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Astragaloside IV inhibits oxidized low-density lipoprotein-induced endothelial damage via upregulation of miR-140-3p

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Abstract. Oxidized low-density lipoprotein (ox-LDL)-mediated endothelial cell injury has an important role in the vascular complications of type 2 diabetes. Astragaloside IV (ASV) is an active component of *Radix Astragali*, which has been demonstrated to exert protective effects against endothelial damage. The present study explored whether microRNAs (miRNAs) are involved in mediating the protective effects of ASV on ox-LDL-induced damage in human umbilical vein endothelial cells (HUVECs). RNA sequencing and reverse transcription-quantitative PCR analyses revealed that ox-LDL treatment significantly downregulated miR-140-3p expression in HUVECs. miR-140-3p overexpression promoted cell proliferation and inhibited apoptosis in ox-LDL-induced HUVECs. However, inhibition of miR-140-3p expression could reverse the effects of ASV on ox-LDL-induced HUVECs and reactivate ASV-inhibited PI3K/Akt signaling in ox-LDL-induced HUVECs. In addition, Krüppel-like factor 4 (KLF4) was identified as a target of miR-140-3p in ox-LDL-treated HUVECs. Subsequent experiments revealed that KLF4 overexpression partially counteracted the protective effects of miR-140-3p or ASV treatment in ox-LDL-induced HUVECs. Taken together, the current findings demonstrated that the protective effects of ASV on HUVECs were dependent on miR-140-3p upregulation and subsequent inhibition of KLF4 expression, which in turn suppressed the PI3K/Akt signaling pathway. The present results shed light to the molecular mechanism by which ASV alleviated ox-LDL-induced endothelial cell damage.

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Abbreviations: Akt, Akt serine/threonine kinase; ASV, astragaloside IV; HUVECs, human umbilical vein endothelial cells; KLF4, Krüppel-like factor 4; ox-LDL, oxidized low-density lipoprotein; PI3K, phosphatidylinositol 3-kinase

Key words: endothelial cells, oxidized low-density lipoprotein, astragaloside IV, miR-140-3p, Krüppel-like factor 4

Introduction

Diabetes mellitus is a metabolic disorder characterized by consistent hyperglycemia and is a global public health concern (1). The vascular complications of type 2 diabetes can lead to microvascular and macrovascular damage and are major causes of disability and death in type 2 diabetes patients (2,3). Endothelial dysfunction renders diabetics vulnerable to limb infections and end-organ damage, such as nephropathy, neuropathy and retinopathy (4). In addition, diabetic macrovascular disease resembles atherosclerotic lesions both morphologically and functionally (5), and oxidized low-density lipoprotein (ox-LDL) is a key component involved in the genesis of atherosclerotic lesions and is cytotoxic to various cell types, such as endothelial cells (ECs), and is therefore suggested to contribute to endothelial dysfunction (6). Therefore, preventing ox-LDL-induced endothelial injury has received considerable attention as a potential therapeutic target for the treatment of diabetic vascular complications (7).

Astragaloside IV (ASV), known as a purified small molecular saponin, is one of the main active components of *Radix Astragali* that possesses comprehensive biological properties, including anti-inflammatory, immunoregulatory, antioxidant

and antiaging properties (8-10). Our previous findings demonstrated that ASV significantly inhibited epithelial-mesenchymal transition induced by transforming growth factor-β1 during the progression of lung fibrosis (11). In addition, previous findings suggested that ASV induced vasodilation by regulating nitric oxide production in the endothelium (12) and it improvesd vascular endothelial dysfunction induced by hyperglycemia via the toll-like receptor 4/nuclear factor (NF)-κB signaling pathway (13). Although the protective effects of ASV on endothelial dysfunction have been reported, the detailed molecular mechanisms remain unclear. microRNAs (miRNAs) have been proposed to serve crucial roles in diverse pathophysiological processes, including endothelial injury (14). A previous study demonstrated that miR-26a expression was downregulated in atherosclerotic mice and ox-LDL-stimulated human aortic ECs, and miR-26a overexpression prevented ox-LDL-induced EC apoptosis (15). In addition, Yin *et al* (16) reported that miR-338-3p downregulation increased cell viability and inhibited cell apoptosis in ox-LDL-induced human umbilical vein endothelial cells (HUVECs). The above findings indicated the involvement of miRNAs in regulating ox-LDL-induced EC damage. However, further studies are required to elucidate the involvement of miRNAs in mediating the protective effects of ASV on ox-LDL-induced ECs.

Based on the above results, the present study conducted RNA sequencing (RNA-Seq) analysis to screen for dysregulated miRNAs in HUVECs under ox-LDL stimulation. Next, the effect of miR-140-3p, one of the most strongly downregulated miRNAs induced by ox-LDL, in the protective role of ASV in ox-LDL-induced HUVEC apoptosis was explored. The mechanisms underlying the effects of ASV in HUVECs were also investigated.

Materials and methods

Cell culture and reagents. HUVECs (Clonetics; Lonza Group, Ltd.) were incubated in Dulbecco's modified Eagle medium (DMEM; HyClone; GE Healthcare Life Sciences) with 5 mM glucose, 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin, and 100 mg/ml streptomycin (Beyotime Institute of Biotechnology). Cells were incubated at 37˚C with 5% CO_2 in an incubator (Thermo Fisher Scientific, Inc.). ASV (Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA). HUVECs were treated with 100 μg/ml ox-LDL (Beijing Solarbio Science & Technology Co., Ltd.) for varying incubation periods (6, 12 and 24 h), as previously described (17,18).

Cell transfection. An overexpression vector (pcDNA3.1/+) containing the human Krüppel-like factor 4 (KLF4) gene and an empty pcDNA3.1/+vector were purchased from Guangzhou RiboBio Co., Ltd. miR-140-3p mimics (5'-UACCACAGGGUA GAACCACGG-3'), miR-negative control (miR-NC; 5'-UGC AAGCACGAAUUAAUUGGCG-3'), miR-140-3p inhibitors (5'-CCGUGGUUCUACCCUGUGGUA-3'), as well as inhibitor control (5'-UGACCGAUCGUACUUAUAGUCUG-3'), were purchased form Guangzhou RiboBio Co., Ltd. Cells were cultured in six-well plates at the density of $2x10^5$ cells/well. A total of 200 nM pcDNA3.1-KLF4 plasmid or empty vector pcDNA3.1 was transiently transfected into HUVECs using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). miR-140-3p mimics, miR-NC, miR-140-3p inhibitors and control inhibitors (50 nM) were transiently transfected into HUVECs using Lipofectamine 2000, according to manufacturer's instructions. Changes in mRNA and protein expression levels were assessed at 24 h post-transfection.

RNA-seq. Normal HUVECs and ox-LDL-stimulated HUVECs were analyzed using RNA-seq. Four separate samples were prepared for each group. Total RNA was isolated from cells using RNAios Plus reagent (Takara Bio, Inc.) and purified using the RNeasy Plant Mini kit (Qiagen GmbH). Sequencing was performed at Guangzhou RiboBio Co., Ltd. Total RNA was sequenced on the Illumina HiSeq 2500 system. RNA-seq reads were aligned to the human transcriptome (UCSC, hg19) using bowtie (http://bowtie-bio.sourceforge.net/index.shtml) and RSEM (https://deweylab.github.io/RSEM/), as previously described (19). $P<0.05$ was considered statistically significant.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). miR-140-3p levels and KLF4 mRNA expression levels were analyzed by RT-qPCR. Total RNA was isolated from HUVECs using RNAios Plus reagent (Takara Bio, Inc.), according to the manufacturer's protocol. To evaluate miR-140-3p expression, cDNA was synthesized from total RNA using a miScript Reverse Transcription kit (Qiagen GmbH). Subsequently, qPCR was conducted using a TaqMan® MicroRNA Reverse Transcription kit (Qiagen GmbH). To measure KLF4 mRNA expression, total RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent kit (Takara Bio, Inc.). Next, qPCR was performed using SYBR Premix Ex Taq™ (Takara Bio, Inc.), following the manufacturer's instructions. Relative miR-140-3p levels and KLF4 mRNA expression levels were normalized to those of U6 RNA and β-actin, respectively. The following primers were designed: miR-140-3p, forward 5'-ACACTCCAGCTGGGAGGCGGG GCGCCGCGGGA-3' and reverse 5'-CTCAACTGGTGT CGTGGA-3'; U6, forward 5'-CTCGCTTCGGCAGCACA-3' and reverse 5'-AACGCTTCACGAATTTGCGT-3'; KLF4, forward 5'-GAACTCACACAGGCGAGAAA-3' and reverse 5'-GAACTCACACAGGCGAGAAA-3'; and β-actin, forward 5'-ATTTCTGAATGGCCCAGGT-3' and reverse 5'-CTGCCT CAACACCTCAACC-3'. The thermocycling conditions were 95˚C for 5 min, followed by 35 cycles of 95˚C for 5 sec, 60˚C for 30 sec and 70˚C for 10 sec. Relative miRNA levels and mRNA expression levels were determined using the $2^{-\Delta\Delta Cq}$ method (20).

Cell proliferation assay. Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc.). Following transfection, HUVECs ($5x10^4$/ml) were seeded into 96-well plates and allowed to grow for 0, 1, 2 and 3 days. The experiment was repeated thrice. Five parallel wells were set in each group after incubation. CCK-8 reagent (10 μl) was added to each well and incubated at 37˚C with 5% CO_2 for another 2 h. Finally, the optical density of each well was measured at 450 nm using a microplate reader (model 680; Bio-Rad Laboratories, Inc.).

Apoptosis analysis. Cell apoptosis was evaluated using a fluorescein isothiocyanate (FITC)-conjugated Annexin V Apoptosis Detection kit I (BD Biosciences). Following cell transfection, HUVECs were seeded into six-well plates and exposed to ox-LDL for 24 h. Apoptotic cells were analyzed using FACScan (BD Biosciences) with CellQuest software version 0.9.3.1 (BD Biosciences).

Luciferase reporter assay. The 3' untranslated region (UTR) fragments of KLF4 containing the predicted binding sites of miR-140-3p were synthesized and cloned into the psiCHECK-2 dual luciferase reporter plasmid (Promega Corporation); this reporter plasmid was designated KLF4 wild-type. Mutation in the putative miR-140-3p target sequences in the 3'UTR of KLF4 was generated using a site-directed gene mutagenesis kit (Takara Bio, Inc.); this reporter plasmid was designated KLF4 mutant-type. For the luciferase reporter assay, HUVECs were seeded into six-well plates and co-transfected with 200 ng KLF4 wild-type or KLF4 mutant-type and 100 nM miR-NC or miR-140-3p mimics using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) reagent, following with the manufacturer's protocol. After 24 h, the cell lysates were assayed for luciferase activity using the Luciferase Assay System (Promega Corporation). Luciferase activity was measured on a luminescence counter (Centro XS3 LB 960; Berthold Technologies). The relative luciferase activity was expressed as the ratio of firefly luciferase to *Renilla* luciferase activity.

Western blotting. Following treatment, HUVECs were collected and lysed using 1% RIPA lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitors (Roche Diagnostics). The protein concentration was quantified using a bicinchoninic acid kit (Beijing Solarbio Science & Technology Co., Ltd.), and equal amounts of proteins (20 μg) were size-fractionated by 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (EMD Millipore). Following blocking with 5% nonfat skim milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies against KLF4 (1:1,000; cat. no. ab215036; Abcam), phosphorylated (p-) PI3K (1:1,000; Tyr458/Tyr199; cat. no. 4228; Cell Signaling Technology, Inc.), PI3K (1:1,000; cat. no. 4249; Cell Signaling Technology, Inc.), p-Akt (1:1,000; Ser/Thr; cat. no. 9611; Cell Signaling Technology, Inc.), Akt (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.), and β-actin (1:1,000; cat. no. ab8226; Abcam). Then, the membranes were incubated with the corresponding secondary horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. ab6721; Abcam) at room temperature for 1 h. Signals were visualized with the enhanced chemiluminescence detection reagents (EMD Millipore), and the band intensities were quantified using Quantity One software version 4.62 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All results were presented as the mean ± standard deviation of three independent experiments. Differences among groups were analyzed by one-way ANOVA, followed by Tukey's test using SPSS version 19.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-140-3p promotes proliferation and inhibits apoptosis in HUVECs. To determine the expression profiles of miRNAs in ox-LDL-induced HUVECs, RNA-Seq analysis was performed to screen for differentially expressed miRNAs with or without 100 μg/ml ox-LDL stimulation for 24 h. The miRNA expression profiles of the ox-LDL-exposed group compared with the control group were visualized using a volcano plot (Fig. 1A) and heat map (Fig. 1B). The analysis identified 120 dysregulated miRNAs, including 60 downregulated miRNAs and 60 upregulated miRNAs, using a cutoff of fold change >2. To investigate the in-depth function of the dysregulated miRNAs, the most strongly downregulated miRNA, miR-140-3p, was selected as a target for validation of the RNA-Seq results. Subsequently, RT-qPCR results confirmed that ox-LDL stimulation downregulated miR-140-3p expression in HUVECs in a time-dependent manner (Fig. 1C). Therefore, the role of miR-140-3p in ox-LDL-induced HUVECs was further investigated. Transfection of HUVECs with miR-140-3p mimics results in a significant increase in miR-140-3p levels, as detected by RT-qPCR (Fig. 1D). Results of the CCK-8 assay revealed that ox-LDL stimulation suppressed the proliferation of HUVECs, whereas miR-140-3p overexpression reversed the inhibitory effects of ox-LDL on cell proliferation (Fig. 1E). Results of flow cytometry assay in Fig. 1F and G demonstrated that ox-LDL-induced HUVECs had significantly higher apoptosis rates compared to those of the control group. However, miR-140-3p overexpression decreased the apoptotic cell rate compared with those of the ox-LDL + miR-NC group (Fig. 1F and G). The current findings suggested that miR-140-3p promoted cell proliferation and inhibited the apoptosis of ox-LDL-induced HUVECs.

ASV relieves ox-LDL-induced HUVECs apoptosis by upregulating miR-140-3p expression and suppressing the PI3K/Akt pathway. Considering that miR-140-3p regulates cell proliferation and apoptosis in ox-LDL-induced HUVECs and that ASV is likely to directly influence miRNA expression levels, the hypothesis that ASV protected HUVECs from ox-LDL by miR-140-3p regulation was investigated next. Results demonstrated that ASV did not affect miR-140-3p levels under normal conditions, but it significantly upregulated miR-140-3p levels in a concentration-dependent manner under ox-LDL stimulation (Fig. 2A). To determine whether miR-140-3p is required for ASV-induced changes in HUVECs, miR-140-3p levels were downregulated by transfecting HUVECs with miR-140-3p inhibitors, and the transfection efficiency was validated via RT-qPCR (Fig. 2B). Based on the observed effects of ASV on miR-140-3p expression (Fig. 2A), the dose of 100 μM ASV was selected for the subsequent experiments. Results demonstrated that miR-140-3p inhibition reversed the protective effects of ASV on ox-LDL-induced proliferation of HUVECs (Fig. 2C). Results of apoptosis assay revealed that ASV significantly inhibited ox-LDL-triggered apoptosis (Fig. 2D and E). However, inhibition of miR-140-3p expression partly reversed

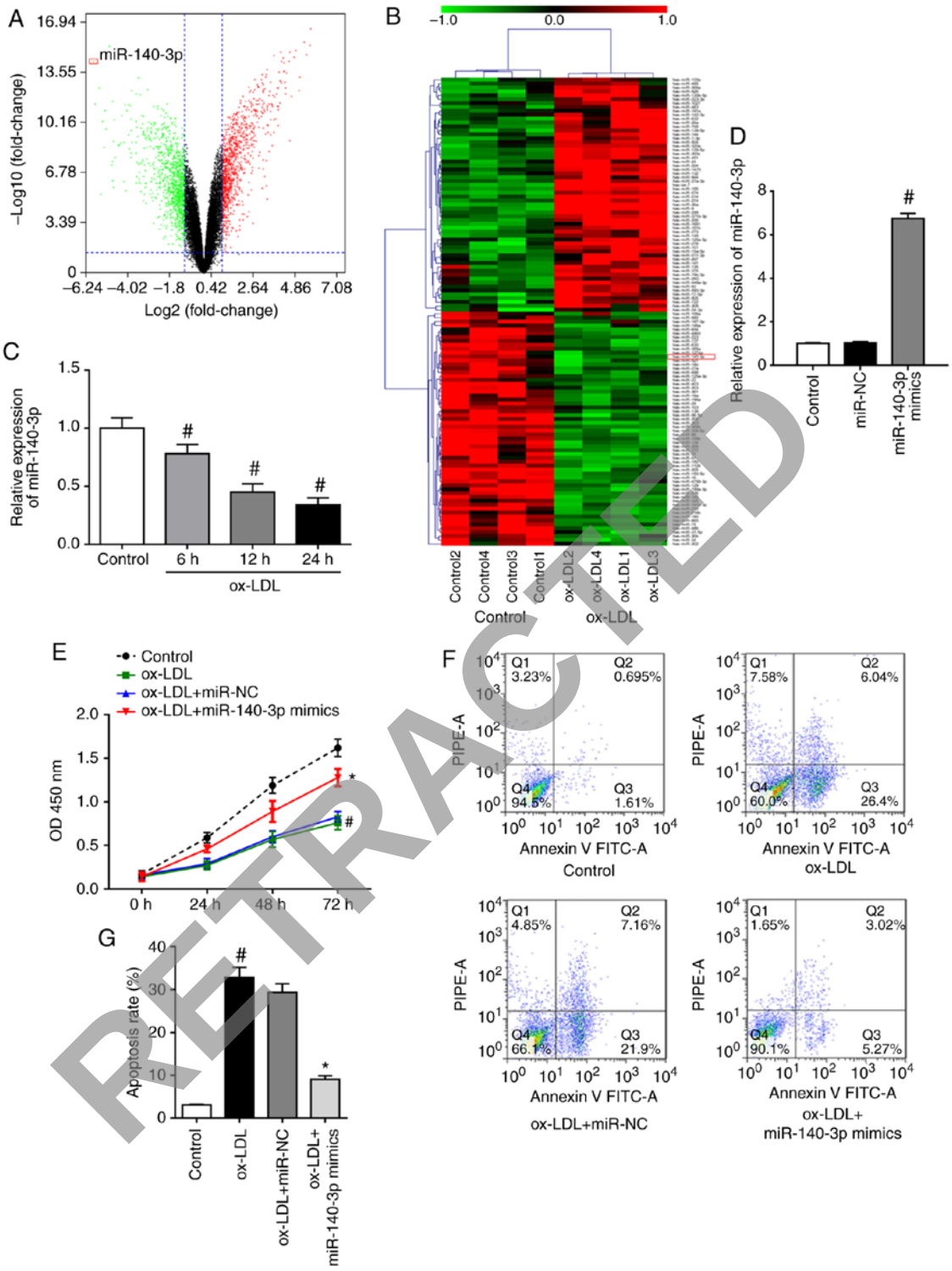

Figure 1. miR-140-3p overexpression promotes proliferation and inhibits apoptosis in ox-LDL induced HUVECs. (A) Volcano plot showing the differential expression of all miRNAs following RNA sequencing analysis. (B) Heat map and clustering analysis identified 120 significantly dysregulated miRNAs, including 60 downregulated miRNAs and 60 upregulated miRNAs at fold change >2 (P<0.05). Red indicates upregulation and green indicates downregulation. (C) miR-140-3p levels were analyzed by RT-qPCR in HUVECs treated with 100 μg/ml ox-LDL for 0, 6, 12 and 24 h (n=3). (D) miR-140-3p levels were analyzed by RT-qPCR following transfection of HUVECs with miR-NC or miR-140-3p mimics (n=3). (E) Cell proliferation of HUVECs was evaluated using Cell Counting Kit-8 agent, following overexpression of miR-140-3p with or without ox-LDL treatment (n=3). (F) Representative plots and (G) quantification of flow cytometry analysis to detect cell apoptosis in HUVECs. Three independent experiments were conducted. Data are presented as the mean ± standard deviation of at least three experiments. #P<0.05 vs. control; *P<0.05 vs. ox-LDL+miR-NC. ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control.

the effects of ASV on HUVECs (Fig. 2D and E). In addition, ASV treatment suppressed the ox-LDL-mediated activation of the PI3K/Akt pathway in HUVECs, as evidenced by the increased levels of phosphorylated PI3K and phosphorylated Akt (Fig. 2F-H). Inhibition of miR-140-3p rescued the ASV-induced downregulation of p-PI3K and p-Akt levels

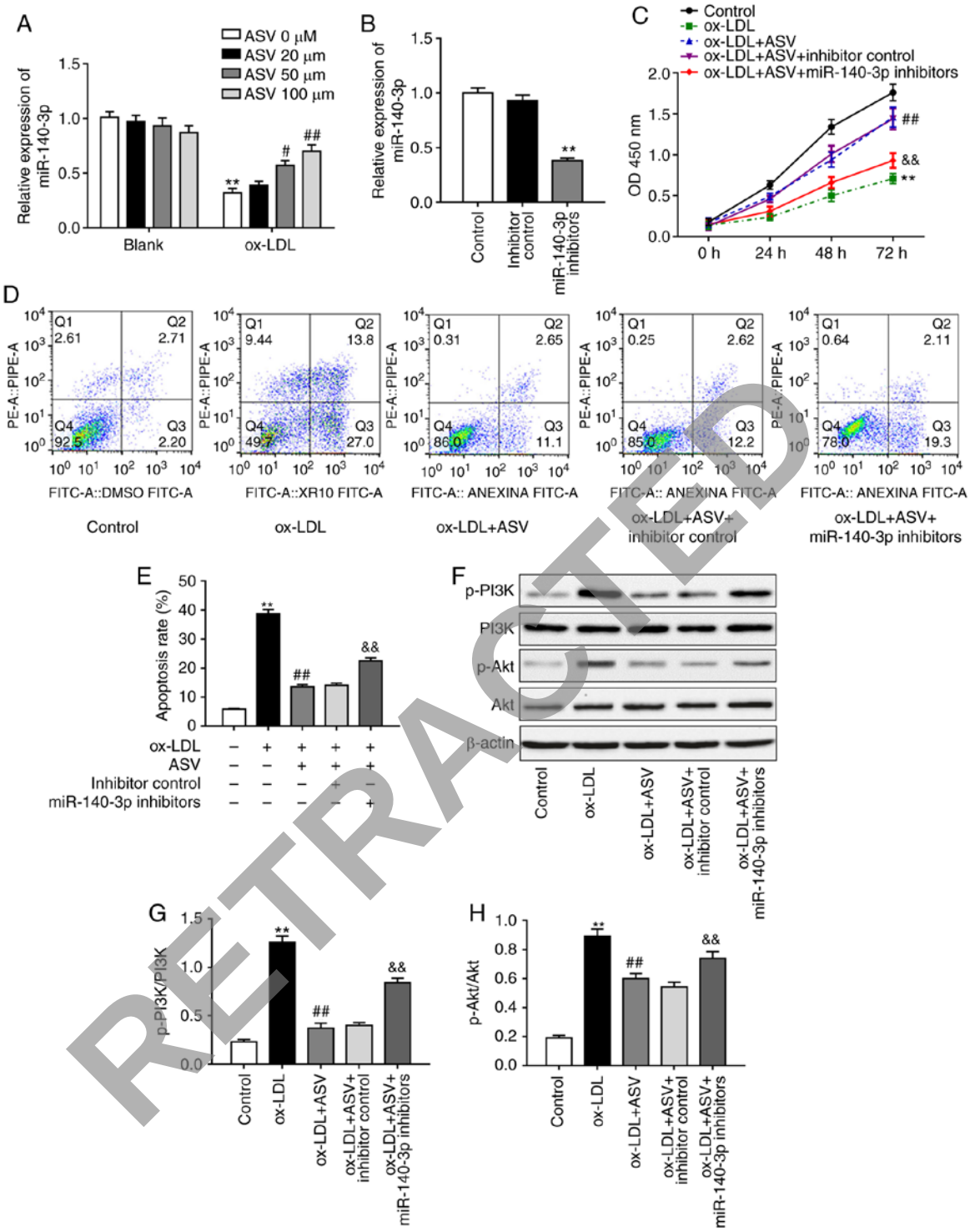

Figure 2. ASV protects HUVECs from ox-LDL-induced damage by upregulating miR-140-3p expression. (A) miR-140-3p levels were analyzed by reverse transcription-quantitative PCR in HUVECs with or without ASV treatment (0, 20, 50, 100 μM for 24 h; n=3). **P<0.01 vs. blank; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. ox-LDL alone. (B) miR-140-3p levels in HUVECs transfected with miR-140-3p inhibitors or inhibitor control (n=3). **P<0.01 vs. inhibitor control. (C) Cell proliferation was evaluated using the Cell Counting Kit-8 agent in HUVECs transfected with miR-140-3p inhibitors or inhibitor control under ASV treatment (n=3). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. ox-LDL alone; $^{\&\&}$P<0.01 vs. ox-LDL+ASV+inhibitor control. (D) Representative plots and (E) quantification of flow cytometry analysis to detect cell apoptosis of ox-LDL-induced HUVECs following ASV treatment and/or miR-140-3p downregulation. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. ox-LDL alone; $^{\&\&}$P<0.01 vs. ox-LDL+ASV+inhibitor control. (F) Representative blots and (G) quantification of PI3K and (H) Akt phosphorylation levels in ox-LDL-induced HUVECs following ASV treatment and/or miR-140-3p downregulation. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. ox-LDL alone; $^{\&\&}$P<0.01 vs. ox-LDL+ASV+inhibitor control. ASV, astragaloside IV; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein; RT-qPCR, reverse transcription-quantitative PCR.

(Fig. 2F-H). These results indicated that ASV protected ox-LDL-induced HUVEC injury by regulating miR-140-3p expression and the PI3K/Akt pathway.

miR-140-3p exerts its function by regulating KLF4 in HUVECs. To elucidate the mechanisms underlying the role of miR-140-3p in the effects of ASV on ox-LDL stimulated

HUVECs, the bioinformatics tool StarBase v3.0 (http://starbase.sysu.edu.cn/) was used to predict the downstream target of miR-140-3p. The complementary binding sites within miR-140-3p and the 3'UTR of KLF4 are illustrated in Fig. 3A. Subsequently, a luciferase reporter assay confirmed the direct binding of miR-140-3p at the KLF4 3'UTR (Fig. 3B). RT-qPCR analysis revealed that KLF4 mRNA expression levels were upregulated in ox-LDL-induced HUVECs in a time-dependent manner (Fig. 3C). miR-140-3p overexpression caused significant downregulation of KLF4 expression in ox-LDL induced HUVECs both at the mRNA (Fig. 3D) and protein levels (Fig. 3E). Thus, the results indicated that KLF4 is a direct target of miR-140-3p in HUVECs. Furthermore, KLF4 overexpression using plasmid transfection significantly upregulated KLF4 expression, both at the mRNA (Fig. 3F) and protein levels (Fig. 3G). Subsequent experiments revealed that KLF4 overexpression reversed the changes induced by miR-140-3p mimics on ox-LDL-induced cell proliferation (Fig. 3H) and apoptosis (Fig. 3I and J) in HUVECs. Taken together, the present results suggested that miR-140-3p regulated cell proliferation and apoptosis in ox-LDL-induced HUVECs by regulating KLF4.

ASV influences ox-LDL-induced HUVEC damage via the KLF4-dependent PI3K/Akt pathway. The present study further investigated the involvement of KLF4 in the protective role of ASV on HUVECs under ox-LDL stimulation. Functional analyses revealed that KLF4 overexpression partially rescued the effects of ASV on ox-LDL-induced cell proliferation (Fig. 4A) and apoptosis (Fig. 4B and C). In addition, KLF4 overexpression increased p-PI3K, and p-Akt levels, although the total protein levels of PI3K and AKT in ASV-treated HUVECs under ox-LDL condition were not affected (Fig. 4D-F). These results confirmed that ASV regulated ox-LDL induced cell proliferation and apoptosis in HUVECs via the KLF4-dependent PI3K/Akt pathway.

Discussion

The present study focused on the molecular mechanism underlying the protective effects of ASV on HUVECs induced by ox-LDL. The current integrated analyses revealed that ASV alleviated ox-LDL-induced HUVEC apoptosis by upregulating miR-140-3p expression and subsequently inhibiting the KLF4/PI3K/Akt signaling pathway.

Emerging evidence suggests that ASV has a protective role against HUVEC injury. A previous study demonstrated that ASV promoted cell proliferation, reduced apoptosis, and downregulated the expression levels of tumor necrosis factor-α and interleukin-1β in HUVECs via inhibition of the JNK pathway (21). In addition, the findings of Ma *et al* (22) suggested that ASV inhibited inflammation induced by phorbol-12-myristate 13-acetate in HUVECs by reducing the phosphorylation levels of JNK and the p38 pathway. Furthermore, ASV could suppress hydrogen peroxide-induced oxidative stress by inhibiting the reactive oxygen species/NF-κB pathway and endothelial nitric oxide synthase uncoupling (23). The present results consistently demonstrated that ASV promoted cell proliferation and inhibited cell apoptosis of ox-LDL-induced HUVECs. However, further studies will be required to fully investigate the exact mechanism underlying the beneficial effects of ASV on HUVECs.

Recently, miRNAs have been reported to have an important role in HUVEC dysfunction (24-26). Multiple studies indicated that non-coding RNAs are also involved in the function of ASV on cell viability (27) and autophagy (28). However, there is currently no evidence on the involvement of miRNAs in anti-apoptosis action of ASV in ox-LDL induced HUVECs. To this end, the present study performed RNA-seq analysis to screen the potential miRNAs involved in ox-LDL induced EC injury. The present results demonstrated that ox-LDL significantly downregulated miR-140-3p expression in a time-dependent manner. A recent study demonstrated that ellagic acid could upregulate miR-140-3p expression and inhibit MAP kinase kinase 6 expression to inhibit apoptosis in cardiomyocytes (29). However, little is known regarding the role of miR-140-3p in the apoptosis of HUVECs. The present study first revealed that miR-140-3p overexpression effectively reversed ox-LDL-triggered cell apoptosis in HUVECs. In addition, ASV treatment was demonstrated to upregulate miR-140-3p expression in ox-LDL-induced HUVECs, and inhibition of miR-140-3p expression could reverse the protective effects of ASV on ox-LDL-induced damage in HUVECs. Although Rasheed *et al* (30) have reported that removal of epigallocatechin-3-O-gallate could upregulate miR-140-3p expression in chondrocytes, the present study is the first to provide evidence that ASV upregulates miR-140-3p expression to alleviate ox-LDL-mediated cell injury. Previous studies reported that ASV could inhibit the PI3K/Akt pathway to alleviate cell dysfunction (9,12,17,31-32). Additionally, a recent study identified that overexpression of miR-9-5p suppressed the PI3K/Akt pathway by inhibiting CXC chemokine receptor-4, thereby reducing high glucose-induced apoptosis in HUVECs (33). The present study identified that ASV suppressed ox-LDL stimulated activation of the PI3K/Akt pathway in HUVECs and inhibition of miR-140-3p could reactivate the PI3K/Akt pathway, which could promote apoptosis. However, the current study also found that Akt was a survival signaling, which helps to protect cells from various stimuli inducing cell death (34,35). Therefore, further studies are required to verify that ASV inhibits apoptosis via Akt suppression. Another limitation of the current study is that only one of the most dysregulated miRNAs during ox-LDL-mediated ECs injury was confirmed and investigated; further experiments are needed to fully identify other specific miRNAs involved in EC damage.

Biological analysis and luciferase reporter assay identified KLF4 as a target of miR-140-3p in ox-LDL-stimulated HUVECs. KLF4 is a member of the Krüppel-like family of transcription factors, which serve important roles in regulating endothelial biology (36). KLF4 has been demonstrated as a downstream effector of ERK5, which contributes to the protection of endothelial cells from oxidative stress-induced cell apoptosis (37). Given the importance of KLF4 in endothelial protection, analyzing the expression profiles in injured HUVECs and the downstream signaling pathways is of significant interest. Recent studies demonstrated that KLF4 overexpression reduced cell viability and increased the proportion of apoptotic cells (38,39). By contrast, another study by Yang *et al* (40) suggested that KLF4 protected cells

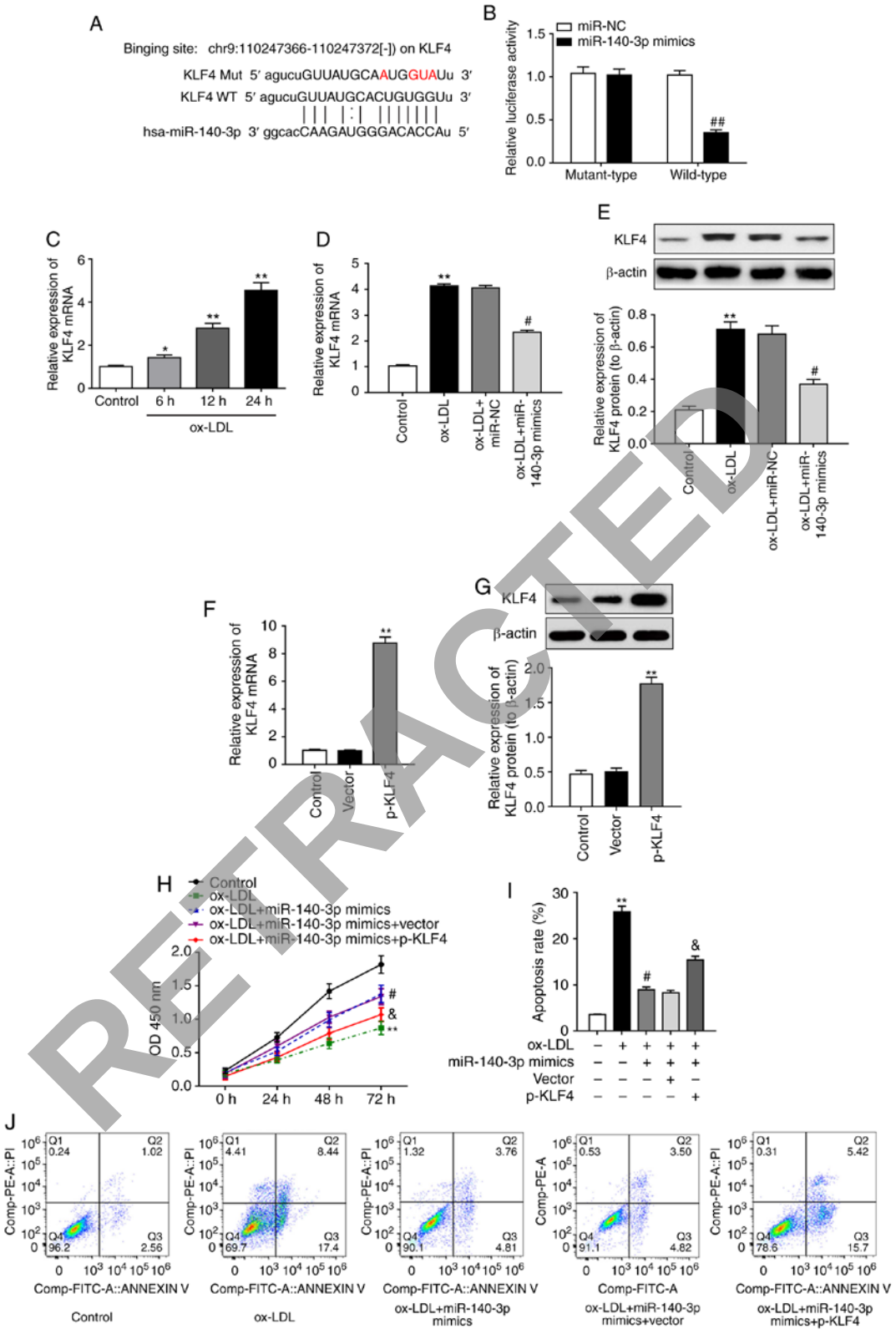

Figure 3. miR-140-3p upregulation promotes cell proliferation and inhibits cell apoptosis by targeting KLF4 in ox-LDL-induced HUVECs. (A) The predicted binding sites between miR-140-3p and KLF4, by using the bioinformatics tool Starbase v3.0. (B) Relative luciferase activity in HUVECs transfected with miR-140-3p mimic or miR-NC together with luciferase reporter constructs containing either the wide-type or mutant-type 3' untranslated region of KLF4. $^{##}$P<0.01 vs. miR-NC. (C) Relative mRNA expression levels of KLF4 in HUVECs stimulated with 100 μg/ml ox-LDL for 0, 6, 12, and 24 h (n=3). *P<0.05 and **P<0.01 vs. control. (D) Relative mRNA expression levels of KLF4 and (E) KLF4 protein expression levels in ox-LDL-induced HUVECs with or without miR-140-3p overexpression (n=3). **P<0.01 vs. control; $^{\#}$P<0.05 vs. ox-LDL+miR-NC. (F) Relative mRNA expression levels of KLF4 in HUVECs transfected with pcDNA (empty vector control) or pcDNA-KLF4 at 24 h post-transfection (n=3). **P<0.01 vs. control. (G) Relative protein expression levels of KLF4 in HUVECs transfected with pcDNA or pcDNA-KLF4 at 24 h post-transfection (n=3). **P<0.01 vs. control. (H) Cell proliferation was evaluated using Cell Counting Kit-8 agent in HUVECs transfected with miR-140-3p mimics or pcDNA-KLF4 (n=3). **P<0.01 vs. control; $^{\#}$P<0.05 vs. ox-LDL alone; and $^{\&}$P<0.05 vs. ox-LDL+miR-140-3p mimics. (I) Representative plots and (J) quantification of flow cytometry analysis to detect cell apoptosis in HUVECs. Data are presented as the mean ± standard deviation of at least three independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05 vs. ox-LDL alone; and $^{\&}$P<0.05 vs. ox-LDL+miR-140-3p mimics. KLF4, Krüppel-like factor 4; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; NC, negative control.

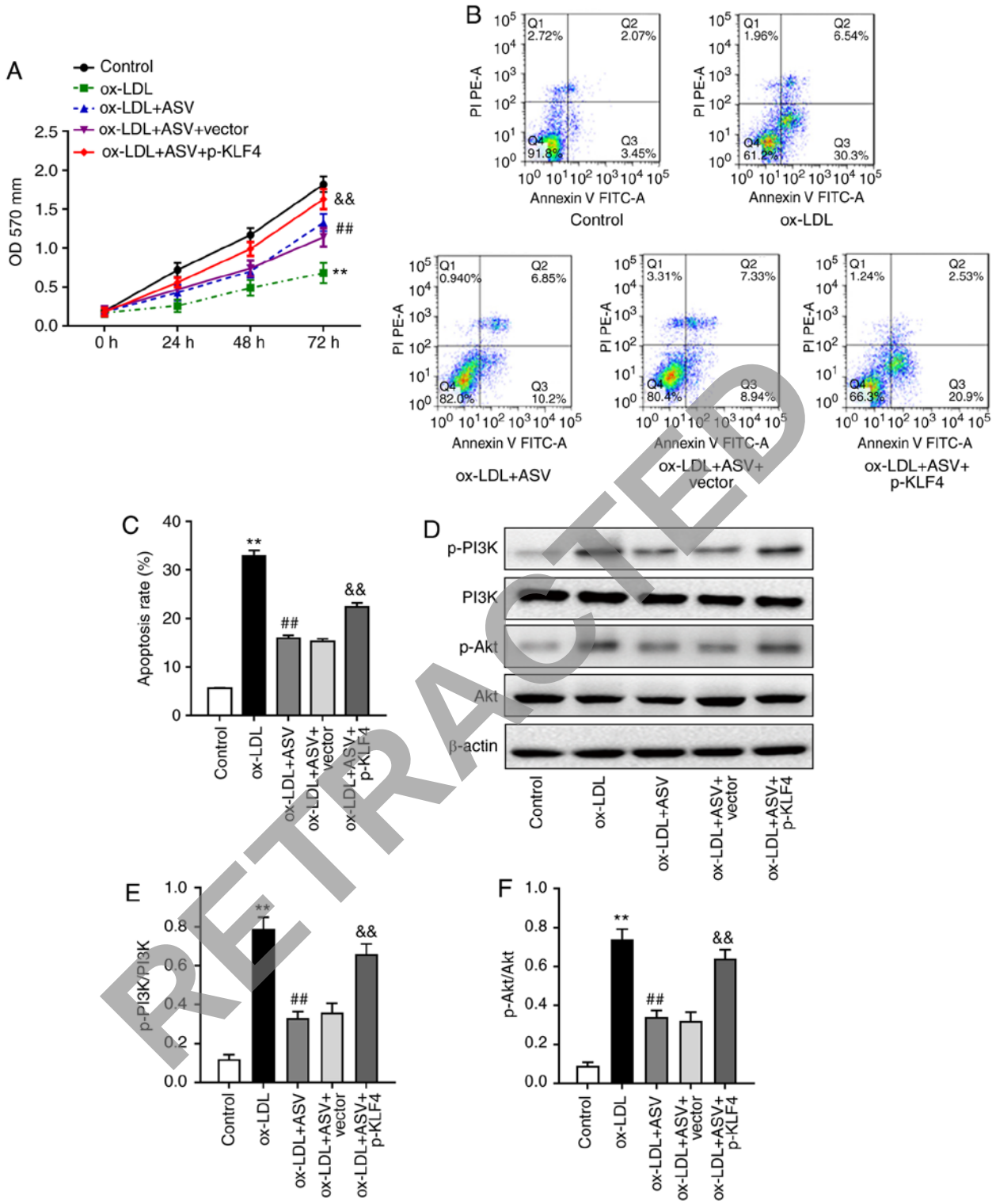

Figure 4. KLF4 overexpression reverses the protective effects of ASV on ox-LDL-induced HUVECs. (A) Cell viability was assessed by Cell Counting Kit-8 assay in ox-LDL-induced HUVECs following ASV treatment and/or KLF4 overexpression (n=3). (B) Representative plots and (C) quantification of flow cytometry analysis to detect cell apoptosis in ox-LDL-induced HUVECs following ASV treatment and/or KLF4 overexpression (n=3). (D) Representative blots and (E) quantification of PI3K and (F) Akt phosphorylation levels in ox-LDL-induced HUVECs following ASV treatment and/or KLF4 overexpression. Data are presented as the mean ± standard deviation of at least three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. ox-LDL alone; $^{\&\&}$P<0.01 vs. ox-LDL+ASV+vector. KLF4, Krüppel-like factor 4; ASV, astragaloside IV; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells.

from ischemic stroke-induced apoptosis via transcriptional activation of metastasis associated lung adenocarcinoma transcript 1. In the present study, results indicated that ox-LDL treatment caused a significant upregulation of KLF4 expression, whereas miR-140-3p overexpression reduced KLF4 expression levels in ox-LDL-induced HUVECs. In addition, restoration of KLF4 levels could reverse the anti-apoptosis effect of miR-140-3p overexpression and

ASV treatment in HUVECs. The conflicting role of KLF4 in cell proliferation and apoptosis could be attributed to the different types of cells and stimulatory conditions, and further studies will be required to determine the role of KLF4 in the pathology of diseases. In addition, KLF4 has been identified as a regulator of the PI3K/Akt signaling pathway in cancer cells (41,42). Similarly, the present results revealed that KLF4 activated the PI3K/Akt signaling pathway in ox-LDL-induced HUVECs. Taken together, these results revealed that miR-140-3p regulated ox-LDL-induced EC injury by targeting KLF4 and the downstream PI3K/Akt pathway.

In summary, the current findings provided evidence that ASV alleviated ox-LDL-induced apoptosis in HUVECs via the upregulation of miR-140-3p expression and subsequent inactivation of the KLF4/PI3K/Akt signaling pathway, thereby shedding light on the molecular mechanism by which ASV alleviates ox-LDL-induced HUVEC apoptosis. Thus, ASV may be a promising therapeutic target to suppress apoptosis of ECs, and fine tuning of the miR-140-3p/KLF4 axis through biological or pharmacological approaches may aid in relieving ox-LDL-induced EC damage.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

WQ, QQ and XC designed the study and performed the statistical analysis. WQ, XC and RH performed western blot analysis and data correction. WY, XZ and HZ isolated and identified EPCs. XC and RZ performed proliferation, migration and tube formation assays. WQ, XZ and QQ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All of the animal procedures, including housing, care and experimental protocols, were approved by the Animal Care and Use Committee of Shandong University of Traditional Chinese Medicine.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Shore AC, Colhoun HM, Natali A, Palombo C, Khan F, Östling G, Aizawa K, Kennbäck C, Casanova F, Persson M, *et al*: Use of vascular assessments and novel biomarkers to predict cardiovascular events in type 2 Diabetes: The SUMMIT VIP study. Diabetes Care 41: 2212-2219, 2018.
2. Rao Kondapally Seshasai S, Kaptoge S, Thompson A, Di Angelantonio E, Gao P, Sarwar N, Whincup PH, Mukamal KJ, Gillum RF, Holme I, *et al*: Diabetes mellitus, fasting glucose, and risk of cause-specific death. N Engl J Med 364: 829-841, 2011.
3. Tousoulis D, Papageorgiou N, Androulakis E, Siasos G, Latsios G, Tentolouris K and Stefanadis C: Diabetes mellitus-associated vascular impairment: Novel circulating biomarkers and therapeutic approaches. J Am Coll Cardiol 62: 667-676, 2013.
4. Shamsaldeen YA, Ugur R, Benham CD and Lione LA: Diabetic dyslipidaemia is associated with alterations in eNOS, caveolin-1, and endothelial dysfunction in streptozotocin treated rats. Diabetes Metab Res Rev 34: e2995, 2018.
5. Gilbert RE: Endothelial loss and repair in the vascular complications of diabetes: Pathogenetic mechanisms and therapeutic implications. Circ J 77: 849-856, 2013.
6. Fu C, Yin D, Nie H and Sun D: Notoginsenoside R1 protects HUVEC against oxidized low-density lipoprotein (Ox-LDL)-induced atherogenic response via down-regulating miR-132. Cell Physiol Biochem 51: 1739-1750, 2018.
7. Pollack RM, Donath MY, LeRoith D and Leibowitz G: Anti-inflammatory agents in the treatment of Diabetes and its vascular complications. Diabetes Care 39 (Suppl 2): S244-S252, 2016.
8. Song MT, Ruan J, Zhang RY, Deng J, Ma ZQ and Ma SP: Astragaloside IV ameliorates neuroinflammation-induced depressive-like behaviors in mice via the PPARγ/NF-κB/NLRP3 inflammasome axis. Acta Pharmacol Sin 39: 1559-1570, 2018.
9. Liu ZH, Liu HB and Wang J: Astragaloside IV protects against the pathological cardiac hypertrophy in mice. Biomed Pharmacother 97: 1468-1478, 2018.
10. Li M, Li H, Fang F, Deng X and Ma S: Astragaloside IV attenuates cognitive impairments induced by transient cerebral ischemia and reperfusion in mice via anti-inflammatory mechanisms. Neurosci Lett 639: 114-119, 2017.
11. Qian W, Cai X, Qian Q, Zhang W and Wang D: Astragaloside IV modulates TGF-β1-dependent epithelial-mesenchymal transition in bleomycin-induced pulmonary fibrosis. J Cell Mol Med 22: 4354-4365, 2018.
12. Lin XP, Cui HJ, Yang AL, Luo JK and Tang T: Astragaloside IV improves vasodilatation function by regulating the PI3K/Akt/eNOS signaling pathway in rat aorta endothelial cells. J Vasc Res 55: 169-176, 2018.
13. Leng B, Tang F, Lu M, Zhang Z, Wang H and Zhang Y: Astragaloside IV improves vascular endothelial dysfunction by inhibiting the TLR4/NF-κB signaling pathway. Life Sci 209: 111-121, 2018.
14. Stępień EŁ, Durak-Kozica M, Kamińska A, Targosz-Korecka M, Libera M, Tylko G, Opalińska A, Kapusta M, Solnica B, Georgescu A, *et al*: Circulating ectosomes: Determination of angiogenic microRNAs in type 2 diabetes. Theranostics 8: 3874-3890, 2018.

15. Liang W, Fan T, Liu L and Zhang L: Knockdown of growth-arrest specific transcript 5 restores oxidized low-density lipoprotein-induced impaired autophagy flux via upregulating miR-26a in human endothelial cells. Eur J Pharmacol 843: 154-161, 2019.
16. Yin J, Hou X and Yang S: microRNA-338-3p promotes ox-LDL-induced endothelial cell injury through targeting BAMBI and activating TGF-β/Smad pathway. J Cell Physiol 234: 11577-11586, 2019.
17. Wang Y, Che J, Zhao H, Tang J and Shi G: Paeoniflorin attenuates oxidized low-density lipoprotein-induced apoptosis and adhesion molecule expression by autophagy enhancement in human umbilical vein endothelial cells. J Cell Biochem 120: 9291-9299, 2019.
18. Yu S, Zhang L, Liu C, Yang J, Zhang J and Huang L: PACS2 is required for ox-LDL-induced endothelial cell apoptosis by regulating mitochondria-associated ER membrane formation and mitochondrial Ca^{2+} elevation. Exp Cell Res 379: 191-202, 2019.
19. Li B and Dewey CN: RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12: 323, 2011.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. You L, Fang Z, Shen G, Wang Q, He Y, Ye S, Wang L, Hu M, Lin Y, Liu M and Jiang A: Astragaloside IV prevents high glucose-induced cell apoptosis and inflammatory reactions through inhibition of the JNK pathway in human umbilical vein endothelial cells. Mol Med Rep 19: 1603-1612, 2019.
22. Ma Y, Zhao Y, Zhang R, Liang X, Yin Z, Geng Y, Shu G, Song X, Zou Y, Li L, *et al*: Astragaloside IV inhibits PMA-induced EPCR shedding through MAPKs and PKC pathway. Immunopharmacol Immunotoxicol 39: 148-156, 2017.
23. Xu C, Tang F, Lu M, Yang J, Han R, Mei M, Hu J and Wang H: Pretreatment with Astragaloside IV protects human umbilical vein endothelial cells from hydrogen peroxide induced oxidative stress and cell dysfunction via inhibiting eNOS uncoupling and NADPH oxidase-ROS-NF-κB pathway. Can J Physiol Pharmacol 94: 1132-1140, 2016.
24. Lin H, Pan S, Meng L, Zhou C, Jiang C, Ji Z, Chi J and Guo H: MicroRNA-384-mediated Herpud1 upregulation promotes angiotensin II-induced endothelial cell apoptosis. Biochem Biophys Res Commun 488: 453-460, 2017.
25. Wu CY, Zhou ZF, Wang B, Ke ZP, Ge ZC and Zhang XJ: MicroRNA-328 ameliorates oxidized low-density lipoprotein-induced endothelial cells injury through targeting HMGB1 in atherosclerosis. J Cell Biochem, 2018.
26. Zhong X, Li P, Li J, He R, Cheng G and Li Y: Downregulation of microRNA-34a inhibits oxidized low-density lipoprotein-induced apoptosis and oxidative stress in human umbilical vein endothelial cells. Int J Mol Med 42: 1134-1144, 2018.
27. Li Y, Ye Y and Chen H: Astragaloside IV inhibits cell migration and viability of hepatocellular carcinoma cells via suppressing long noncoding RNA ATB. Biomed Pharmacother 99: 134-141, 2018.
28. Song Z, Wei D, Chen Y, Chen L, Bian Y, Shen Y, Chen J and Pan Y: Association of astragaloside IV-inhibited autophagy and mineralization in vascular smooth muscle cells with lncRNA H19 and DUSP5-mediated ERK signaling. Toxicol Appl Pharmacol 364: 45-54, 2019.
29. Wei DZ, Lin C, Huang YQ, Wu LP and Huang MY: Ellagic acid promotes ventricular remodeling after acute myocardial infarction by up-regulating miR-140-3p. Biomed Pharmacother 95: 983-989, 2017.
30. Rasheed Z, Rasheed N and Al-Shaya O: Epigallocatechin-3-O-gallate modulates global microRNA expression in interleukin-1β-stimulated human osteoarthritis chondrocytes: Potential role of EGCG on negative co-regulation of microRNA-140-3p and ADAMTS5. Eur J Nutr 57: 917-928, 2018.
31. Wei R, Liu H, Chen R, Sheng Y and Liu T: Astragaloside IV combating liver cirrhosis through the PI3K/Akt/mTOR signaling pathway. Exp Ther Med 17: 393-397, 2019.
32. Tang F and Yang TL: MicroRNA-126 alleviates endothelial cells injury in atherosclerosis by restoring autophagic flux via inhibiting of PI3K/Akt/mTOR pathway. Biochem Biophys Res Commun 495: 1482-1489, 2018.
33. Yi J and Gao ZF: MicroRNA-9-5p promotes angiogenesis but inhibits apoptosis and inflammation of high glucose-induced injury in human umbilical vascular endothelial cells by targeting CXCR4. Int J Biol Macromol 130: 1-9, 2019.
34. Kawasaki Y, Fujiki M, Uchida S, Morishige M, Momii Y and Ishii K: A single oral dose of Geranylgeranylacetone upregulates vascular endothelial growth factor and protects against Kainic acid-induced neuronal cell death: Involvement of the Phosphatidylinositol-3 kinase/Akt pathway. Pathobiology 84: 184-191, 2017.
35. Giordano A, Romano S, D'Angelillo A, Corcione N, Messina S, Avellino R, Biondi-Zoccai G, Ferraro P and Romano MF: Tirofiban counteracts endothelial cell apoptosis through the VEGF/VEGFR2/pAkt axis. Vasc Pharmacol 80: 67-74, 2016.
36. Zhou Z, Rawnsley DR, Goddard LM, Pan W, Cao XJ, Jakus Z, Zheng H, Yang J, Arthur JS, Whitehead KJ, *et al*: The cerebral cavernous malformation pathway controls cardiac development via regulation of endocardial MEKK3 signaling and KLF expression. Dev Cell 32: 168-180, 2015.
37. Ohnesorge N, Viemann D, Schmidt N, Czymai T, Spiering D, Schmolke M, Ludwig S, Roth J, Goebeler M and Schmidt M: Erk5 activation elicits a vasoprotective endothelial phenotype via induction of Kruppel-like factor 4 (KLF4). J Biol Chem 285: 26199-26210, 2010.
38. Choi H and Roh J: Role of Klf4 in the regulation of apoptosis and cell cycle in rat granulosa cells during the periovulatory period. Int J Mol Sci 20, 2018.
39. Wang J, Wang B, Chen LQ, Yang J, Gong ZQ, Zhao XL, Zhang CQ and Du KL: miR-10b promotes invasion by targeting KLF4 in osteosarcoma cells. Biomed Pharmacother 84: 947-953, 2016.
40. Yang H, Xi X, Zhao B, Su Z and Wang Z: KLF4 protects brain microvascular endothelial cells from ischemic stroke induced apoptosis by transcriptionally activating MALAT1. Biochem Biophys Res Commun 495: 2376-2382, 2018.
41. Lv S, Ji L, Chen B, Liu S, Lei C, Liu X, Qi X, Wang Y, Lai-Han Leung E, Wang H, *et al*: Histone methyltransferase KMT2D sustains prostate carcinogenesis and metastasis via epigenetically activating LIFR and KLF4. Oncogene 37: 1354-1368, 2018.
42. Liu CH, Huang Q, Jin ZY, Zhu CL, Liu Z and Wang C: miR-21 and KLF4 jointly augment epithelial-mesenchymal transition via the Akt/ERK1/2 pathway. Int J Oncol 50: 1109-1115, 2017.