

SESN1 attenuates the Ox-LDL-induced inflammation, apoptosis and endothelial-mesenchymal transition of human umbilical vein endothelial cells by regulating AMPK/SIRT1/LOX1 signaling

FENG GAO, YONGCHENG ZHAO, BIN ZHANG, CHUNWEI XIAO,
ZHANFA SUN, YUAN GAO and XUEYONG DOU

Department of Cardiovascular Surgery, Xuzhou Cancer Hospital, Xuzhou, Jiangsu 221005, P.R. China

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Abstract. Endothelial cells are an important component of the heart and vasculature and form a crucial link between the cardiovascular system and the immune system. Sestrin 1 (SESN1) has an important role in atherosclerosis by inhibiting NOD-like receptor family pyrin domain containing 3 inflammasome activation. However, whether SESN1 is involved in human umbilical vein endothelial cell (HUVEC) injury caused by atherosclerosis has remained to be elucidated. The present study aimed to investigate the functions of SESN1 in the inflammatory response, apoptosis and endothelial-mesenchymal transition (EndMT) of HUVECs following stimulation with oxidized low-density lipoprotein (Ox-LDL). SESN1 expression at the mRNA and protein levels was detected using reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. Following SESN1 overexpression in Ox-LDL-stimulated HUVECs, cell viability was determined using a Cell Counting Kit-8 assay. Terminal deoxynucleotidyl transferase-mediated nick-end labeling staining was employed to detect cell apoptosis and western blot analysis was used to determine the levels of apoptosis-related proteins. RT-qPCR, ELISA and western blot were utilized to determine the levels of inflammatory factors. Immunofluorescence staining, RT-qPCR and western blot analysis were employed to assess the EndMT of Ox-LDL-stimulated HUVECs. The results revealed that SESN1 exhibited a low expression in HUVECs following Ox-LDL stimulation. SESN1 overexpression suppressed inflammation, apoptosis and EndMT in Ox-LDL-induced HUVECs. In addition, SESN1 stimulated adenosine monophosphate-activated protein kinase catalytic

subunit α 1/sirtuin 1 signaling to suppress Ox-LDL receptor-1 expression. An AMPK and SIRT1 inhibitor reversed the effects of SESN1 overexpression on the inflammatory response, apoptosis and EndMT of HUVECs exposed to Ox-LDL. Taken together, the present study demonstrated that SESN1 exerts a suppressive effect on Ox-LDL-induced inflammation, apoptosis and EndMT of HUVECs, suggesting that SESN1 may be used as a novel biomarker for endothelial injury-related disorders.

Introduction

Atherosclerosis, the leading cause of cardiovascular disease, is a chronic inflammatory disorder, during which the dysfunction of endothelial cells is the first step (1-3). Accumulating evidence has confirmed that endothelial cells have a major role in the development of atherosclerosis and that endothelial dysfunction and damage have an initial role (4,5). Under pathological conditions, injured endothelial cells display great plasticity by differentiating into healthy and functional endothelial cells (6). Atherosclerosis is a persistent inflammatory condition and vascular endothelial cell apoptosis is considered the initiation factor of the occurrence, development and pathogenesis of atherosclerosis (7,8). Furthermore, endothelial-mesenchymal transition (EndMT) helps endothelial cells to acquire a mesenchymal phenotype (9). Oxidized low-density lipoprotein (Ox-LDL) is a common factor in the establishment of experimental models of atherosclerosis, which may induce endothelial cell apoptosis and act as an essential risk factor for the formation of atherosclerosis (10,11).

Sestrins (SESNs) are a family of highly conserved stress-inducible proteins that regulate multiple cell homeostatic mechanisms (12). A deficiency in endogenous SESNs may lead to metabolic disorders, including insulin resistance, fat accumulation, mitochondrial dysfunction and oxidative damage (13). In addition, the SESN family are antioxidant enzymes and transcriptional targets of tumor suppressor protein p53 (14). As a primary member of the SESN family, SESN1 functions as a vital mediator in multiple human diseases, including human maxillary cancer, myoblast differentiation and diabetes (15-17). Furthermore, SESN1 has been reported to activate the adenosine monophosphate-activated

Correspondence to: Dr Feng Gao, Department of Cardiovascular Surgery, Xuzhou Cancer Hospital, 131 Huancheng Road, Xuzhou, Jiangsu 221005, P.R. China
E-mail: gaofenggf45@163.com

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protein kinase catalytic subunit $\alpha 1$ (AMPK) signaling pathway and to function as a suppressor of the mechanistic target of rapamycin complex 1 kinase (18,19). Recent evidence has demonstrated that SESN1 suppresses the inflammation of macrophages in a murine atherosclerosis model (20). In addition, Zhang *et al.* (21) revealed that SESN1 is expressed, at low levels, in endothelial cells subjected to mild shear stress. However, whether SESN1 is involved in human umbilical vein endothelial cell (HUVEC) injury induced by atherosclerosis has remained largely elusive.

SESN1 has been indicated to reduce myocardial hypertrophy and activate AMPK signaling (22). As metabolic sensors, AMPK and sirtuin 1 (SIRT1) have been identified as master regulators of metabolism (23). In a previous study, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that AMPK is able to regulate the expression of downstream SIRT1 genes, while SIRT1 may inhibit the expression of Ox-LDL receptor-1 (LOX1) (24). It is well established that endothelial cell injury is induced by Ox-LDL to drive the progression of atherosclerosis and LOX1 is the primary OxLDL receptor of endothelial cells, which may be activated by Ox-LDL, and it mediates cell damage in atherosclerosis (25). Therefore, blocking LOX1 expression is considered to be a major strategy in the management of atherosclerosis.

In the present study, the effects of SESN1 on the inflammation, apoptosis and EndMT of HUVECs exposed to Ox-LDL were investigated. In addition, the latent regulatory mechanisms among SESN1, AMPK/SIRT1 signaling and LOX1 were identified. The present results may highlight a novel target for the treatment of atherosclerosis.

Materials and methods

Bioinformatics analysis. The Human Protein Atlas (<https://www.proteinatlas.org/>) was used to assess SESN1 expression in HUVECs.

Cells and cell treatment. HUVECs (cat. no. KCB2012087YJ) obtained from the Kunming Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences were maintained in Dulbecco's modified Eagle's medium Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (RWDLS) in a humidified atmosphere with 5% CO₂ at 37°C. For the subsequent experiments, various concentrations (0, 25, 50, 75 and 100 μ g/ml) of Ox-LDL (cat. no. 20605ES05; Yeasen Biotechnology Co., Ltd.) were used to pre-treat the HUVECs for 24 h according to a previous study (26). In addition, 15 μ M SIRT1 inhibitor, nicotinamide (NAM; MilliporeSigma) (27) and 8 μ M AMPK inhibitor (compound C; MilliporeSigma) were respectively utilized to pre-treat the cells for 30 min (28).

Cell Counting Kit-8 (CCK-8) assay. Cell viability was evaluated by the CCK-8 reagent (Beyotime Institute of Biotechnology) according to manufacturer's protocol. The HUVECs were seeded into 96-well plates at the density of 4×10^4 cells/well and cultured at 37°C overnight. Following the indicated treatments, CCK-8 solution (10 μ l) was added to each well. Following incubation for 2 h at 37°C, cell proliferation

was detected using a microplate reader (Bio-Rad Laboratories, Inc.) at an absorbance wavelength of 450 nm.

Reverse transcription-quantitative PCR (RT-qPCR). Isolation of total RNA was performed using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in compliance with the manufacturer's instructions. The temperature protocol for this step was as follows: 70°C for 5 min, 37°C for 5 min and 42°C for 1 h. Subsequently, complementary DNA was synthesized using the PrimeScript RT reagent (Takara Bio, Inc.). An ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a SYBR Green Master Mix Kit (Toyobo Co. Ltd.) were used for PCR analysis. The following thermocycling conditions were used: Initial denaturation at 95°C for 7 min; and 40 cycles of 95°C for 15 sec and 60°C for 30 sec; and a final extension at 72°C for 30 sec. The primers sequences were as follows: SESN1 forward, 5'-GCG ACCAGGACGAGGAACCTT-3' and reverse, 5'-TGCATCTGT GCGTCTTCACT-3'; tumor necrosis factor (TNF)- α forward, 5'-CTGGGCAGGTCTACTTTGGG-3' and reverse, 5'-CTG GAGGCCCCAGTTTGAAT-3'; interleukin (IL)-6 forward, 5'-TCCACAAGCGCCTTCGGTC-3' and reverse, 5'-GGT CAGGGGTGGTTATTGCAT-3'; IL-1 β forward, 5'-GCTCGC CAGTGAAATGATGG-3' and reverse, 5'-TCGTGCACATAA GCCTCGTT-3'; α smooth muscle actin (α -SMA) forward, 5'-AAAGCAAGTCCTCCAGCGTT-3' and reverse, 5'-TTA GTCCCGGGGATAGGCAA-3'; vimentin forward, 5'-AAC TTAGGGGCGCTCTTGTC-3' and reverse, 5'-ATTCAAGTC TCAGCGGGCTC-3'; CD31 (also known as platelet endothelial cell adhesion molecule 1) forward, 5'-AGAGAGGCT GCTGTCATTGC-3' and reverse, 5'-GGCCCTCAGAAGAC AACAT-3'; von Willebrand factor (vWF) forward, 5'-CAA CACCTGCATTTGCCGAA-3' and reverse, 5'-ATGCGGAGG TCACCTTTCAG-3'; GAPDH forward, 5'-AATGGGCAG CCGTTAGGAAA-3' and reverse, 5'-GCGCCCAATACGACC AAATC-3'. Relative gene expression was calculated using the 2^{- $\Delta\Delta$ C_q} method using GAPDH as an internal control (29).

Western blot analysis. Total protein was extracted from the HUVECs using lysis buffer (Beyotime Institute of Biotechnology). Total protein was quantified using a bicinchoninic acid kit (Beyotime Institute of Biotechnology). Subsequently, 40 μ g of total protein was loaded per lane on a 10% SDS-PAGE gel, electroblotted onto polyvinylidene difluoride membranes (Thermo Fisher Scientific, Inc.) and blocked using 5% skimmed milk (MilliporeSigma) for 1 h at room temperature. The membranes were then incubated overnight at 4°C with the following primary antibodies: Anti-SESN1 (1:1,000 dilution; cat. no. ab134091), anti-inducible nitric oxide synthase (iNOS) (1:1,000 dilution; cat. no. ab178945), anti-total NF- κ B p65 (t-NF- κ B p65) (1:1,000 dilution; cat. no. ab32536), anti-phosphorylated NF- κ B p65 (p-NF- κ B p65) (1:2,000 dilution; cat. no. ab86299), anti-BCL2 associated X, apoptosis regulator (Bax) (1:1,000 dilution; cat. no. ab182733), anti-cleaved caspase-3 (1:400 dilution; cat. no. ab32042), anti-caspase-3 (1:5,000 dilution; cat. no. ab32351), anti-poly(adenosine diphosphate ribose) polymerase (PARP) (1:1,000 dilution; cat. no. ab191217) anti-cleaved PARP (1:1,000 dilution; cat. no. ab32064), anti- α -SMA (1:50 dilution; cat. no. ab150301), anti-CD31 (1:1,000 dilution; cat. no. ab9498),

anti-vimentin (1:1,000 dilution; cat. no. ab92547), anti-vWF (1:1,000 dilution; cat. no. ab287962), anti-p-AMPK (1:500 dilution; cat. no. ab131357), anti-AMPK (1:500 dilution; cat. no. ab3759), anti-SIRT1 (1:1,000 dilution; cat. no. ab189494), anti-LOX1 (1:1,000 dilution; cat. no. ab214427) and anti- β -actin (1:1,000 dilution; cat. no. ab8227; all from Abcam) and anti-B cell lymphoma-2 (Bcl-2) (1:1,000 dilution; cat. no. 15071, Cell Signaling Technology, Inc.). Following primary antibody incubation, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:3,000 dilution; cat. no. 7074S; Cell Signaling Technology, Inc.) for 1 h at room temperature and washed three times with PBS. Proteins bands were visualized using enhanced chemiluminescence (Thermo Fisher Scientific, Inc.) and ImageJ software (v6; National Institutes of Health) was used to analyze the protein bands.

Cell transfection. To overexpress SESN1, transfection of the pcDNA3.1 vector (Shanghai GenePharma, Co., Ltd.) containing the SESN1 gene (Ov-SESN1; 4 μ g) or the empty vector plasmid (Ov-NC; 4 μ g) was performed using Lipofectamine 2000[®] (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in accordance with the manufacturer's protocol. After 48 h, the transfected cells were collected for use in subsequent experiments.

Test for inflammatory factors contents. The cell culture medium was collected and the supernatant was obtained via centrifugation. The ELISA sandwich method was employed to evaluate the concentrations of TNF- α (cat. no. F02810), IL-6 (cat. no. F01310) and IL-1 β (cat. no. F01220) in culture medium supernatant in accordance with the manufacturer's protocols. These kits were acquired from Shanghai Xitang Biotechnology. The optical density at 450 nm was measured with a microplate reader (Bio-Rad Laboratories, Inc.).

Terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining. A TUNEL staining kit (Beyotime Institute of Biotechnology) was utilized according to the manufacturer's instructions. In brief, following incubation with 4% paraformaldehyde at 4°C for 20 min, the cells were treated with 0.5% Triton X-100. They were then incubated with 50 μ l TUNEL reaction buffer for 1 h at 37°C. Cell nuclei were stained with 2 μ g/ml DAPI solution for 10 min at room temperature in the dark. Images were acquired under a fluorescence microscope (Olympus Corporation) and cells were counted in five randomly selected microscopic fields.

Immunofluorescence staining. HUVECs were first cultured on sterilized coverslips. Subsequently, the transfected HUVECs were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.5% Triton X-100 and blocked with 1% bovine serum albumin (MilliporeSigma) for 1 h at room temperature. The cells were then incubated with primary antibodies against CD31 (1:1,000 dilution; cat. no. ab9498; Abcam) or α -SMA (1:50 dilution; cat. no. ab150301; Abcam) overnight at 4°C. Subsequently, cells were incubated with Alexa Fluor[®] 488-conjugated secondary antibody (1:400 dilution; cat. no. A11008; Molecular Probes; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. After washing with PBS three times, DAPI was used to stain the coverslips

for 5 min at room temperature. The results were imaged using a fluorescence microscope (Olympus Corporation).

Statistical analysis. Values are expressed as the mean \pm standard deviation and analyzed using GraphPad Prism software (version 8.0; GraphPad Software, Inc.). All experiments were performed as three independent replicates. An unpaired Student's t-test was used to compare differences between two groups, while differences between more than two groups were analyzed using a one-way ANOVA followed by Tukey's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SESN1 expression is downregulated in Ox-LDL-stimulated HUVECs. To evaluate the role of SESN1 in HUVECs, SESN1 expression was first examined in HUVECs. Using the Human Protein Atlas, SESN1 was indicated to be highly expressed in HUVECs (Fig. 1A). Subsequently, various concentrations (0, 25, 50, 75 and 100 μ g/ml) of Ox-LDL were utilized to stimulate the HUVECs and the viability was examined using a CCK-8 assay. The results suggested that the viability of HUVECs was decreased upon exposure to Ox-LDL in a concentration-dependent manner (Fig. 1B). In addition, RT-qPCR and western blot analysis demonstrated that SESN1 expression at both the mRNA and protein level was decreased as the concentration of Ox-LDL increased; when the Ox-LDL concentration was 100 μ g/ml, SESN1 expression was the lowest (Fig. 1C and D). Therefore, 100 μ g/ml Ox-LDL was used in the subsequent experiments. Taken together, the results demonstrated that SESN1 exhibits low expression in Ox-LDL-stimulated HUVECs.

SESN1 overexpression mitigates the decrease in the viability and inflammation of HUVECs stimulated with Ox-LDL. For the purpose of assessing the function of SESN1 in HUVECs, SESN1 was first overexpressed by transfection with the Ov-SESN1 plasmid and the transfection efficiency was examined using RT-qPCR and western blot analysis (Fig. 2A and B). Subsequently, CCK-8 assays demonstrated that SESN1 overexpression led to an increase in cell viability following stimulation with Ox-LDL (Fig. 2C). Furthermore, RT-qPCR revealed that the enhanced mRNA levels of inflammatory factors, including TNF- α , IL-6 and IL-1 β , in the HUVECs stimulated with Ox-LDL were decreased following the overexpression of SESN1 (Fig. 2D-F). Consistently, Ox-LDL stimulation led to significant increases in the contents of TNF- α , IL-6 and IL-1 β compared to the control group, which were restored by overexpression of SESN1 (Fig. 2G). Furthermore, western blot analysis indicated that overexpression of SESN1 attenuated the iNOS and p/t-NF- κ B p65 protein levels triggered by Ox-LDL (Fig. 2H). In summary, these results demonstrated that SESN1 exerted protective effects against the Ox-LDL-induced decrease in the viability and inflammation of HUVECs.

SESN1 overexpression suppresses the apoptosis of Ox-LDL-stimulated HUVECs. The detection of cell apoptosis was performed using a TUNEL assay. The results revealed that

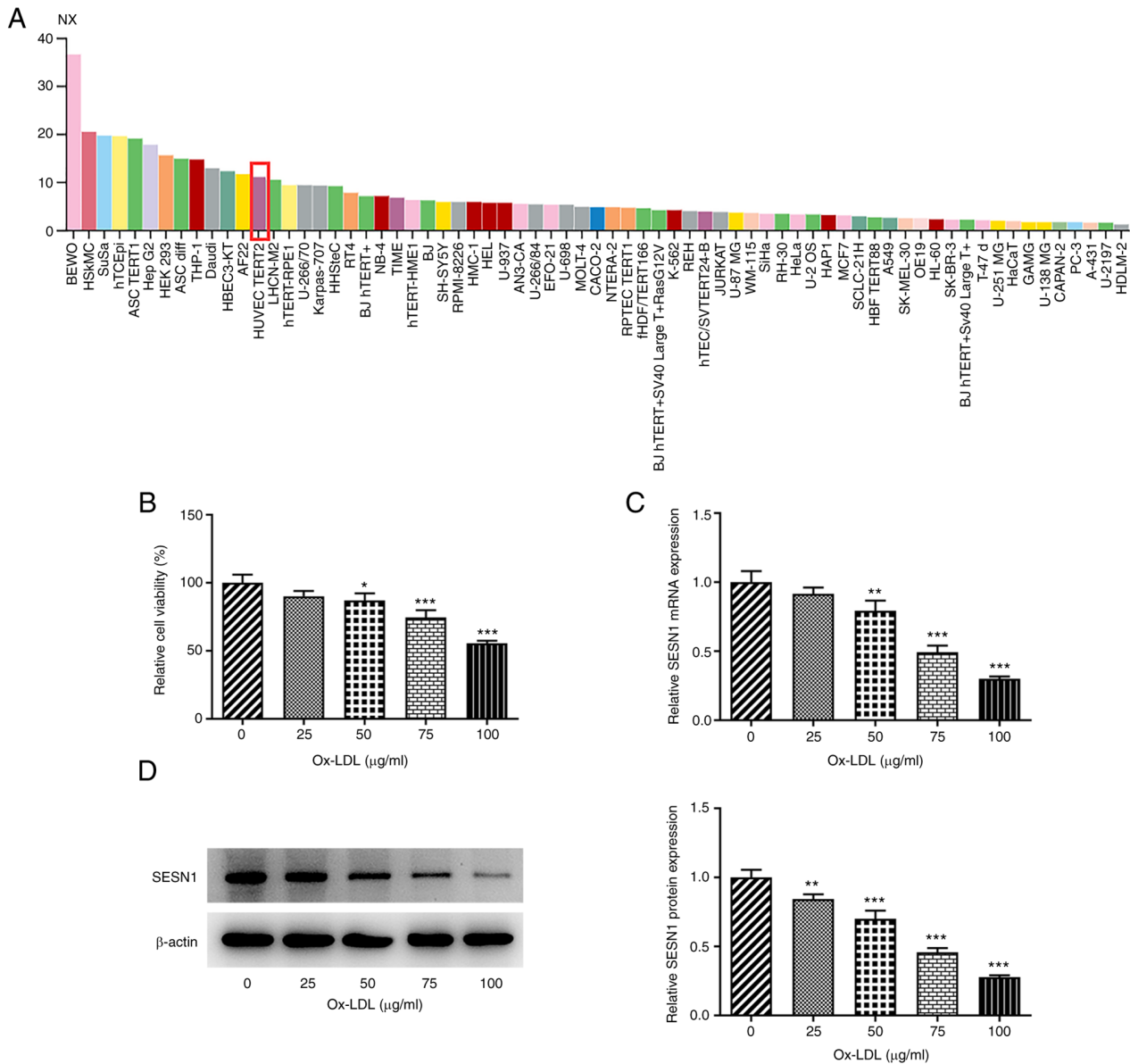


Figure 1. SESN1 is downregulated in Ox-LDL-stimulated HUVECs. (A) SESN1 expression was examined in endothelial cells through the Human Protein Atlas. (B) Cell viability was detected using a Cell Counting Kit-8 assay. (C) Reverse transcription-quantitative PCR and (D) western blot analysis were used to analyze SESN1 expression in HUVECs exposed to various concentrations of Ox-LDL. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 0 $\mu\text{g/ml}$ Ox-LDL. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells.

the Ox-LDL-induced apoptosis of HUVECs was inhibited by SESN1 (Fig. 3A and B). In addition, western blot analysis was used to examine the protein levels of Bax, cleaved caspase-3, caspase-3, cleaved PARP, PARP and Bcl-2, all of which are apoptosis-related factors. It was noted that following stimulation with Ox-LDL, the Bax, cleaved caspase-3/caspase-3 and cleaved PARP/PARP protein levels were increased, while the Bcl-2 protein level was decreased, indicating that Ox-LDL induced apoptosis of HUVECs. However, when SESN1 was overexpressed, HUVEC apoptosis was impeded (Fig. 3C). These results suggested that SESN1 exerted a suppressive effect against HUVEC apoptosis following stimulation with Ox-LDL.

SESN1 attenuates the Ox-LDL-induced EndMT of HUVECs. Using immunofluorescence staining, it was demonstrated that Ox-LDL decreased the expression of the endothelial marker

CD31 (Fig. 4A) and increased that of the mesenchymal marker α -SMA (Fig. 4B) in HUVECs; these effects were mitigated by SESN1 overexpression. In addition, the increased expression of mesenchymal markers (α -SMA and vimentin) and the decreased expression of endothelial markers (CD31 and vWF) in Ox-LDL-stimulated HUVECs were both reversed by SESN1 overexpression (Fig. 4C and D). Thus, SESN1 prevented the Ox-LDL-induced EndMT of HUVECs.

SESN1 mediates AMPK/SIRT1/LOX1 signaling in Ox-LDL-stimulated HUVECs. It is well established that SESN1 is capable of stimulating the AMPK signaling pathway (16). Using KEGG pathway enrichment analysis, it was indicated that SIRT1 was a critical regulator of AMPK activation and it inhibited the expression of LOX1, which has been identified as a major receptor for Ox-LDL

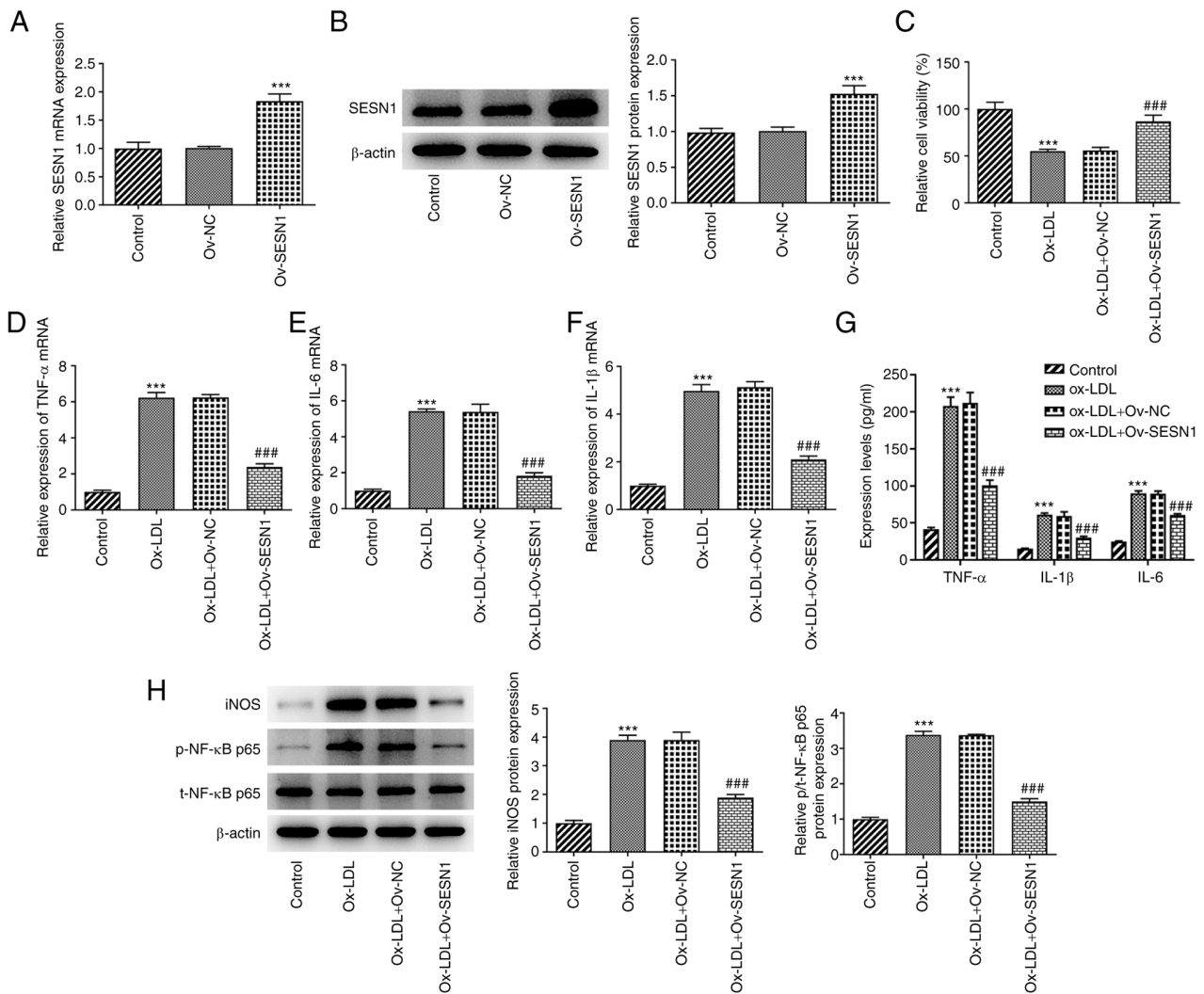


Figure 2. SESN1 overexpression mitigates the decrease in the viability and inflammatory response of human umbilical vein endothelial cells stimulated with Ox-LDL. (A and B) SESN1 overexpression efficiency was examined using (A) RT-qPCR and (B) western blot analysis. *** $P < 0.001$ vs. Ov-NC. (C) Cell Counting Kit-8 assays were used to assess cell proliferation. (D-F) RT-qPCR was used to analyze the mRNA levels of (D) TNF- α , (E) IL-6 and (F) IL-1 β . (G) The contents of TNF- α , IL-6 and IL-1 β in cell culture supernatant were assessed with ELISA kits. (H) Western blot analysis was used to examine the protein levels of iNOS, p-NF- κ B p65 and t-NF- κ B p65. *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. Ox-LDL + Ov-NC. RT-qPCR, reverse transcription-quantitative PCR; SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; NC, negative control; iNOS, inducible nitric oxide synthase; p-, phosphorylated protein; t-, total protein.

in endothelial cells (30,31). Hence, whether SESN1 was associated with the activation of AMPK/SIRT1 signaling to suppress LOX1, thus participating in the inflammation, apoptosis and EndMT of HUVECs, was next assessed. Western blot analysis revealed that Ox-LDL stimulation decreased the protein levels of p-AMPK/AMPK and SIRT1, whereas it increased the protein levels of LOX1. However, these effects were reversed by SESN1 overexpression (Fig. 5A). Of note, the addition of the AMPK inhibitor compound C notably downregulated p-AMPK/AMPK and SIRT1 expression and upregulated LOX1 expression when compared with the Ox-LDL + Ov-SESN1 group (Fig. 5B). In addition, the SIRT1 inhibitor NAM decreased SIRT1 expression and increased LOX1 expression. However, the inhibitors had no effect on p-AMPK/AMPK expression. In conclusion, SESN1 activated AMPK/SIRT1 signaling to suppress LOX1 in HUVECs following stimulation with Ox-LDL.

SESN1 exerts protective effects on the viability and inflammation of Ox-LDL-stimulated HUVECs via activation of AMPK/SIRT1/LOX1 signaling. Subsequently, the results of a CCK-8 assay demonstrated that the promotion of the viability of Ox-LDL-stimulated HUVECs by SESN1 overexpression was attenuated by the inhibitors of AMPK or SIRT1 (Fig. 6A). Similarly, the decreased expression of the inflammatory factors, TNF- α , IL-6 and IL-1 β [tested by RT-qPCR (Fig. 6B) and ELISA (Fig. 6C)], as well as iNOS and p-p65/p65 of NF- κ B [detected by western blot analysis (Fig. 6D)] due to SESN1 overexpression was abrogated by AMPK or SIRT1 inhibitor. These results suggested that AMPK/SIRT1 signaling reversed the protective effects of SESN1 against the decrease in the viability and inflammation of HUVECs mediated by Ox-LDL.

Overexpression of SESN1 prevents Ox-LDL-mediated HUVEC apoptosis by regulating AMPK/SIRT1/LOX1 signaling. Analysis of cell apoptosis using the TUNEL assay revealed

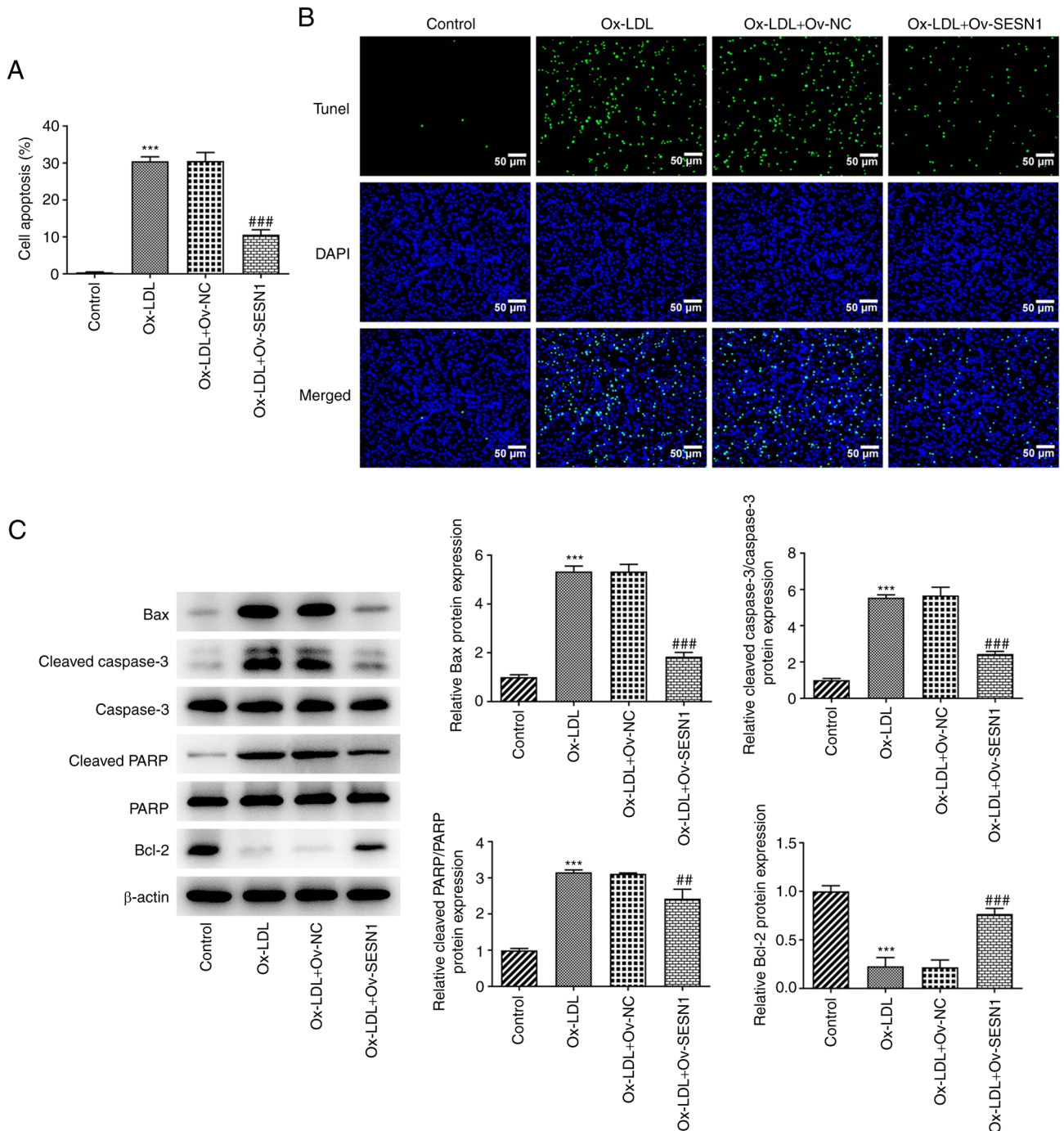


Figure 3. SESN1 overexpression suppresses Ox-LDL-induced human umbilical vein endothelial cell apoptosis. (A and B) Cell apoptosis was measured using a TUNEL assay. (A) Quantified apoptotic rates and (B) representative TUNEL staining images (scale bars, 50 μ m). (C) Western blot analysis was used to examine the protein levels of apoptosis-related factors. *** P <0.001 vs. control; ## P <0.01, ### P <0.001 vs. Ox-LDL + Ov-NC. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; NC, negative control; PARP, poly(adenosine diphosphate ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling.

that the suppression of apoptosis of Ox-LDL-stimulated HUVECs associated with SESN1 overexpression was abrogated by AMPK or SIRT1 inhibitor (Fig. 7A and B). Western blot analysis indicated that the decrease in the protein levels of Bax, cleaved caspase-3/caspase-3 and cleaved PARP/PARP, and the increase in the protein level of Bcl-2 in Ox-LDL-stimulated HUVECs induced by SESN1 overexpression were all reversed by treatment with an AMPK or SIRT1 inhibitor; these observations were consistent with the results of

TUNEL assay (Fig. 7C). Taken together, it was indicated that SESN1 impeded Ox-LDL-induced HUVEC apoptosis via the activation of AMPK/SIRT1/LOX1 signaling.

Inhibition of AMPK/SIRT1/LOX1 signaling attenuates the protective effects of SESN1 against the Ox-LDL-induced EndMT of HUVECs. Immunofluorescence staining confirmed that SESN1 enhanced CD31 expression and decreased α -SMA expression upon exposure to Ox-LDL; this effect was reversed

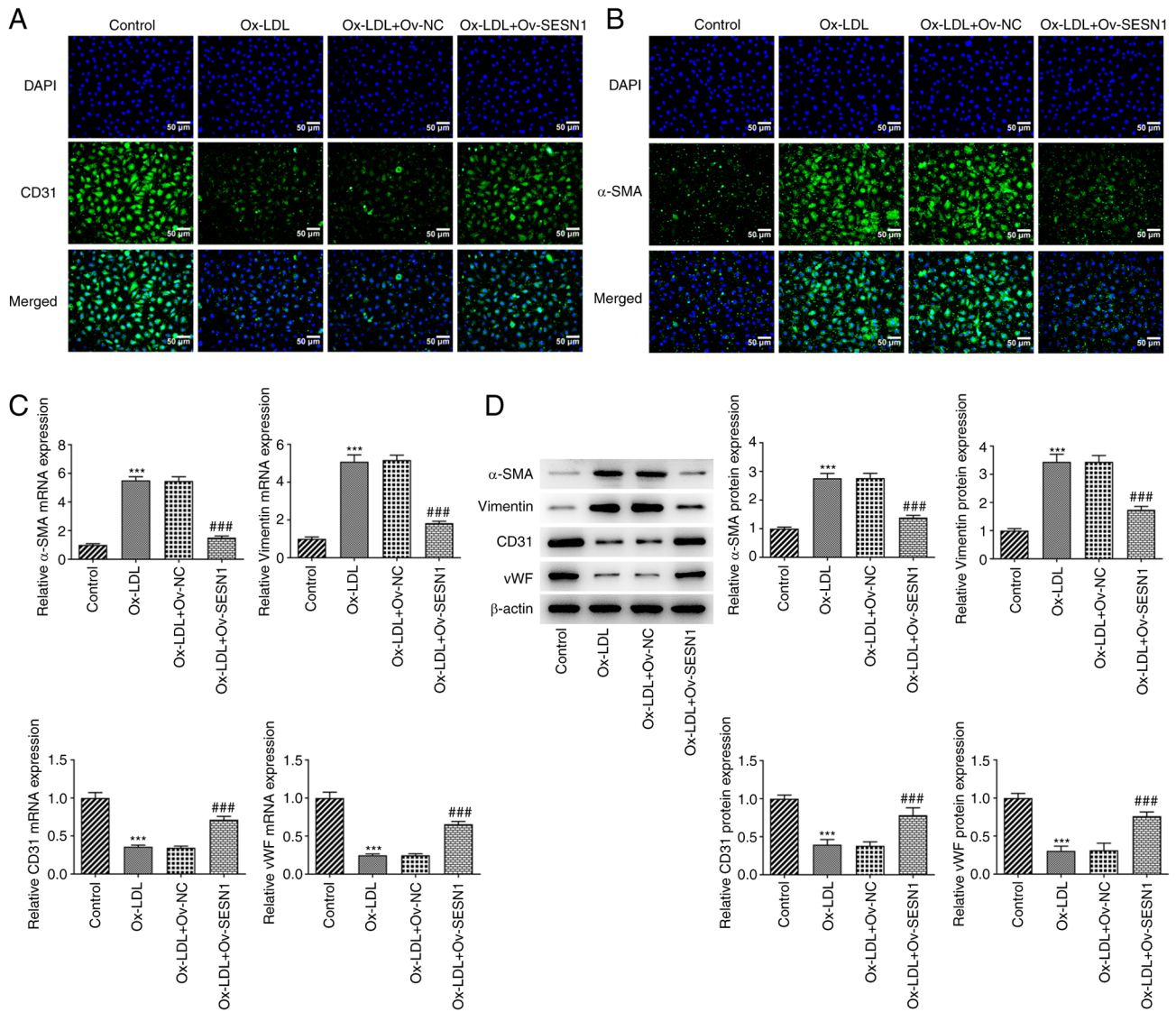


Figure 4. SESN1 attenuates Ox-LDL-induced endothelial-mesenchymal transition of human umbilical vein endothelial cells. (A) CD31 and (B) α -SMA expression was assessed by immunofluorescence staining (scale bars, 50 μ m). (C) Reverse transcription-quantitative PCR and (D) western blot analysis were used to examine the expression of CD31, vWF, α -SMA and vimentin. *** P <0.001 vs. control; ### P <0.001 vs. Ox-LDL + Ov-NC. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; NC, negative control; SMA, smooth muscle actin; vWF, von Willebrand factor.

by AMPK or SIRT1 inhibitor (Fig. 8A and B). Furthermore, AMPK or SIRT1 inhibitor reversed the promoting effects of SESN1 on the expression of CD31 and vWF, and abrogated the suppressive effects of SESN1 on α -SMA and vimentin expression in Ox-LDL-stimulated HUVECs (Fig. 8C and D). Collectively, SESN1 impeded the Ox-LDL-mediated EndMT of HUVECs via the activation of AMPK/SIRT1/LOX1 signaling.

Discussion

Endothelial cells are considered active metabolic components of biological tissue that have crucial physiological functions (32). It is widely acknowledged that endothelial cell injury is highly associated with various human diseases, including chronic cardiovascular, renal and metabolic diseases (33). With regard to cardiovascular diseases, endothelial cell injury is considered to be an early and enduring feature,

preceding the development of cardiovascular disease (34). As a risk factor for atherosclerosis, Ox-LDL contributes to atherosclerotic plaque formation and progression via several mechanisms, including endothelial cell dysfunction (25). Under pathological conditions, Ox-LDL is known to induce endothelial cell injury during atherosclerosis (35). SESN1 has been reported to be involved in cell metabolism and cardiovascular and age-related diseases (36). Furthermore, SESN1 has been demonstrated to inhibit NOD-like receptor family pyrin domain containing 3 inflammasome activation in lipopolysaccharide-primed macrophages induced by Ox-LDL via the inactivation of NF- κ B signaling (20). The present study confirmed the low expression of SESN1 in Ox-LDL-stimulated HUVECs and Ox-LDL stimulation decreased cell viability in a concentration-dependent manner.

Endothelial cell apoptosis has a key role in the process of atherosclerosis, which is hallmarked by the inflammatory response (37). For instance, iNOS, a pro-inflammatory

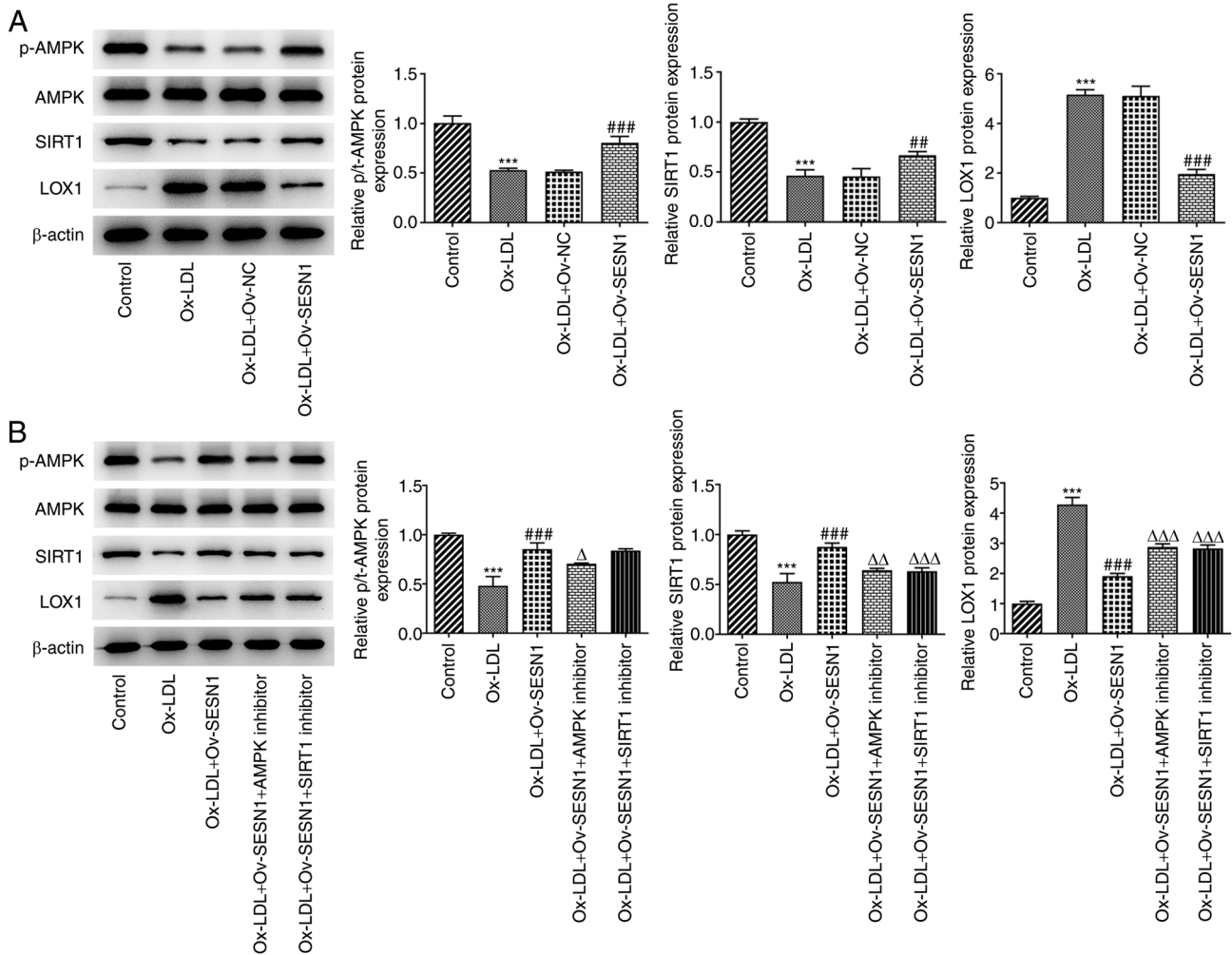


Figure 5. SESN1 mediates AMPK/SIRT1/LOX1 signaling in Ox-LDL-stimulated HUVECs. (A) Western blot analysis was used to examine p-AMPK, AMPK, SIRT1 and LOX1 protein levels in HUVECs co-treated with Ox-LDL and Ov-SESN1. *** $P < 0.001$ vs. control; ** $P < 0.01$, *** $P < 0.001$ vs. Ox-LDL + Ov-NC. (B) Western blot analysis was used to examine p-AMPK, AMPK, SIRT1 and LOX1 protein levels in the control, Ox-LDL, Ox-LDL + Ov-SESN1, Ox-LDL + Ov-SESN1 + AMPK inhibitor and Ox-LDL + Ov-SESN1 + SIRT1 inhibitor groups. *** $P < 0.001$ vs. control; *** $P < 0.001$ vs. Ox-LDL; $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs. Ox-LDL + Ov-SESN1. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; NC, negative control; LOX1, low-density lipoprotein receptor-1; p-AMPK, phosphorylated adenosine monophosphate-activated protein kinase catalytic subunit α ; SIRT1, sirtuin 1; HUVECs, human umbilical vein endothelial cells.

cytokine, is considered to be a major contributor to atherosclerosis (38). NF- κ B transcription factors formed by the dimerization of Rel proteins [RelA (p65), c-Rel, RelB, p50, p52] have been confirmed to have crucial roles in inflammation, immunity, cell proliferation and apoptosis (39,40). In addition, as has been demonstrated, EndMT is the process through which endothelial cells undergo a series of molecular events, which leads to the adoption of a mesenchymal-like phenotype (41). Furthermore, endothelial cells undergoing EndMT lose expression of endothelial cell-specific proteins, including CD31 and vWF, and this initiates the expression of mesenchymal cell-specific genes and the production of their encoded proteins, including α -SMA and vimentin (42). For instance, α -SMA, an EndMT-related marker, has been reported to be involved in cardiogenesis and cardiovascular diseases (43). As an EndMT-related marker, CD31 has also been confirmed as a potential therapeutic target in atherosclerosis (44). Furthermore, apoptosis, inflammation and EndMT induced by Ox-LDL are vital for the development of atherosclerosis (45).

Previous studies have indicated that SESN1 has a crucial role in cell apoptosis and the inflammatory response. For instance, SESN1 is targeted by microRNA-16-5p and influences myoblast proliferation and apoptosis (16). SESN1 also inhibits macrophage-mediated inflammation of the aorta (20). SESN1 has a crucial role in aerobic exercise and suppresses the activation of inflammatory signaling (46). In the present study, functional experiments demonstrated that stimulation with 100 μ g/ml Ox-LDL promoted apoptosis of HUVECs. Furthermore, Ox-LDL stimulation elevated the TNF- α , IL-6 and IL-1 β mRNA levels, as well as the iNOS, p/t-NF- κ B p65 protein levels. In addition, Ox-LDL increased α -SMA and vimentin expression, whereas it decreased CD31 and vWF expression. All these aforementioned effects were reversed by SESN1 overexpression.

AMPK is a central regulator of endothelial cell metabolism and has a crucial role in diabetes, cancers and vascular diseases (47,48). SIRT1 protein, the product of the longevity gene, is involved in a wide variety of cellular processes (49).

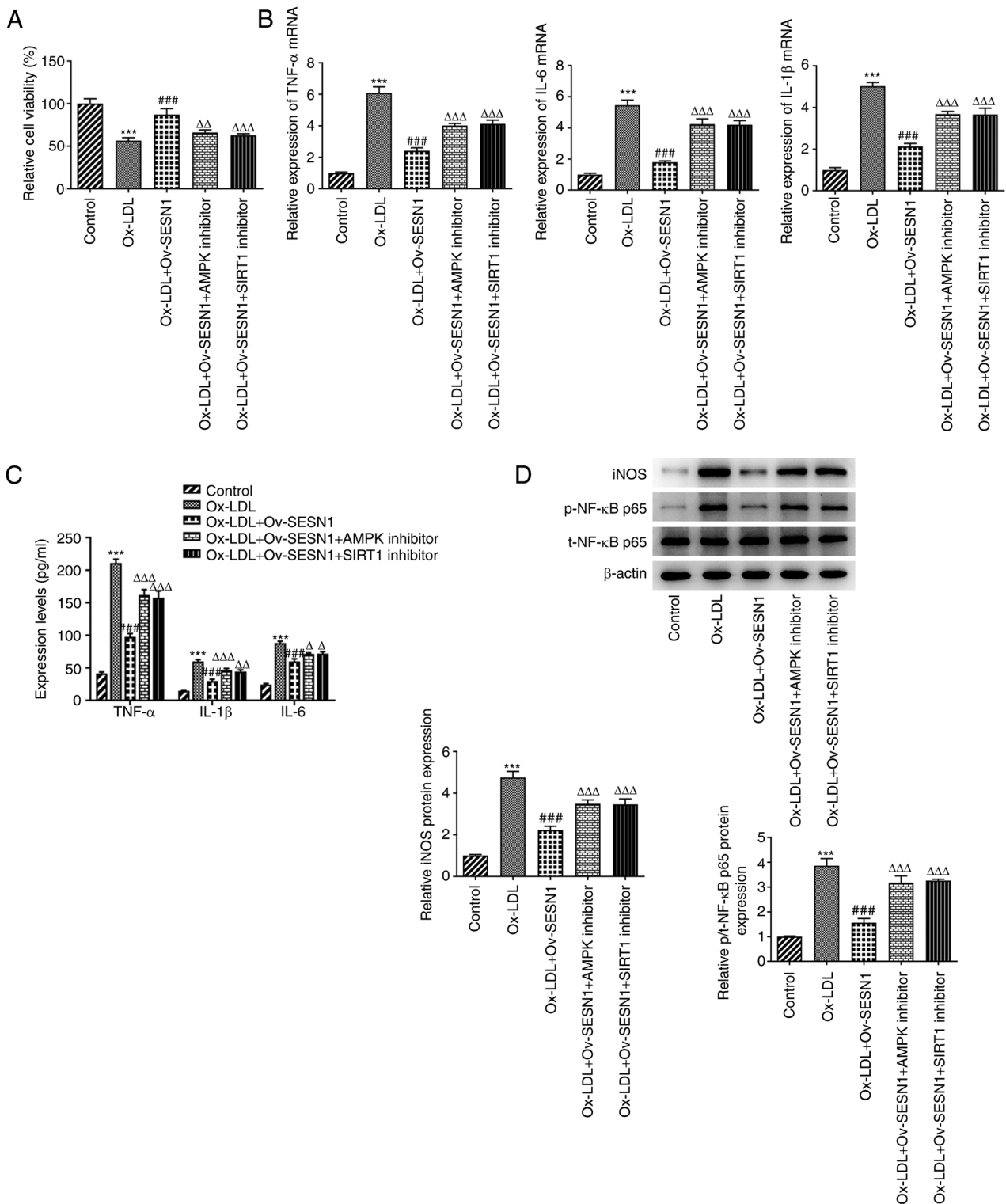


Figure 6. SESN1 attenuates the decrease in cell viability and inflammation in Ox-LDL-stimulated human umbilical vein endothelial cells via activating AMPK/SIRT1/LOX1 signaling. (A) Cell viability was detected using a Cell Counting Kit-8 assay. (B) Reverse transcription-quantitative PCR was used to analyze the expression of inflammatory factors. (C) ELISA kits were used to determine the levels of TNF- α , IL-6 and IL-1 β in cell culture supernatant. (D) Western blot analysis was used to examine the iNOS, p-NF- κ B p65 and t-NF- κ B p65 protein levels. ***P<0.001 vs. control; ###P<0.001 vs. Ox-LDL; Δ P<0.05, $\Delta\Delta$ P<0.01, $\Delta\Delta\Delta$ P<0.001 vs. Ox-LDL + Ov-SESN1. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; LOX1, low-density lipoprotein receptor-1; SIRT1, sirtuin 1; AMPK, adenosine monophosphate-activated protein kinase catalytic subunit α 1; iNOS, inducible nitric oxide synthase; p-, phosphorylated protein; t-, total protein.

It has been reported that SIRT1 mediates endothelial functions (50). Furthermore, SIRT1 is an important activator of AMPK (30). Of note, SIRT1 suppresses the expression

of LOX1, which is a 50-kDa transmembrane glycoprotein that serves as a receptor for Ox-LDL (30,51). As has been previously reported, SESNs are the primary regulators of

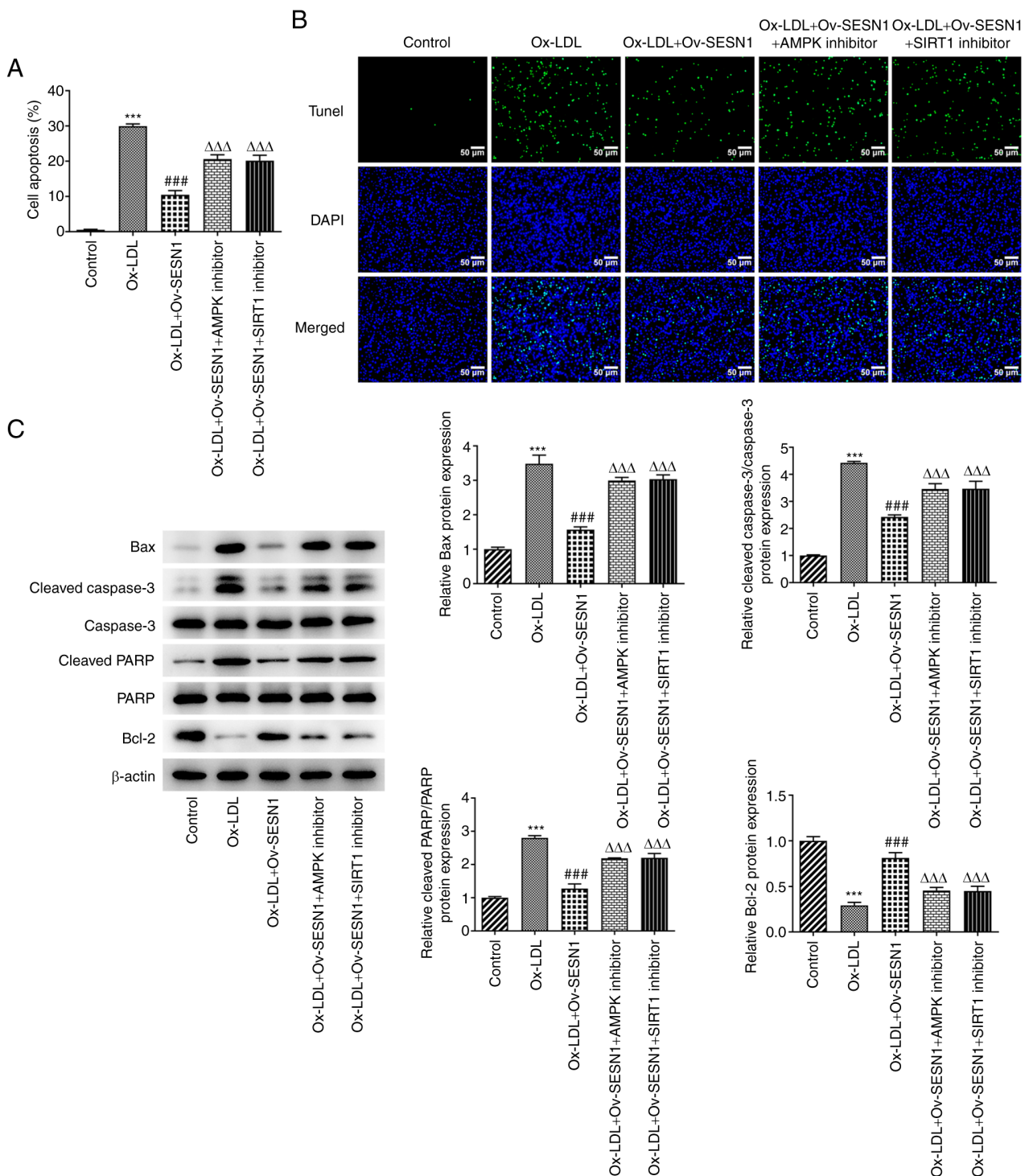


Figure 7. Overexpression of SESN1 prevents Ox-LDL-mediated human umbilical vein endothelial cell apoptosis by regulating AMPK/SIRT1/LOX1 signaling. (A and B) Cell apoptosis was measured using TUNEL assays. (A) Quantified apoptotic rates and (B) representative TUNEL staining images (scale bars, 50 μ m). (C) Western blot analysis was employed to quantify apoptosis-associated proteins. *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. Ox-LDL; $\Delta\Delta\Delta P < 0.001$ vs. Ox-LDL + Ov-SESN1. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; SIRT1, sirtuin 1; LOX1, low-density lipoprotein receptor-1; AMPK, adenosine monophosphate-activated protein kinase catalytic subunit α 1; TUNEL, terminal deoxynucleotidyl transferase-mediated nick-end labeling; PARP, poly(adenosine diphosphate ribose) polymerase.

AMPK (52). SESN1 has been indicated to directly interact with AMPK, thereby participating in several human diseases. For instance, Li *et al.* (53) demonstrated that SESN1 protected cardiomyocytes from doxorubicin-induced damage by upregulating AMPK expression. The SESN1-2/AMPK/mTOR axis is regulated by AMPK phosphorylation, which has a crucial

role in pancreatic cancer cell autophagy (54). In the present study, it was observed that the overexpression of SESN1 enhanced the decreased protein levels of p-AMPK/AMPK and SIRT1, and decreased the elevated protein levels of LOX1 following exposure to Ox-LDL. This indicated that SESN1 led to the activation of AMPK/SIRT1 signaling to inhibit LOX1

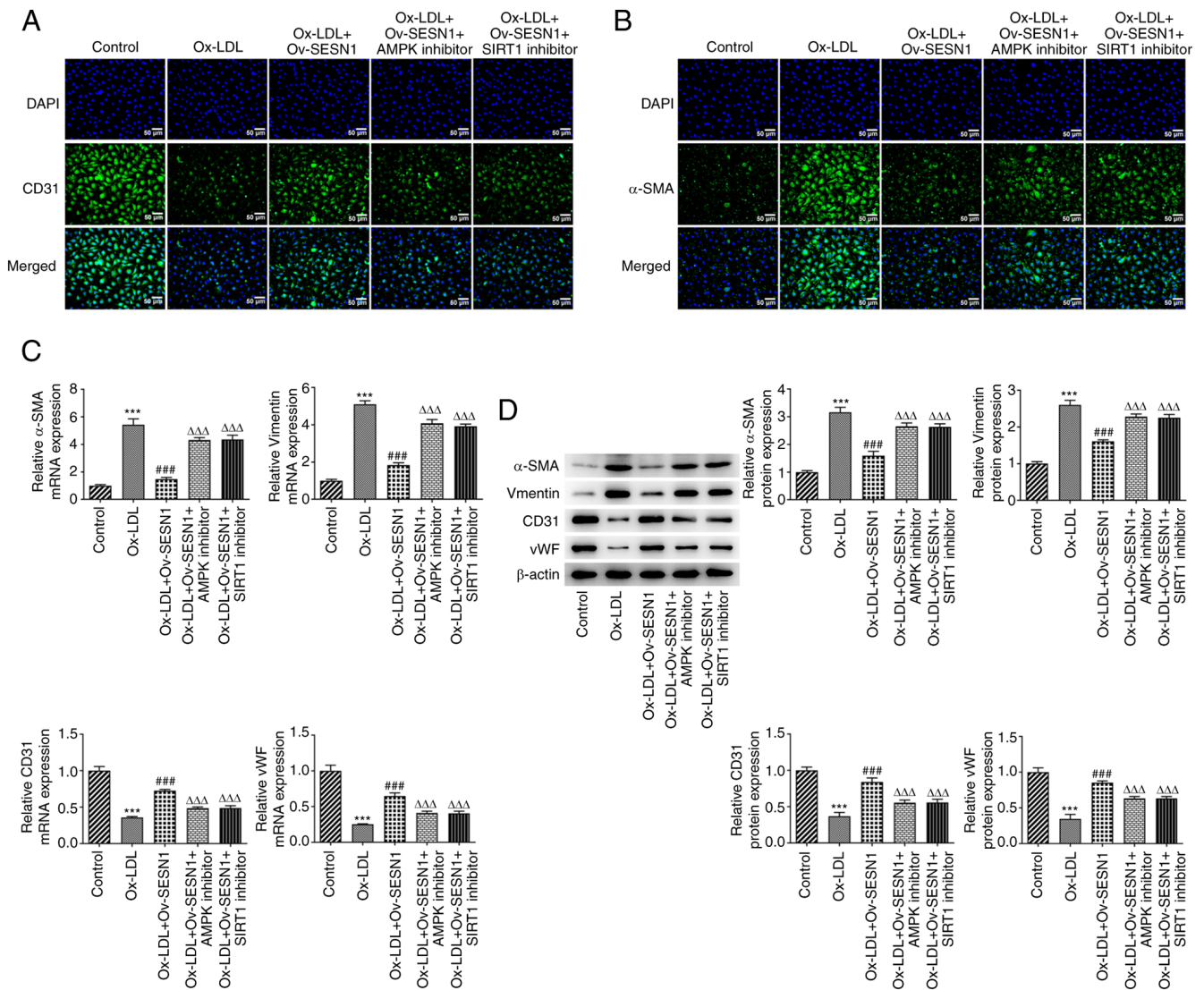


Figure 8. Inhibition of AMPK/SIRT1/LOX1 signaling attenuates the protective effects of SESN1 against the Ox-LDL-induced endothelial-mesenchymal transition of human umbilical vein endothelial cells. (A) CD31 and (B) α -SMA expression was assessed using immunofluorescence staining (scale bars, 50 μ m). (C) Reverse transcription-quantitative PCR and (D) western blot analysis were used to examine the expression of CD31, vWF, α -SMA and vimentin. *** P <0.001 vs. control; ### P <0.001 vs. Ox-LDL; $\Delta\Delta\Delta P$ <0.001 vs. Ox-LDL + Ov-SESN1. SESN1, sestrin 1; Ox-LDL, oxidized low-density lipoprotein; Ov, overexpression; LOX1, low-density lipoprotein receptor-1; SIRT1, sirtuin 1; SMA, smooth muscle actin; vWF, von Willebrand factor; AMPK, adenosine monophosphate-activated protein kinase catalytic subunit α 1.

expression in Ox-LDL-stimulated HUVECs. At the same time, it was noted that AMPK or SIRT1 inhibitor attenuated the protective effects of SESN1 against the Ox-LDL-induced apoptosis, inflammation and EndMT of HUVECs.

In conclusion, the present study demonstrated that SESN1 suppressed Ox-LDL-induced endothelial EndMT, inflammation and apoptosis by regulating the AMPK/SIRT1/LOX1 signaling pathway. SESN1 may thus prove to be an effective biomarker for protection from endothelial cell injury, thus highlighting a novel target for the treatment of atherosclerosis. The lack of elucidation of upstream mechanisms of SESN1 and an *in vivo* animal study are limitations of the present study and a comprehensive analysis is required in the future.

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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

FG and YZ conceived and designed the study. BZ and CX performed the experiments. ZS, YG and XD analyzed and interpreted the experimental data. FG and XD wrote and revised the manuscript. YZ and BZ confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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