KLF5/LINC00346/miR-148a-3p axis regulates inflammation and endothelial cell injury in atherosclerosis

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Abstract. Atherosclerosis (AS) is the main pathological basis of cardiovascular diseases, which are related to high morbidity and mortality rates. The present study aimed to investigate the role of the Krüppel-like factor 5 (KLF5)/LINC00346/miR-148a-3p loop in AS. The expression levels of KLF5 in serum and of KLF5/LINC00346/miR-148a-3p in human umbilical vein endothelial cells (HUVECs) were detected by RT-qPCR analysis. The protein expression levels of KLF5, phosphorylated (p-)endothelial nitric oxide synthase (eNOS) and eNOS in HUVECs were analyzed by western blot analysis. Changes in the levels of TNF-α, IL-1β, IL-6 and nitric oxide (NO) were determined in the supernatant through the application of available commercial kits. The binding of KLF5 to the promoter region of LINC00346 was verified by chromatin immunoprecipitation (ChIP)-PCR assay. The combinatory interaction between KLF5 and LINC00346, LINC00346 and miR-148a-3p, and miR-148a-3p and KLF5 was confirmed by luciferase reporter assay. The results revealed that KLF5 expression was increased in the serum of patients with AS and also in oxidized low-density lipoprotein (OX-LDL)-stimulated HUVECs. The transcription factor KLF5 promoted the transcription of LINC00346. KLF5 interference or LINC00346 interference inhibited the expression of inflammatory factors and functional injury in OX-LDL-stimulated HUVECs. LINC00346 functioned as a sponge of miR-148a-3p. miR-148a-3p overexpression inhibited the expression of inflammatory factors and functional injury in OX-LDL-stimulated HUVECs and miR-148a-3p targeted KLF5 expression. On the whole, the present study demonstrates that KLF5 interference induces the downregulation of LINC00346 and also inhibits inflammation and functional injury in OX OX-LDL-stimulated HUVECs by upregulating miR-148a-3p expression.

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Introduction

Atherosclerosis (AS) is a chronic inflammatory reaction of the arterial walls in response to vascular endothelial cell injury. AS pathogenesis involves endothelial cell injury, lipid accumulation in the vascular wall, monocyte adhesion and transformation, the release of inflammatory factors and finally, the proliferation and migration of smooth muscle cells, which eventually leads to AS development (1). AS is the main pathological basis of cardiovascular diseases, which are associated with high morbidity and mortality rates (2,3). Significant progress has been made concerning the treatment options of patients with AS; however, the majority of therapeutics used are often associated with chronic side-effects (4). Therefore, it is necessary to elucidate the molecular mechanisms of AS in order to identify novel diagnostic and therapeutic modalities, that can more treat AS more efficiently.

Krüppel-like factor 5 (KLF5) is a protein, encoded in humans by the KLF5 gene, and belongs to the Krüppel-like factor subfamily of zinc finger proteins. KLF5 can regulate the expression of a number of downstream target genes, including cyclin D1, cyclin B1, fibroblast growth factor-binding protein and other coding genes (5). KLF5 is expressed in a wide variety of cells, including vascular smooth muscle cells, lipocytes, neurons and white blood cells, and its expression is particularly high in intestinal epithelial cells (6). Therefore, KLF5 is involved in the regulation of inflammatory stress response and intestinal development, which is caused by cardiovascular remodeling in embryonic development (5). KLF5 promotes angiogenesis through the upregulation of vascular endothelial growth factor (VEGFA), myosin heavy chain kinase (MHC), myosin light chain kinase (MLCK), calponin, smooth muscle actin (SMA) and transgelin (SM22-a), thereby increasing the proliferation and migration of vascular smooth muscle cells (7-9). MicroRNA (miRNA/miR)-152 prevents AS progression and reduces β-catenin expression through the downregulation of KLF5 (10). KLF5 promotes the proliferation of vascular smooth muscle cells and subsequently promotes the formation of atherosclerotic plaques (11). However, the role of

KLF5 in endothelial cell damage caused by AS, at least to the best of our knowledge, has not yet been studied.

Long non-coding RNAs (lncRNAs) are a group of transcripts of >200 nucleotides in length, which lack protein coding potential (12). lncRNAs may play an important role in the treatment of AS. lncRNA non-coding RNA activated by DNA damage (NORAD) expression has been observed to be increased in human umbilical vein endothelial cells (HUVECs). This is induced by oxidized low-density lipoprotein (OX-LDL) and NORAD knockdown and has been shown to function as a promoter of OX-LDL-induced HUVEC injury and AS (13). In a previous study, lncRNA forkhead box C2-antisense RNA 1 (FOXC2-AS1) expression was found to be markedly increased in patients with AS, and FOXC2-AS1 overexpression promoted the proliferation and inhibited apoptosis of vascular smooth muscle cells (VSMCs) (14). lncRNA CDKN2B antisense RNA 1 expression has been noted to be elevated in human atherosclerotic plaques and OX-LDL-stimulated HUVECs, and can promote cell proliferation and migration by sponging miR-399-5p (15).

The present study aimed to explore the important role of the KLF5/p53 regulated carcinoma associated Stat3 activating long intergenic non-protein coding transcript (LINC00346)/miR-148a-3p axis in AS. It was found that the transcription factor, KLF5, promoted LINC00346 transcription. In addition, the role of LINC00346 in AS progression was confirmed and the positive feedback loop of KLF5/LINC00346/miR-148a-3p in AS was revealed, providing a potential therapeutic target for AS.

Materials and methods

Serum samples. A total of nine patients with AS and nine healthy volunteers were recruited from Changzhou Second People's Hospital (Jiangsu, China) between July, 2019 and October, 2019. The inclusion criteria were as follows: i) An age 25-75 years; ii) patients exhibiting hypertension, coronary heart disease and hyperlipidemia; and iii) patients provided informed consent for participation. The exclusion criteria were a history of stroke, transient ischemic attack, coronary instability, congestive heart failure, chronic or acute inflammatory conditions, cancer and recent intracranial hemorrhage. From the health check-up center, nine healthy donor control samples, (25-75 years) were selected as the control group.

Approval for the study was obtained from the Ethics Committee of Changzhou No. 2 People's Hospital, Affiliated Nanjing Medical University. Informed consent and relevant clinical information were obtained from all participants. Blood samples (5 ml) were collected from all participants and were centrifuged at 3,000 x g for 10 min at 4˚C. Serum samples were then stored at -80˚C.

Cell culture and transfection. HUVECs were obtained from the American Type Culture Collection (ATCC). HUVECs were routinely cultured in DMEM (Thermo Fisher Scientific, Inc.) containing 15% FBS (Gibco; Thermo Fisher Scientific, Inc.) in an incubator under humidity conditions of 5% CO_2 and 37˚C, and cells in logarithmic growth phase were used. HUVECs were treated with 100 μmol/l OX-LDL for 24 h. The short hairpin RNA (shRNA)-negative control (NC), shRNA-KLF5-1/2, shRNA-LINC00346-1/2 were obtained from Shanghai GenePharma Co., Ltd. A KLF5 overexpression plasmid, pcDNA3.1-KLF5 (OV-KLF5), was commercially constructed by Shanghai GenePharma Co., Ltd., and an empty pcDNA 3.1 vector (OV-NC) was used as the control. For miRNA transfection, miR-148-5p mimic, mimic-NC were obtained from Sangon Biotech Co., Ltd. OX-LDL-stimulated HUVECs (2nd generation) were transfected with shRNA-NC (500 μM), shRNA-KLF5-1/2 (500 μM), OV-NC (500 μM), OV-KLF5 (500 μM), shRNA-LINC00346-1/2 (500 μM), miR-NC (500 μM) and miR-148-5p mimic (500 μM) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 48 h of transfection, the cells were harvested for downstream assays.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the serum samples and HUVECs in each group using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, and 2 μg RNA per sample were reverse transcribed into cDNA by First-Stand cDNA Synthesis Super Mix (TransGen Biotech Co., Ltd.). Each gene was amplified by qPCR. The following thermocycling conditions were used: The reaction conditions were 95˚C pre-denaturation for 30 sec, 95˚C denaturation for 5 sec, 60˚C annealing for 30 sec, 72˚C extension for 30 sec, and 40 cycles were repeated. The primer sequences of all genes were as follows: KLF5 forward, 5'-ACGCTTGGCCTA TAACTTGGT-3' and reverse, 5'-TGGAGGAAGCTGAGG TGTCA-3'; LINC00346 forward, 5'-AGCTTGAATGGCGTT GGAACCTATAG-3' and reverse, 5'-ATAGTCCCTTCCTCG AATCCTAGT-3'; GAPDH forward, 5'-AAGGTGAAGGTC GGAGTCA-3' and reverse, 5'-GGAAGATGGTGATGGGAT TT-3'; miR-148a-3p forward, 5'-TCAGTGCACTACAGAACT TTGT-3' and reverse, 5'-GTCACCCCTGTTTCTGGCAC-3'; U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. GAPDH was used as the endogenous control of KLF5 and LINC00346, and U6 was used as the endogenous control of miR-148a-3p. The relative expression of KLF5, LINC00346 and miR-148a-3p was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. HUVECs in each group were digested and collected by trypsin. Subsequently, total protein was extracted from the cells in each group with the addition of cell lysis solution and centrifugation at 3,000 x g at 4˚C for 10 min. The quantity measurement of the total isolated protein was performed using the BCA method. Protein samples were denatured in a boiling water bath for 5 min. Denatured proteins were obtained and 80 μg protein per lane was separated via 12% SDS-PAGE electrophoresis. The PVDF membrane was then blocked with 5% skimmed milk powder on a shaking table at room temperature for 2 h, and then incubated with anti-KLF5 (dilution, 1:1,000; ab137676), phosphorylated (p-)endothelial nitric oxide synthase (eNOS; dilution, 1:1,000; ab184154), eNOS (dilution, 1:1,000; ab76198) and GAPDH (dilution, 1:25,00; ab9485) (all from Abcam) antibodies overnight at 4˚C. Following primary antibody incubation, the membranes were incubated with horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:2,000; ab6721; Abcam) for 1 h at room temperature. Protein bands were visualized using

ECL reagent (Thermo Fisher Scientific, Inc.) and densitometric analysis was performed using Quantity One software (Version 4.6.6; Bio-Rad Laboratories, Inc.).

ELISA. HUVECs at the logarithmic growth stage were inoculated into a 10-cm culture dish at an appropriate density. After the indicated treatments, the HUVECs, together with the culture medium, were centrifuged at 3,000 x g for 5 min at 4˚C, in order to obtain the supernatant. The contents of TNF-α, IL-1β and IL-6 in the supernatant were determined according to the instructions of TNF-α (cat. no. PT518), IL-1β (cat. no. PI305) and IL-6 (cat. no. PI330) ELISA kits (Beyotime Institute of Biotechnology).

Detection of nitric oxide (NO) levels. HUVECs were digested to prepare a $1x10^8$/l cell suspension, which was inoculated into a 24-well plate. According to the experimental groups, the corresponding treatment was performed. The cell culture supernatant was obtained to detect NO levels according to the instructions provided with the NO kit (cat. no. S0021S; Beyotime Institute of Biotechnology).

Chromatin immunoprecipitation (ChIP)-PCR assay. HUVECs were fixed at room temperature with formaldehyde for 10 min, washed with PBS, and the collected cells were placed in an ultrasonic water bath. Ultrasonic treatment was then carried out. The long strand DNA of the gene was broken into 200-1,000 bp DNA fragments by ultrasonic treatment, which was centrifuged at 16,000 x g for 15 min at room temperature to obtain the supernatant. A total of 20 μl supernatant was removed as the input. KLF5 antibody (dilution, 2.5-5 μg/10^6 cells; ab277773, Abcam) and corresponding IgG antibody (A7016; Beyotime Institute of Biotechnology) were added into the remaining supernatant, which was incubated overnight at 4˚C. Magnetic beads were added the following day for 2 h at room temperature. Following centrifugation at 16,000 x g for 2 min at 4˚C, the precipitate was eluted step by step with low-salt buffer solution and high-salt buffer solution, respectively, and NaCl solution was then added followed by incubation at 65˚C overnight to remove chromatin fixation. EDTA, Tris-HCl and protease were added followed by incubation at 65˚C for 1 h. Finally, phenol-chloroform method was used to extract the 50 μl purified product for PCR detection.

Luciferase reporter assay. HUVECs were transfected with LINC00346 (full)-L and OV-NC/OV-KLF5, LINC00346 (site2)-L and OV-KLF5 using Lipofectamine 2000®. The relative luciferase activity was detected using a Dual Luciferase Reporter assay system (Promega Corporation) to confirm the binding between KLF5 and LINC00346.

Wild-type (WT)-LINC00346, mutant (Mut)-LINC00346, WT-KLF5 or Mut-KLF5 were co-transfected into HUVECs with miR-148a-3p mimic or miR-NC using Lipofectamine 2000® and the luciferase activity was detected to confirm the binding between LINC00346/KLF5 and miR-148a-3p after transfection for 48 h. Relative luciferase activity was normalized to *Renilla* luciferase activity (control).

Statistical analysis. SPSS 22.0 software (IBM Corp.) was used for the statistical analysis and the data are presented as the mean ± SD. One-way ANOVA was used for the statistical comparisons between groups, followed by Tukey's post hoc test for multiple comparisons between groups. An independent samples unpaired Student's t-test was used for comparisons between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

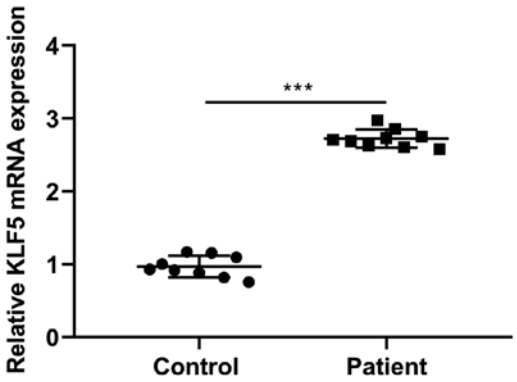

Figure 1. KLF5 expression in serum of patients with atherosclerosis. Serum KLF5 expression was detected by RT-qPCR analysis. ***P<0.001; n=3. KLF5, Krüppel-like factor 5.

Results

KLF5 expression in AS patient serum. As shown in Fig. 1, KLF5 expression levels were increased in serum from patients with AS in comparison with the respective healthy donor expression levels.

KLF5 interference inhibits OX-LDL-induced inflammatory factor expression in HUVECs. KLF5 mRNA expression was increased in OX-LDL-stimulated HUVECs and was decreased when the OX-LDL-stimulated HUVEC cells were transfected with shRNA-KLF5-1/2. KLF5 mRNA expression was decreased further in the OX-LDL-stimulated HUVECs transfected with shRNA-KLF5-1 in comparison with HUVECs transfected with shRNA-KLF5-2 (Fig. 2A). KLF5 mRNA expression was also decreased further in HUVECs transfected with shRNA-KLF5-1 as compared with HUVECs transfected with shRNA-KLF5-2 (Fig. 2B). Changes in KLF5 protein expression in these groups were similar to those obtained for KLF5 mRNA expression (Fig. 2C). Therefore, shRNA-KLF5-1 was selected for use in subsequent experiments. The TNF-α, IL-1β and IL-6 levels in OX-LDL-stimulated HUVECs were upregulated and were subsequently suppressed by KLF5 interference (Fig. 2D).

KLF5 interference inhibits OX-LDL-induced injury to HUVECs. NO expression was decreased in the OX-LDL-stimulated HUVECs, while KLF5 interference increased NO expression (Fig. 3A). p-eNOS/eNOS expression in OX-LDL-stimulated HUVECs was decreased. This effect was reversed by KLF5 interference (Fig. 3B and C).

Transcription factor KLF5 promotes LINC00346 transcription. LINC00346 expression was increased in the serum of patients with AS (Fig. 4A). LINC00346 expression was increased in OX-LDL-stimulated HUVECs and was

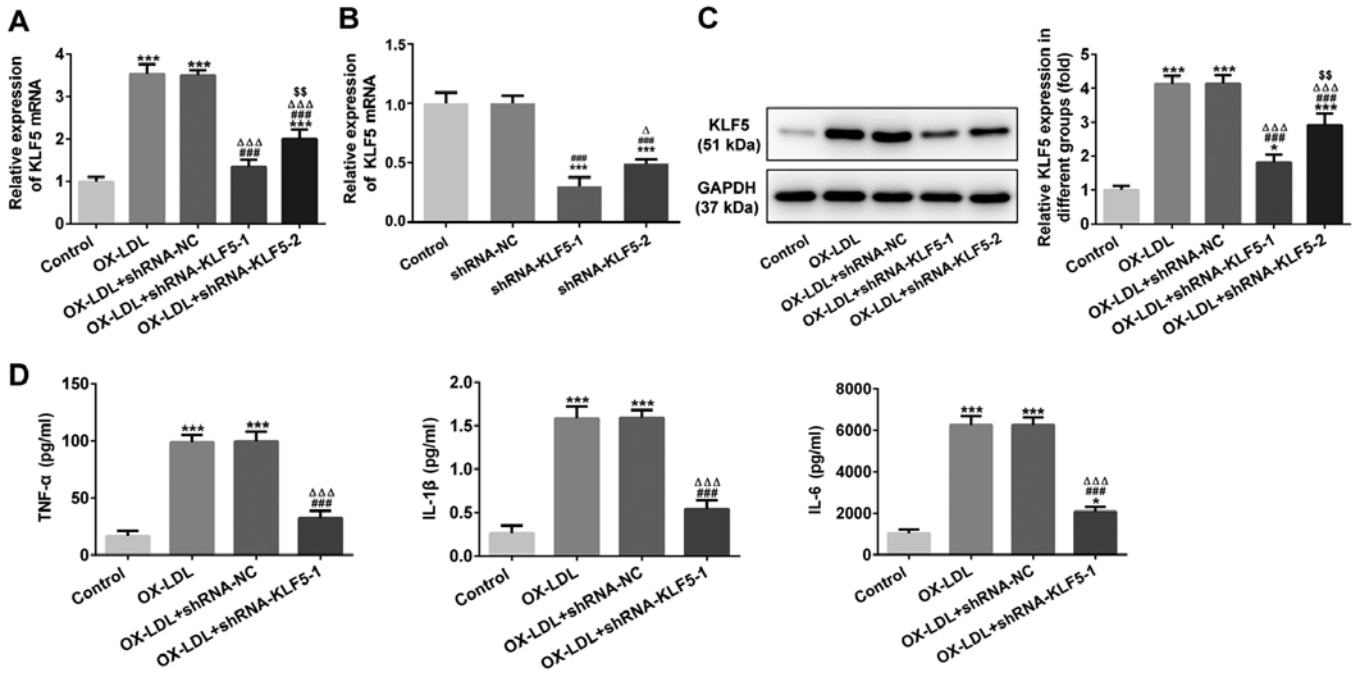

Figure 2. KLF5 interference inhibits OX-LDL-induced inflammatory factor expression in HUVECs. (A) KLF5 mRNA expression in HUVECs following OX-LDL induction and shRNA-KLF5 transfection was detected by RT-PCR analysis. ***P<0.001 vs. control; $^{###}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; $^{\$\$}$P<0.01 vs. OX-LDL + shRNA-KLF5-1. (B) KLF5 mRNA expression in HUVECs following shRNA-KLF5 transfection was detected by RT-PCR analysis. ***P<0.001 vs. control; $^{###}$P<0.001 vs. shRNA-NC; $^{\Delta}$P<0.05 vs. shRNA-KLF5-1. (C) KLF5 protein expression in HUVECs following OX-LDL induction and shRNA-KLF5 transfection was examined by western blot analysis. *P<0.05 and ***P<0.001 vs. control; $^{###}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; $^{\$\$}$P<0.01 vs. OX-LDL + shRNA-KLF5-1. (D) The levels of TNF-α, IL-1β and IL-6 in the supernatant of HUVECs following OX-LDL induction and shRNA-KLF5 transfection were detected by ELISA. *P<0.05 and ***P<0.001 vs. control; $^{###}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; n=3. KLF5, Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein.

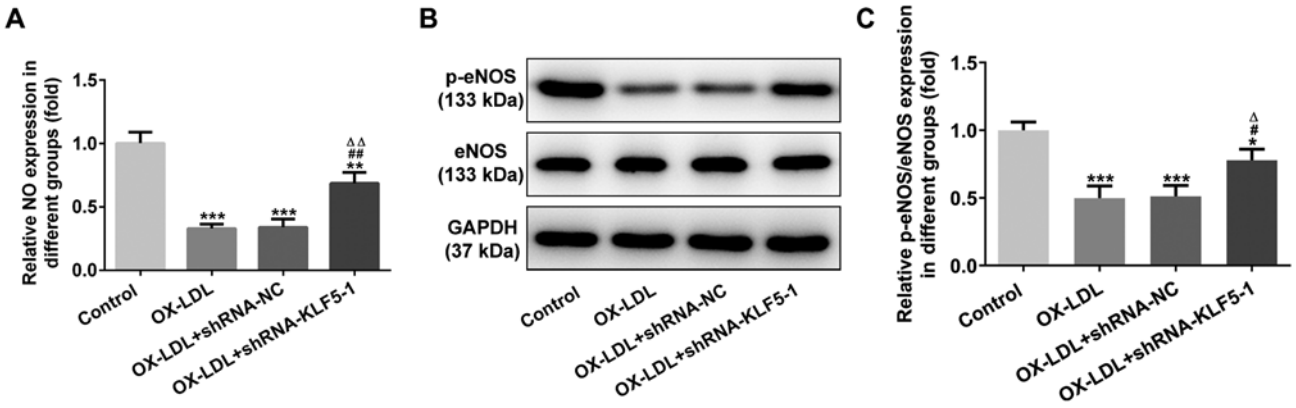

Figure 3. KLF5 interference inhibits OX-LDL-induced function injury to HUVECs. (A) The NO level in the supernatant of HUVECs following OX-LDL induction and shRNA-KLF5 transfection was detected using a NO kit. (B) Protein bands of p-eNOS and eNOS. (C) OX-LDL-induced p-eNOS/eNOS expression in HUVECs following shRNA-KLF5 transfection was examined by western blot analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.05 vs. OX-LDL; $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 vs. OX-LDL + shRNA-NC; n=3. KLF5, Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

subsequently suppressed by KLF5 interference (Fig. 4B). KLF5 expression was increased in OX-LDL-stimulated HUVECs, and was further promoted by KLF5 overexpression (Fig. 4C). KLF5 overexpression further increased LINC00346 expression in OX-LDL-stimulated HUVECs (Fig. 4D). The results presented in Fig. 4E illustrate that KLF5 can bind to the four promoter regions of LINC00346. Mutated site2 LINC00346 and full LINC00346 were respectively co-transfected into HUVECs with OV-KLF5 plasmid and the results revealed that KLF5 (site2) could bind to LINC00346 (Fig. 4F).

LINC00346 interference inhibits the expression of inflammatory factors in HUVECs, induced by OX-LDL. LINC00346 expression was increased in OX-LDL-stimulated HUVECs (Fig. 5A) and LINC00346 expression was decreased in OX-LDL-stimulated HUVECs transfected with shRNA-LINC00346-1/2. LINC00346 expression was decreased to a greater extent in shRNA-LINC00346-1-transfected OX-LDL-stimulated HUVEC cells in comparison with the shRNA-LINC00346-2-transfected cells (Fig. 5B); LINC00346 expression was further down-regulated in shRNA-LINC00346-1-transfected HUVECs in

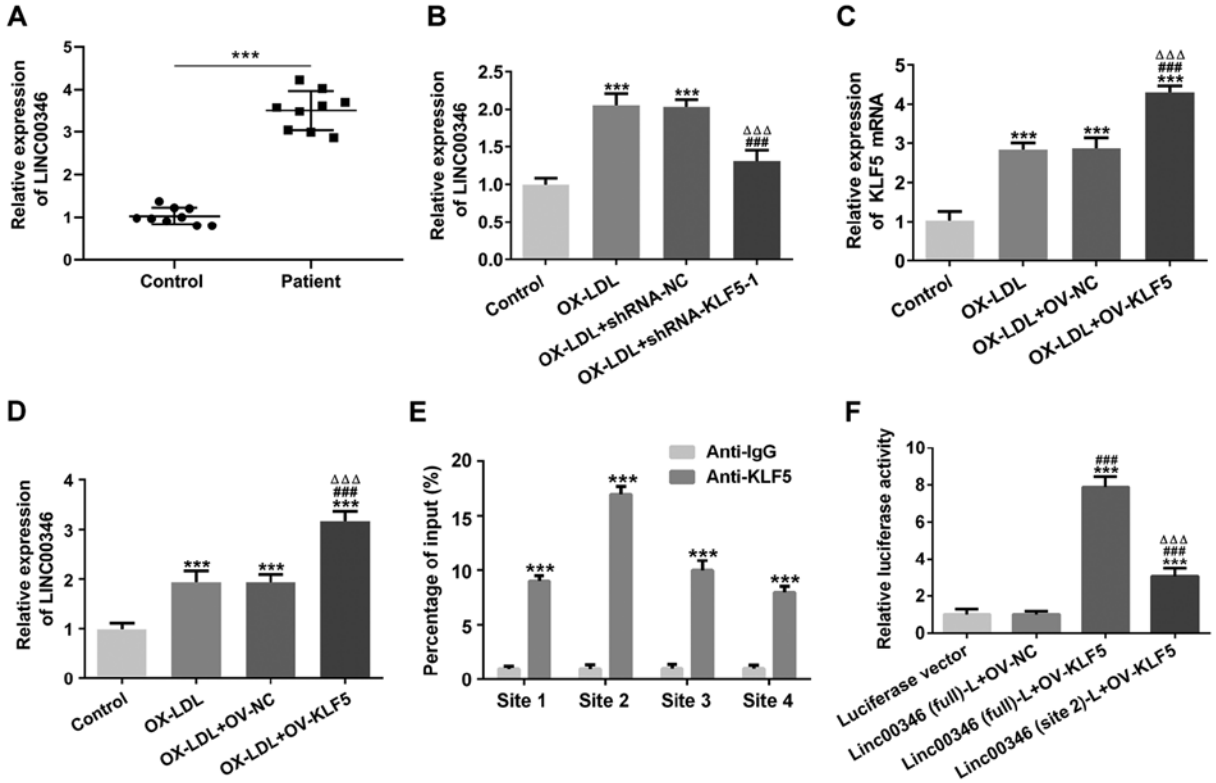

Figure 4. Transcription factor KLF5 promotes LINC00346 transcription. (A) LINC00346 expression in serum of patients with atherosclerosis was detected by RT-PCR analysis. ***P<0.001 vs. control. (B) LINC00346 expression in HUVECs following OX-LDL induction and shRNA-KLF5 transfection was detected by RT-PCR analysis. ***P<0.001 vs. control; ###P<0.001 vs. OX-LDL; ΔΔΔP<0.001 vs. OX-LDL + shRNA-NC. (C) KLF5 expression in HUVECs following OX-LDL induction and ov-KLF5 transfection was detected by RT-PCR analysis. (D) LINC00346 expression in HUVECs following OX-LDL induction and ov-KLF5 transfection was detected by RT-PCR analysis. ***P<0.001 vs. control; ###P<0.001 vs. OX-LDL; ΔΔΔP<0.001 vs. OX-LDL + OV-NC. (E) KLF5 bound to four promoter regions of LINC00346, which was confirmed by ChIP-PCR assay. ***P<0.001 vs. Anti-IgG. (F) Detection of luciferase activity. ***P<0.001 vs. luciferase vector; ###P<0.001 vs. Linc00346(full)-L + OV-NC; ΔΔΔP<0.001 vs. Linc00346(full)-L + OV-KLF5; n=3. KLF5, Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein.

comparison with the shRNA-LINC00346-2-transfected cells (Fig. 5C); thus, shRNA-LINC00346-1 was selected for use in subsequent experiments. OX-LDL induction upregulated the TNF-α, IL-1β and IL-6 levels, which were then decreased by LINC00346 interference (Fig. 5D).

LINC00346 interference inhibits OX-LDL-induced injury and KLF5 expression in HUVECs. NO expression was decreased in OX-LDL-stimulated HUVECs and was promoted by LINC00346 interference (Fig. 6A). p-eNOS/eNOS expression was downregulated in OX-LDL-stimulated HUVECs, while LINC00346 interference increased p-eNOS/eNOS expression (Fig. 6B). KLF5 expression was also increased in OX-LDL-stimulated HUVECs, which was suppressed by LINC00346 interference (Fig. 6C).

LINC00346 functions as a sponge for miR-148a-3p. LINC00346 expression in the cytoplasm was increased compared with that in the nucleus (Fig. 7A). The binding sites between LINC00346 and miR-148a-3p shown in Fig. 7B and C confirmed that LINC00346 could bind to miR-148a-3p. miR-148a-3p expression was decreased in OX-LDL-stimulated HUVECs. LINC00346 interference or KLF5 interference promoted miR-148a-3p expression, while KLF5 overexpression suppressed miR-148a-3p expression in OX-LDL-stimulated HUVECs (Fig. 7D-F).

miR-148a-3p overexpression inhibits OX-LDL-induced injury and OX-LDL-induced inflammatory factor expression in HUVEC cells and miR-148a-3p targets KLF5. miR-148a-3p expression was increased in HUVECs transfected with miR-148a-3p mimic (Fig. 8A). miR-148a-3p overexpression decreased the TNF-α, IL-1β and IL-6 levels (Fig. 8B) and increased NO expression in OX-LDL-stimulated HUVECs (Fig. 8C). miR-148a-3p overexpression increased the expression of p-eNOS/eNOS (Fig. 8D) and inhibited the expression of KLF5 in OX-LDL-stimulated HUVECs (Fig. 8E). As shown in Fig. 8F, binding sites were predicted between miR-148a-3p and KLF5, and dual luciferase reporter assay confirmed that miR-148a-3p could bind with KLF5.

Discussion

AS is a chronic inflammatory disease, in which lipids and cholesterol accumulate in large and medium-sized arteries and develop into fibrous plaques (17). The pathogenesis of AS is complex, and involves an inflammatory reaction, abnormal cholesterol levels and activation of the damage response (18,19). After decades of research, the inflammatory response has been found to play an important role in the occurrence and development of AS, and even in the later stage of plaque formation (18,20). The dysfunction of vascular endothelial cells occurs in the impaired area of the arterial vascular

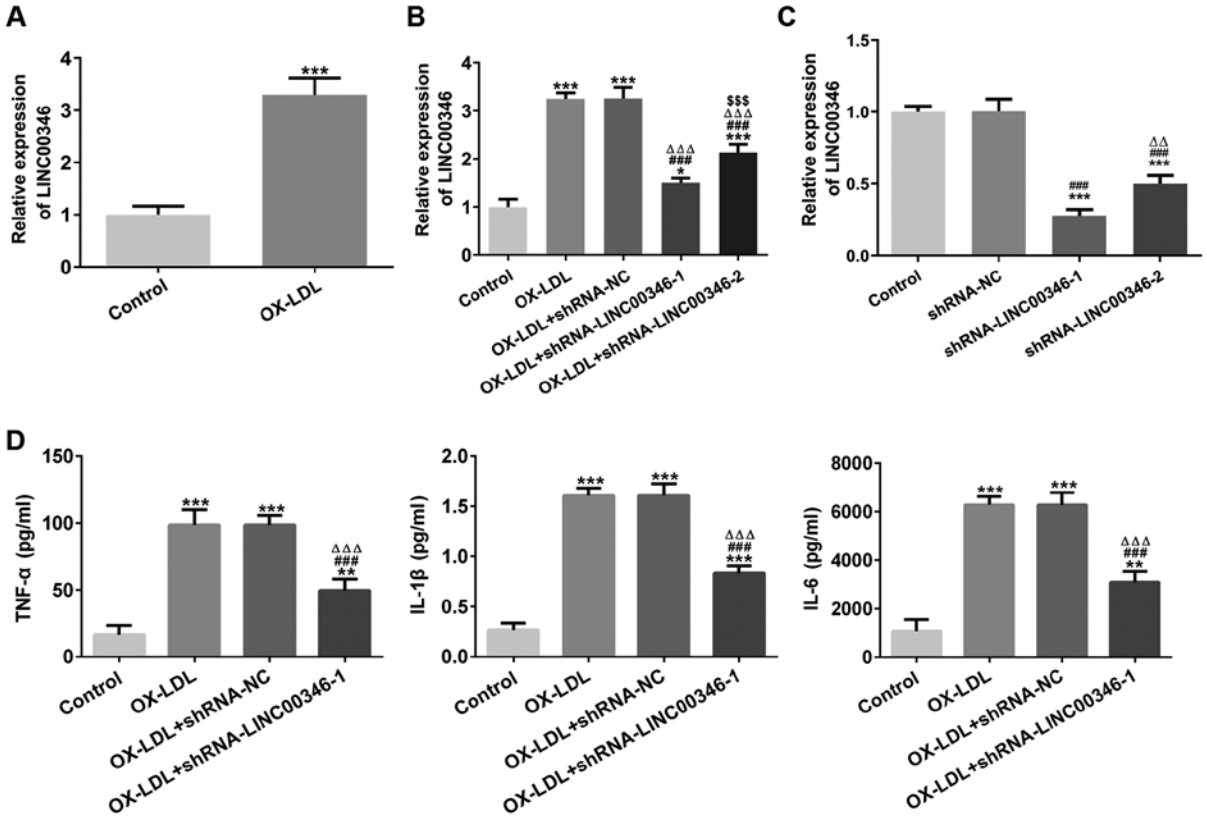

Figure 5. LINC00346 interference inhibits OX-LDL-induced inflammatory factor expression in HUVECs. (A) LINC00346 expression in HUVEC induced by OX-LDL was detected by RT-PCR analysis. ***P<0.001 vs. control. (B) LINC00346 expression in HUVECs following OX-LDL induction and shRNA-LINC00346 transfection was analyzed by RT-PCR analysis. *P<0.05 and ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; $^{\$\$\$}$P<0.001 vs. OX-LDL + shRNA-LINC00346-1. (C) LINC00346 expression in HUVECs following shRNA-LINC00346 transfection was analyzed by RT-PCR analysis. ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. shRNA-NC; $^{\Delta\Delta}$P<0.01 vs. shRNA-LINC00346-1. (D) The levels of TNF-α, IL-1β and IL-6 in the supernatant of HUVECs following OX-LDL induction and shRNA-LINC00346 transfection were detected by ELISA. **P<0.01 and ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; n=3. KLF5, Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein.

system (21,22). The earliest pathological change that can be detected in AS lesions is the focal infiltration of circulating lipoprotein particles into the subendothelial layer following physicochemical modification (23). Vascular lumen obstruction may be caused by erosion of the surface intima (24), which may be triggered by endothelial cell apoptosis, local endothelial denudation and thrombosis (25). The aforementioned studies demonstrated that vascular endothelial cell dysfunction is an important factor leading to AS progression and adverse outcomes. HUVEC cells are often used as the cellular model of AS in previous studies (26-28). In the present study, OX-LDL induced inflammation and injury, and KLF5 interference inhibited the inflammatory response and improved the function of HUVECs induced by OX-LDL.

There is increasing evidence to indicate that lncRNAs can regulate vascular remodeling, lipid metabolism and the inflammatory response (29-31). A previous study indicated that KLF5 transcription factor, promoted the transcription of LINC00346 (32). Current research on LINC00346 focuses on its role in multiple tumors. LINC00346 is highly expressed in gastric cancer and is considered a tumor inducer *in vitro* and *in vivo* (32). In pancreatic cancer, LINC00346 expression has been found to be increased in tumor tissue samples, while the knockdown of LINC00346 suppresses pancreatic cancer cell proliferation, migration, invasion and tumor formation (33). LINC00346 has been shown to be upregulated in tissues and hepatocellular carcinoma cell lines, and LINC00346 may promote the viability, proliferation, migration and invasion of hepatocellular carcinoma cells (34). LINC00346 has been observed to be overexpressed in colorectal cancer tissues and cell lines, and the inhibition of LINC00346 impairs the proliferative, migratory, and invasive abilities of colorectal cancer cells (35). The present study explored the role of LINC00346 in AS. LINC00346 expression was increased in OX-LDL-stimulated HUVECs, and LINC00346 interference resulted in the suppression of OX-LDL-induced inflammation, decreased of OX-LDL-induced injury and the downregulation of KLF5 expression in HUVECs.

In recent years, studies have demonstrated that miRNAs are involved in AS lesions, and AS lesions also cause miRNA changes *in vivo*. miRNAs are more than clinically detectable lesion indicators and may also represent possible novel future therapeutic targets. A recent study on miRNA expression profiles in AS demonstrated that miR-21 expression was found to be significantly increased (36). AS-related inflammation has been found to be inhibited by the expression of miR-146 in macrophages, endothelial cells and hematopoietic cells (37-39). miR-126 is expressed in vascular endothelial cells participating in the inflammatory process of AS, inhibits inflammatory factors, promotes the autophagy of endothelial cells, and thus plays an anti-AS role in maintaining vascular integrity (40,41). In the present study, it was confirmed that LINC00346 could bind to miR-148a-3p, and that it could

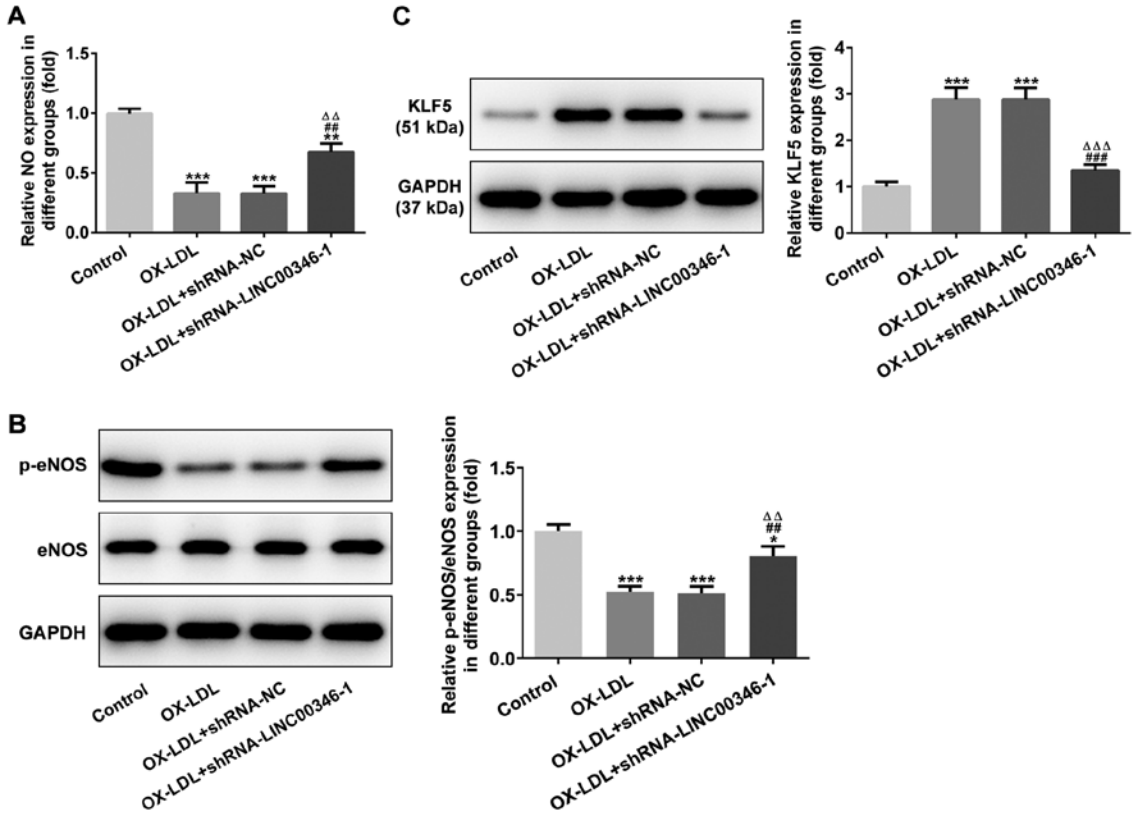

Figure 6. LINC00346 interference inhibits functional injury to HUVECs induced by OX-LDL and KLF5 expression. (A) The NO level in the supernatant of HUVECs following OX-LDL induction and shRNA-LINC00346 transfection was detected using a NO kit. (B) KLF5 expression in HUVECs following OX-LDL induction and shRNA-LINC00346 transfection was examined by western blot analysis. (C) The expression of p-eNOS/eNOS in HUVECs following OX-LDL induction and shRNA-LINC00346 transfection was examined by western blot analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. OX-LDL; $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; n=3. KLF5, Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

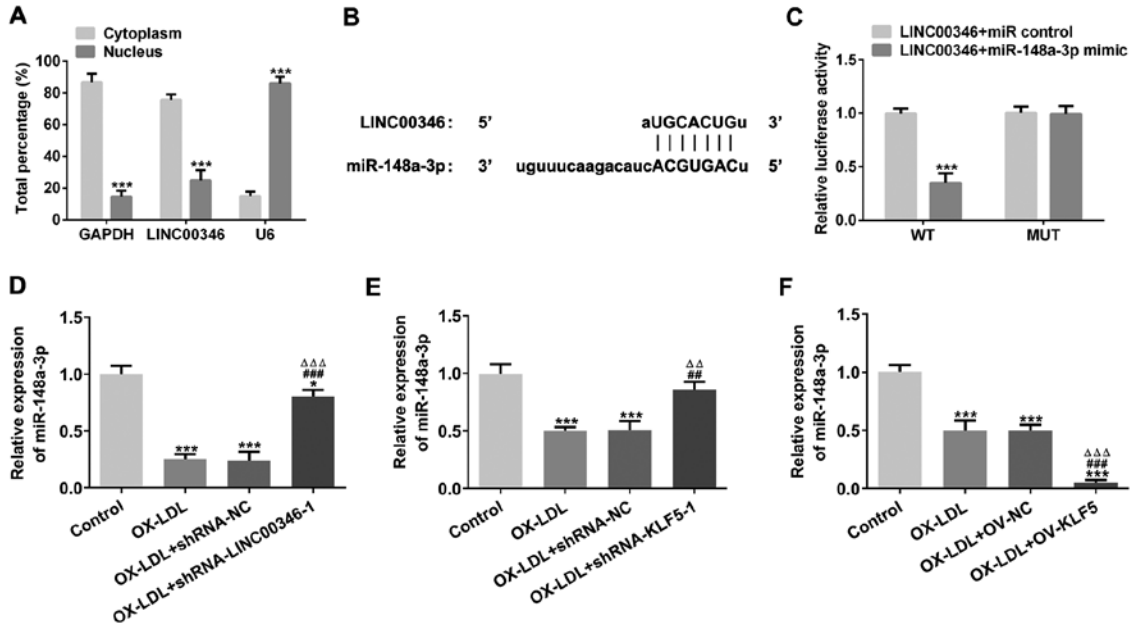

Figure 7. LINC00346 functions as a sponge of miR-148a-3p. (A) LINC00346 expression in cytoplasm and nucleus was detected by RT-PCR analysis. ***P<0.001 vs. cytoplasm. (B) Binding sites between LINC00346 and miR-148a-3p. (C) Detection of luciferase activity. ***P<0.001 vs. LINC00346 + miR control. (D) miR-148a-3p expression in HUVECs following OX-LDL induction and shRNA-LINC00346 transfection was detected by RT-PCR analysis. *P<0.05 and ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC. (E) miR-148a-3p expression in HUVECs following OX-LDL induction and shRNA-KLF5 transfection was detected by RT-PCR analysis. ***P<0.001 vs. control; $^{\#\#}$P<0.01 vs. OX-LDL; $^{\Delta\Delta}$P<0.01 vs. OX-LDL + shRNA-NC. (F) miR-148a-3p expression in HUVECs following OX-LDL induction and OV-KLF5 transfection was detected by RT-PCR analysis. ***P<0.001 vs. control; $^{\#\#\#}$P<0.001 vs. OX-LDL; $^{\Delta\Delta\Delta}$P<0.001 vs. OX-LDL + shRNA-NC; n=3. KLF5, Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein.

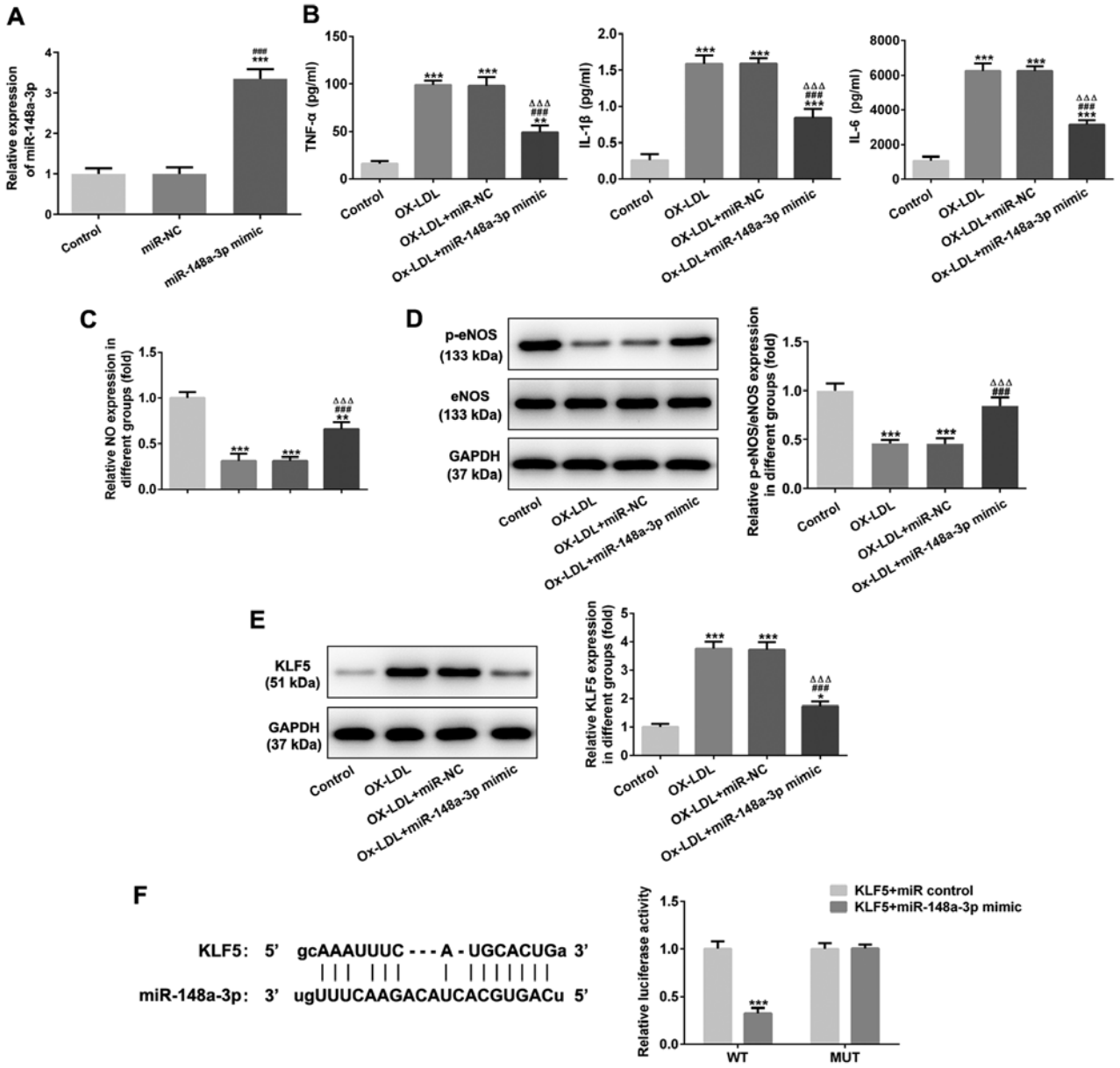

Figure 8. miR-148a-3p overexpression inhibits the expression of inflammatory factors and functional injury to HUVECs induced by OX-LDL and miR-148a-3p targets KLF5. (A) miR-148a-3p expression in HUVECs transfected with miR-148a-3p mimic was detected by RT-PCR analysis. ***P<0.001 vs. control; $^{###}$P<0.001 vs. miR-NC. (B) The levels of TNF-α, IL-1β and IL-6 in the supernatant of HUVECs following OX-LDL induction and miR-148a-3p mimic transfection were determined by ELISA. (C) The NO level in the supernatant of HUVECs following OX-LDL induction and miR-148a-3p mimic transfection was determined using a NO kit. (D) The expression levels of p-eNOS and eNOS in HUVECs following OX-LDL induction and miR-148a-3p mimic transfection were examined by western blot analysis. (E) KLF5 expression in HUVECs following OX-LDL induction and miR-148a-3p mimic transfection were examined by western blot analysis. (F) The binding sites between KLF5 and miR-148a-3p, and detection of luciferase activity. *P<0.05, **P<0.01 and ***P<0.001 vs. control; $^{###}$P<0.001 vs. OX-LDL; ^{ΔΔΔ}P<0.001 vs. OX-LDL + miR-NC; n=3. Krüppel-like factor 5; HUVECs, human umbilical vein endothelial cells; OX-LDL, oxidized low-density lipoprotein; NO, nitric oxide; eNOS, endothelial nitric oxide synthase.

also bind to KLF5. miR-148a-3p expression has been shown to be highly expressed in patients with AS in comparison with healthy individuals, and miR-148a-3p overexpression promotes proliferation and migration, whereas on the other hand it suppresses endothelial cell apoptosis (42). Furthermore, miR-148a-3p overexpression inhibited OX-LDL-induced inflammatory factors expression and injury in HUVECs; the corresponding findings of the present study were consistent with those of the aforementioned previous study.

In conclusion, the present study demonstrated that the expression of KLF5 and LINC00346 was increased in AS patient serum. Additionally, KLF5 interference inhibited inflammation and attenuated injury to HUVECs stimulated with OX-LDL, which was also suppressed by LINC00346 interference. In addition, OX-LDL-induced inflammatory factor expression and injury in HUVECs were also impaired by miR-148a-3p overexpression. The KLF5/LINC00346/miR-148a-3p axis may therefore enhance current understanding of AS pathogenesis and may represent potential targets for the development of novel therapeutics for cardiovascular diseases.

However, there are also some limitations to the present study. OX-LDL through lectin-type oxidized LDL receptor 1 (LOX-1), may cause an increase in leukocyte

adhesion molecules, the activation of apoptotic pathways and an increase in reactive oxygen species in endothelial cells and cause endothelial dysfunction (43). OX-LDL and LOX-1, in combination, play a role in the pathogenesis of atherosclerosis. The authors aim to explore the association between KLF5/LINC00346/miR-148a-3p loop and LOX-1 in future studies. In addition, the effects of disease duration, serum lipids such as cholesterol and medication on the expression of KLF5 were not investigated in the present study; thus, this is also a future research objective. Furthermore, more advanced techniques are required, in order to elucidate further the possibility of KLF5 originating also from blood cells.

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Availability of data and materials

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

Authors' contributions

FW and YJ conceived and designed the study. FW, JG, SH and CZ performed the experiments. FW, ZS, YS and YX analyzed the data. FW wrote the manuscript. YJ revised the manuscript. All authors gave read and approved the final manuscript. FW, JG and SH are responsible for confirming the authenticity of the raw data.

Ethics approval and consent to participate

Approval for the study was obtained from the Ethics Committee of Changzhou No. 2 People's Hospital, Affiliated Nanjing Medical University. Informed consent and relevant clinical information were obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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