by inhibiting miR-515-5p. HUVEC cells were transfected with sh-NC or sh-HOXA11-AS single or cotransfected with inhibitor-NC or miR-515-5p inhibitors, followed by the treatment of vehicle or ox-LDL for 24 h. (A). Cell proliferation was detected by MTT assay. (B). The cell apoptosis was detected by flow cytometry. (C). The ROS was measured by flow cytometry. (D,E). ELISA assays measured malondialdehyde (MDA) and superoxide dismutase (SOD). (F) The levels of apoptosis-related proteins Bax, Bcl-2, cleaved Caspase-3 and p-endothelial nitric oxide synthase (eNOS), total eNOS were detected by western blot. GAPDH was the internal control.



through the increased eNOS activity in both HUVECs and atherosclerosis mice. The further experiment confirmed that miR-515-5p was a direct target of HOXA11-AS, and ROCK1 was a direct target of miR-515-5p. Additional *in vitro* and *in vivo* experiments revealed that knockdown of HOXA11-AS protected endothelial cells from AS via up-regulating miR-515-5p and down-regulating ROCK1. The experiments disclosed that HOXA11-AS promoted atherosclerosis, possibly via regulating the miR-515-5p/ROCK1 axis.

A previous report pointed out that lncRNAs could regulate atherosclerosis events by influencing endothelial cells and macrophages.³ High-level lncRNA H19 expression induced cell proliferation and caused apoptosis inhibition through up-regulating p38 MAPK and p65 NF-κB.²⁶ Another report indicated that patients with atherosclerosis exhibited increased lncRNA LOXL1-AS1 expression with the activated miR-515-5p/STAT3 axis.²⁷ Recently, lncRNA HOXA11-AS was a significant cause of cardiac fibrogenesis by the up-regulation

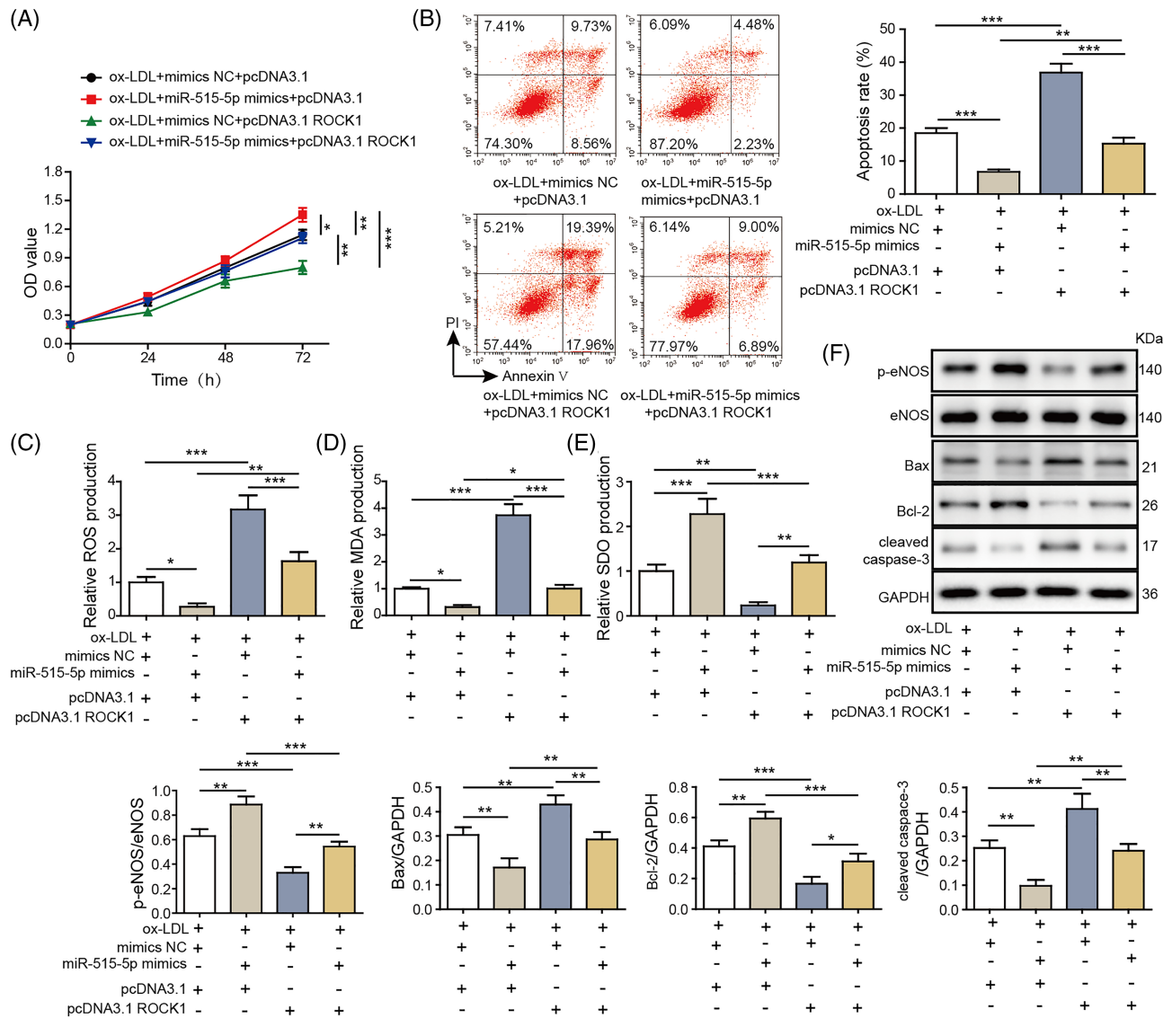
Figure 6 miR-515-5p directly inhibited ROCK1. (A) The binding sequence in ROCK1 for miR-515-5p was predicted by StarBase. (B) The ROCK1 protein levels were detected by western blot in control and atherosclerosis aortic tissues. (C) The ROCK1 protein levels were detected in ox-low-density lipoprotein (LDL)-treated HUVEC. (D,E) The protein levels of ROCK1 proteins were measured in AS aortic tissues (D), and ox-LDL-treated HUVECs (E) with or without knockdown of HOXA11-AS. F. The ROCK1 protein levels were detected 48 h after transfection of miR-15p-5p mimics, miR-15p-5p inhibitors, or their corresponding control. G. The luciferase activity was evaluated after transfection of HOXA11-AS 3' UTR-WT or -MUT vector single or together with mimics NC or miR-515-5p mimics. H. The ROCK1 mRNA level was detected by qRT-PCR in HUVEC with or without overexpressing ROCK1.



of TGF β .¹⁰ The above-mentioned reports highlighted that HOXA11-AS might be a potential candidate for heart diseases. Using the bioinformatics method, we found HOXA11-AS had a putative binding sequence of miR-515-5p. Therefore, we wondered whether HOXA11-AS participated the atherosclerosis development by regulating miR-515-5p. Here, our experiments confirmed that HOXA11-AS was up-regulated in the aortic tissue of HFD-fed atherosclerosis mice and ox-LDL-induced HUVECs. Meanwhile, knockdown of HOXA11-AS could significantly alleviate ox-LDL-induced inhibitory effect on cell proliferation and diminished cell apoptosis of HUVECs.

Additionally, HOXA11-AS knockdown could attenuate the ROS and MDA releases, increase the expression of SOD, and remarkably inhibit ox-LDL-induced overexpression of Bax and cleaved caspase-3, decreasing Bcl-2. Further *in vivo* experiments indicated that HOXA11-AS knockdown attenuated the severity of atherosclerosis by the decreased thickness of the vascular wall, the less foam cell accumulation, and vascular ruptures. Meanwhile, the blood lipids TC, TG, LDL-c were also decreased. The life-threatening risk of atherosclerosis is closely related to plaque formation. Although the plaque formation is multifaceted, the lipoprotein-driven inflammation was the initiator of atherosclerosis. High LDL levels, particu-

Figure 7 miR-515-5p antagonized ox-low-density lipoprotein (LDL)-induced injuries by directly suppressing ROCK1. Human Umbilical Vein Endothelial Cells (HUVEC) were transfected with mimics NC or miR-515-5p together with pc-DNA 3.1 or pc-DNA3.1-ROCK1, followed by the treatment of vehicle or ox-LDL for 24 h. (A). Cell proliferation was measured by MTT assay. (B). The cell apoptosis was measured by flow cytometry. (C). The ROS was measured by flow cytometry. (D,E) ELISA assays measured malondialdehyde (MDA) and superoxide dismutase (SOD). (F) The apoptosis-related proteins Bax, Bcl-2, cleaved Caspase-3 and p-endothelial nitric oxide synthase (eNOS), total eNOS were detected by western blot.



larly its derivate ox-LDL, are modulators of the immune response, which further induced endothelial cells and SMCs to express the adhesion molecules, growth factors, and chemoattractants. Those inflammatory responses further recruit macrophages and dendritic cells to deposit lipids and become foam cells.²⁸ Our results indicated that AS accumulated macrophages and lipids but decreased smooth muscle cells and collagens. However, knockdown of HOXA11-AS could significantly inhibit the accumulation of macrophages in the internal membrane of blood vessels and the subse-

quent generation of foam cells. All these data indicated that HOXA11-AS knockdown had a protective role during atherosclerosis progress.

During the past decade, miRNAs have been identified as significant regulators of atherosclerosis by regulating genes involved with atherosclerosis and modifying their post-transcriptional modification.²⁹ A recent study confirmed that lncRNAs could regulate the miRNAs.³⁰ The experiment showed LOXL1-AS1 directly sponged miR-515-5p and subsequently promoted the expression of STAT3.²⁷ We identified

that miR-515-5p is directly bound to lncRNA HOXA11-AS and ROCK1. One of the significant causes of atherosclerotic plaques is endothelial dysfunction.³¹ Further study showed that atherogenesis is due to abnormal activation of the ROCK pathway.³² The experiment confirmed that the abnormal active ROCK1 could inhibit eNOS phosphorylation and NO production, which contributed to the dysfunction of endothelial cells.³² Several miRNAs, such as miR-101-3p³³ and miR-124-3p,³⁴ could regulate the expression of ROCK1. We used luciferase reporter, and RIP assays further found miR-515-5p directly bound to HOXA11-AS. Additionally, knockdown of HOXA11-AS could diminish ox-LDL-induced inhibition of miR-515-5p. Additionally, inhibition of miR-515-5p could reverse the inhibition of cell apoptosis, the promotion of cell proliferation, the decreased production of ROS and MDA as well as the increased Bcl-2 expression by HOXA11-AS silencing. Furthermore, we also found ROCK1 was overexpressed in atherosclerosis compared with control. ROCK1 overexpression could reverse miR-515-5p-induced increased cell proliferation and SOD synthesis and decreased cell apoptosis, ROS, and MDA productions. Therefore, the down-regulation of HOXA11-AS could influence the progress of atherosclerosis by up-regulating miR-515-5p and inactivating the ROCK1 signalling pathway.

HOXA11-AS has significantly up-regulated in atherosclerosis mice aortic tissues and ox-LDL-induced HUVECs. In this article, we first validated that HOXA11-AS promoted the progress of atherosclerosis via regulating the miR-515-5p/ROCK1 axis. Therefore, the knockdown of HOXA11-AS might be a candidate target to treat atherosclerosis. Because it lacks clinical data, additional evaluation of HOXA11-AS,

miR-515-5p, and ROCK1 in atherosclerosis is being carried out in patient samples.

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Conflict of interest

The authors declare that there is no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supporting information.

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