FOXP1-induced DUSP12 alleviates vascular endothelial cell inflammation and oxidative stress injury induced by ox-LDL via MAP3K5 signaling pathway

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Abstract. Atherosclerosis (AS) is a type of chronic inflammatory disease and the main pathological basis of cardiovascular and cerebrovascular diseases, which seriously threaten the health of patients. The dual specificity phosphatase 12 (DUSP12) protein is known as regulator of inflammatory diseases. Nonetheless, at present, there are only a few reports on the regulatory role of DUSP12 in AS. Human umbilical vein endothelial cells (HUVECs) were induced using oxidized low-density lipoprotein (ox-LDL). Subsequently, cell transfection experiments were performed to overexpress DUSP12 in ox-LDL-induced HUVECs. Cell Counting Kit-8, TUNEL western blotting, 2',7'-dichlorofluorescein diacetate assays, ELISA and other techniques were used to measure cell viability, apoptosis, inflammation, oxidative stress and endothelial function-related indicators. Subsequently, the relationship between DUSP12 and Forkhead box P1 (FOXP1) was predicted using the JASPAR database and verified using luciferase reporter and chromatin immunoprecipitation assays. Finally, the regulatory mechanism was investigated by simultaneously overexpressing DUSP12 and knocking down FOXP1 in ox-LDL-induced HUVECs and MAP3K5-related proteins of the DUSP12 downstream pathway were measured by western blotting. The expression of DUSP12 in ox-LDL-induced HUVECs was significantly decreased. Overexpression of DUSP12 inhibited apoptosis, inflammation and oxidative stress damage and alleviated endothelial dysfunction in ox-LDL-induced HUVECs. FOXP1 promoted the transcription of DUSP12. Moreover, FOXP1 alleviated ox-LDL-induced apoptosis, inflammation and oxidative stress damage in HUVECs by regulating the expression of DUSP12, probably acting through the MAP3K5 pathway. Collectively, the present study revealed that FOXP1-induced DUSP12 alleviated vascular endothelial cell inflammation and oxidative stress injury in ox-LDL-induced HUVECs via the MAP3K5 signaling pathway, which might shed novel insights into the targeted treatment for AS in the clinic.

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Introduction

Atherosclerosis (AS) is a type of chronic inflammatory disease involving large and medium arteries. Atherosclerotic plaques composed of lipids, cholesterol, calcium and abnormal surrounding cells deposited on the vascular wall can lead to the narrowing of the corresponding blood vessels. At the same time, the plaques can also rupture to form a local thrombus, thus causing a series of ischemic diseases (1) AS is the main pathological basis of cardiovascular and cerebrovascular diseases, which are characterized by high morbidity, disability and mortality and seriously threaten human life and health (2,3). Currently, it is generally accepted that chronic inflammation and oxidative stress are key factors in AS (4). Therefore, by taking vascular endothelial cells and their secreted inflammatory factors as the main research topic in AS, an in-depth study of the molecular mechanism of the occurrence and development of AS has far-reaching clinical significance for early detection, diagnosis and treatment of this disease, and decrease of the associated mortality.

Dual specificity phosphatase (DUSP) is a family of tyrosine-specific phosphatase proteins discovered in the last decade. These proteins are mainly responsible for signal transduction through selective dephosphorylation of MAPK proteins, thus participating in a variety of biological functions including protein ubiquitination, proteasome degradation, oxidation, phosphorylation and methylation (5). DUSP12 is a member of the DUSP family. A previous study showed that DUSP12 has a regulatory role in the process of cellular inflammation and oxidative stress. DUSP12 was shown to

prevent hepatic steatosis and inflammation in L02 cells after palmitic/oleic acid treatment. Overexpression of DUSP12 in hepatocytes could reduce high-fat diet-induced hepatic steatosis, insulin resistance and inflammation (6). DUSP12 alleviates oxidative stress damage and apoptosis in diabetic cardiomyopathy through the MAP3K5-JNK/p38 signaling pathway (7). The same study also showed that DUSP12 expression was significantly decreased in ischemia-reperfusion (IR) following major liver surgery and DUSP12 negatively regulated the pro-inflammatory and pro-apoptotic pathways MAP3K5/JNK-p38 and MAPK signaling pathways during liver IR, which could be used as a potential therapeutic target for liver IR (8). However, to the best of the authors' knowledge, the regulatory effects of DUSP12 on inflammation and oxidative stress injury of vascular endothelial cells in AS remain to be elucidated.

The current study found that Forkhead box P1 (FOXP1) could bind to the DUSP12 promoter through bioinformatics binding site prediction. The transcriptional regulatory factor FOXP1 is distributed in different tissues and cell types of the cardiovascular system and plays an important role in regulating cardiovascular system homeostasis (9). Gene deletion of FOXP1 could lead to severe congenital heart defects and embryonic death, pathological myocardial fibrosis and myocardial hypertrophy, exacerbation of atherosclerotic lesions and prolonged elimination of thrombosis (10). It has been shown that FOXP1 transcription activated SESN1 to reduce oxidized low-density lipoprotein (ox-LDL)-induced macrophage inflammation and lipid accumulation (11). However, to the best of the authors' knowledge, the regulatory effects of FOXP1-induced DUSP12 on inflammation and oxidative stress injury of vascular endothelial cells in AS and its mechanisms have not been reported.

The present study aimed to investigate and discuss the regulatory effects and mechanism of action of DUSP12 on ox-LDL-induced inflammation and oxidative stress injury of vascular endothelial cells in AS. Its results provided a theoretical basis for DUSP12 as a potential therapeutic target for AS-related diseases.

Materials and methods

Database. Prediction of promoter binding sites of transcription factors FOXP1 and DUSP12 were obtained from the JASPAR database (https://jaspar.genereg.net/) (12).

Cell culture. Human umbilical vein endothelial cells (HUVECs; cat. no. QC-0122) were purchased from Shanghai Qincheng Biotechnology Co., Ltd. The cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and maintained in a humidified 5% CO_2 atmosphere in a cell incubator at 37˚C. For the establishment of the AS *in vitro* model, HUVECs were treated with 100 μg/ml ox-LDL added to the cells for 24 h at 37˚C (13).

Cell transfection. DUSP12 plasmid (Oe-DUSP12), FOXP1 plasmid (Oe-FOXP1) and the empty vector plasmid (Oe-NC), small interfering (si)RNAs targeting FOXP1 (si-FOXP1#1 or si-FOXP1#2) and the scrambled siRNA negative control (si-NC) were synthesized by Genepharm, Inc. HUVECs were seeded on 6-well plates ($2x10^5$ cells/well) and cultured until the cell confluence reached 80%. A total of 100 nM plasmids were transfected into HUVECs using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. After 48 h of transfection, reverse transcription-quantitative (RT-q) PCR and western blotting were used to measure cell transfection efficiency. All transfections in the present study were transient. The sequences of two human siRNA-FOXP1 and the siRNA-NC were as follows: siRNA-FOXP#1 sense: 5'-UCAAAGGUCACGUCUUACCC-3', and siRNA-FOXP#1 antisense: 5'-GUAAGACGUGACCUUUUGAGG-3'; siRNA-FOXP#2 sense: 5'-UUAUAGUCACCUCAAAAGGUC-3', and siRNA-FOXP#2 antisense: 5'-CCUUUUGAGGUGACUAUAACU-3'; siRNA-NC-sense: 5'-UUCUCCGAACGUGUCACGUTT-3', and siRNA-NC-antisense: 5'-ACGUGACACGUUCGGAGAATT-3'.

Reverse transcription-quantitative (RT-q) PCR. Total RNA from HUVECs ($1x10^4$ cells) was extracted using TRIzol® (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The total RNA was then reverse transcribed into cDNA using a cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Subsequently, a qPCR assay was performed to measure the mRNA levels of DUSP12 and FOXP1 using SYBR Green Master Mix (Takara Bio, Inc.) according to recommended instructions. The following thermocycling conditions were used for qPCR: Pre-denaturation at 95˚C for 1 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 40 sec and extension at 72˚C for 15 sec. Relative expression changes were calculated using the $2^{-\Delta\Delta Cq}$ method (14). Sequences of the primers were: DUSP12 forward, 5'-TGTCATGCAGGAGTCAGTCG-3' and reverse, 5'-CCTCCCTGTGGTAAGCATGG-3'; FOXP1 forward, 5'-AGGCTTCCCTCTGTGTGTTG-3' and reverse, 5'-ACTCCTAGAGGGCTGATGGT-3' and GAPDH forward, 5'-CATGAGAAGTATGACAACAGCCT-3' and reverse, 5'-AGTCCTTCCACGATACCAAAGT-3'.

Western blotting. Total protein was extracted from HUVECs using RIPA buffer (Cell Signaling Technology, Inc.) and quantified using a BCA kit (Beyotime Institute of Biotechnology). Total protein (30 μg/lane) was separated by SDS-PAGE on 10% gel and transferred to PVDF membranes. The membranes were blocked with 5% BSA (Beyotime Institute of Biotechnology) for 1 h at 37˚C and incubated with primary antibodies (Abcam) overnight at 4˚C. The primary antibodies were DUSP12 (1:1,000; cat. no. ab228987; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam), Bax (1:1,000; cat. no. 182733; Abcam), Cleaved PARP (1:1,000; cat. no. ab32064; Abcam), PARP (1:1,000; cat. no. ab191217; Abcam), VCAM-1 (1:1,000; cat. no. ab134047; Abcam), ICAM-1 (1:1,000; cat. no. ab282575; Abcam), p-eNOS (1:1,000; cat. no. ab215717; Abcam), eNOS (1:1,000; cat. no. ab300071; Abcam), FOXP1 (1:1,000; cat. no. ab134055; Abcam), MAP3K5 (1:1,000; cat. no. ab45178; Abcam), p-MAP3K5 (1:1,000; cat. no. ab278547; Abcam) or GAPDH (1:1,000; cat. no. ab9485; Abcam). The membranes were then treated with HRP-linked secondary antibodies (goat anti-rabbit; 1:5,000; cat. no. ab6721; Abcam) for 2 h at 37˚C and were visualized using an ECL kit (MilliporeSigma) and semi-quantified using ImageJ software (version 1.42; National Institutes of Health).

Cell Counting Kit-8 assay. For cell viability assay, HUVECs were plated in triplicate at an initial density of 2,000 cells/well into 96-well plates. DMEM medium containing 10 μl CCK-8 was then added for 4 h. Cell viability was determined at 24 h using the Cell Counting Kit-8 assay (Dojindo Laboratories, Inc.) at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions.

TUNEL staining. Cell apoptosis in HUVECs was measured using the TUNEL staining assay (Beyotime Institute of Biotechnology). Briefly, cells were permeabilized using proteinase K solution (20 μg/ml) at 37˚C for 30 min. Subsequently, the terminal deoxynucleotidyl transferase and fluorescein were added to the cells and incubated in a humidified box at 37˚C for 1 h. A fluorescence microscope (ECLIPSE C1; Nikon Corporation) was used to capture images of the TUNEL-positive cells.

ELISA. The levels of IL-6 (cat. no. H007-1-2), IL-1β (cat. no. H002-1-1) and TNF-α (cat. no. H052-1-2) were measured in cell lysates of HUVECs using ELISA kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. The levels of 8-hydroxy-2-deoxyguanosine (8-OHdG; cat. no. H165-1-2) and nitric oxide (NO; cat. no. A013-2-1) were measured in cell lysates of HUVECs with the corresponding kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions.

Oxidative stress index detection. The reactive oxygen species (ROS) level was measured with a 2',7'-dichlorofluorescein diacetate (DCFH-DA; cat. no. E004, Jiancheng Bioengineering, Nanjing, China) staining assay, according to the manufacturer's instructions. Commercial kits (Nanjing Jiancheng Bioengineering Institute) were used to measure superoxide dismutase (SOD; cat. no. A001-3-2), malondialdehyde (MDA; cat. no. A003-1-2) and catalase (CAT; cat. no. A007-1-1) activity.

Dual-luciferase reporter assay. The mutant (MUT) or wild-type (WT) 3'-untranslated region (UTR) sequence of DUSP12 was cloned into the dual luciferase reporter vectors (DUSP12-WT or DUSP12-MUT). Subsequently, DUSP12-WT or DUSP12-MUT 3'UTR plasmids containing Oe-FOXP1 or Oe-NC that were constructed by GenePharma were co-transfected into HUVECs using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h after transfection, the luciferase activities were determined through a dual-luciferase reporter assay kit (Promega Corporation).

Chromatin immunoprecipitation (ChIP)-seq analysis. A ChIP assay kit (Beyotime Institute of Biotechnology) was used to perform the ChIP-seq analysis, according to the manufacturer's instructions. Briefly, HUVECs were fixed with 1% formaldehyde for 10 min at room temperature. Next, $1x10^6$ HUVECs were collected via centrifugation at 300 x g for 3 min at 25˚C and washed twice with phosphate-buffered saline. Subsequently, HUVECs were lysed RIPA buffer (Cell Signaling Technology, Inc.) and sonicated for 30 min. The sonicated cell lysates (2 μl) were immuno-precipitated with 4 μg antibodies against FOXP1 (1:800; cat. no. ab134055; Abcam) or IgG (negative control, 1:800; cat. no. ab109489; Abcam) at 4˚C overnight. The next day, the samples were conjugated with Protein A agarose (Invitrogen; Thermo Fisher Scientific, Inc.) for 6 h at 4˚C. Finally, the immunoprecipitated DNAs were purified with a ChIP DNA purification kit (Beyotime Institute of Biotechnology) and amplified by virtue of qPCR as aforementioned. RT-qPCR was applied to analyze the enriched DNA. The primers used for the ChIP assay were the same as those used in RT-qPCR.

Statistical analysis. All experiments were replicated for three times. Statistical analyses were performed using SPSS software (version no. 22.0; IBM Corp. USA). Data are presented as the mean ± standard deviation. Data comparisons were made with one-way ANOVA followed by Tukey's post hoc test. $P<05$ was considered to indicate a statistically significant difference.

Results

Overexpression of DUSP12 inhibits apoptosis in ox-LDL-induced HUVECs. DUSP12 was overexpressed in HUVECs and the transfection efficiency was measured by RT-qPCR and western blotting. The results showed that compared with that in the Oe-NC group, the expression of DUSP12 was significantly increased in the Oe-DUSP12 group, indicating successful transfection (Fig. 1A). The cells were then divided into control, ox-LDL, ox-LDL + Oe-NC and ox-LDL + Oe-DUSP12 groups. The results of RT-qPCR and western blotting showed that DUSP12 expression in the ox-LDL group was significantly decreased compared with that in the control group. Compared with that in the ox-LDL + Oe-NC group, DUSP12 expression was decreased in the ox-LDL + Oe-DUSP12 group (Fig. 1B). CCK-8 results showed that ox-LDL significantly inhibited cell viability. After further overexpression of DUSP12, cell viability was recovered (Fig. 1C). TUNEL and western blot assays found that ox-LDL significantly promoted apoptosis, decreased the expression of Bcl-2 and increased expression of Bax and cleaved PARP. Compared with that in the ox-LDL + Oe-NC group, apoptosis was significantly decreased in ox-LDL + Oe-DUSP12 group (Fig. 1D and E).

Overexpression of DUSP12 inhibits inflammation and oxidative stress damage in ox-LDL-induced HUVECs. The effect of DUSP12 expression on ox-LDL-induced cellular inflammation was investigated and ELISA results showed that the levels of TNF-α, IL-1β and IL-6 were significantly increased in the ox-LDL group compared with those in the control group. Compared with those in the ox-LDL + Oe-NC group, the levels of IL-6, IL-1β and TNF-α were inhibited in the ox-LDL + Oe-DUSP12 group (Fig. 2A). Western blotting of the inflammatory proteins vascular cell adhesion molecule 1 (VCAM-1) and intracellular cell adhesion molecule 1 (ICAM-1) showed that the expression levels of these two proteins were significantly increased after ox-LDL induction. The effect on the expression of VCAM-1 and ICAM-1 was reversed after overexpression of DUSP12 (Fig. 2B). DCFH-DA fluorescent probe was used to measure ROS levels in HUVECs. The results showed that the ROS levels in the ox-LDL group were significantly higher than those in the control group. The levels

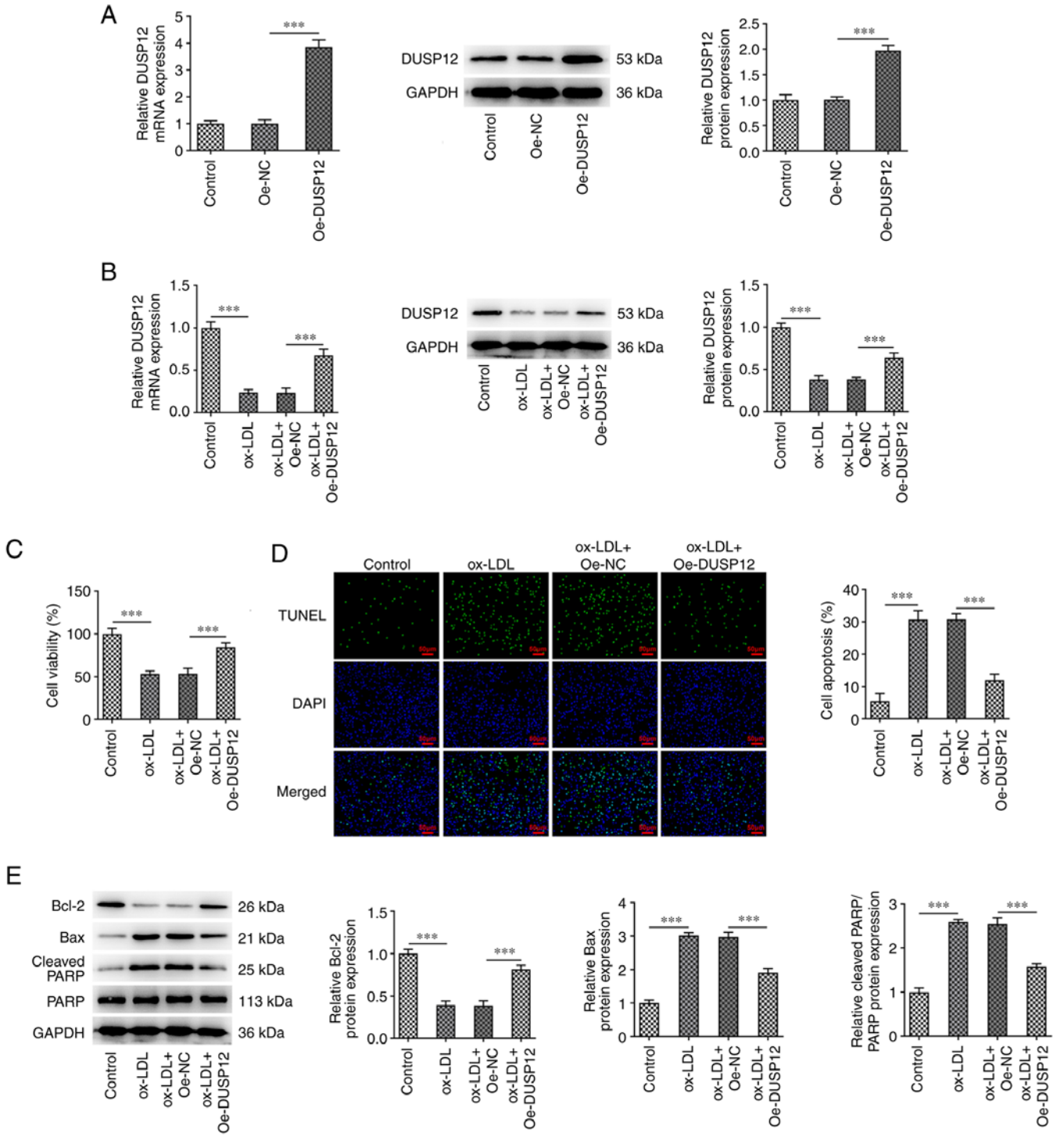

Figure 1. Overexpression of DUSP12 inhibits apoptosis in ox-LDL-induced HUVECs. (A) DUSP12 was overexpressed in HUVEC, and the transfection efficiency was measured using RT-qPCR and western blotting. (B) Cells were then divided into control, ox-LDL, ox-LDL + Oe-NC and ox-LDL + Oe-DUSP12 groups and the expression of DUSP12 was measured using RT-qPCR and western blotting. (C) Cell Counting Kit-8 assay was used to measure cell viability. (D) TUNEL assay was used to measure cell apoptosis. (E) Apoptosis-related proteins Bcl-2, Bax and cleaved PARP were measured by western blotting. ***P<0.001. DUSP12, dual specificity phosphatase 12; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; RT-qPCR, reverse transcription-quantitative PCR; Oe, overexpression; NC, negative control; PARP, poly (ADP-ribose) polymerase.

of ROS in the ox-LDL + Oe-DUSP12 group were significantly decreased compared with those in the ox-LDL + Oe-NC group (Fig. 3A). Subsequently, the levels of oxidative stress markers MDA, SOD and CAT were measured using commercial kits. The results showed that the activities of SOD and CAT were significantly decreased after ox-LDL induction, while the levels of MDA were significantly increased. Compared with those in the ox-LDL + Oe-NC group, SOD and CAT activities in the ox-LDL + Oe-DUSP12 group were significantly increased, while the MDA level was significantly decreased (Fig. 3B). The 8-OHdG level was measured using a specific commercial the kit and the results showed that ox-LDL

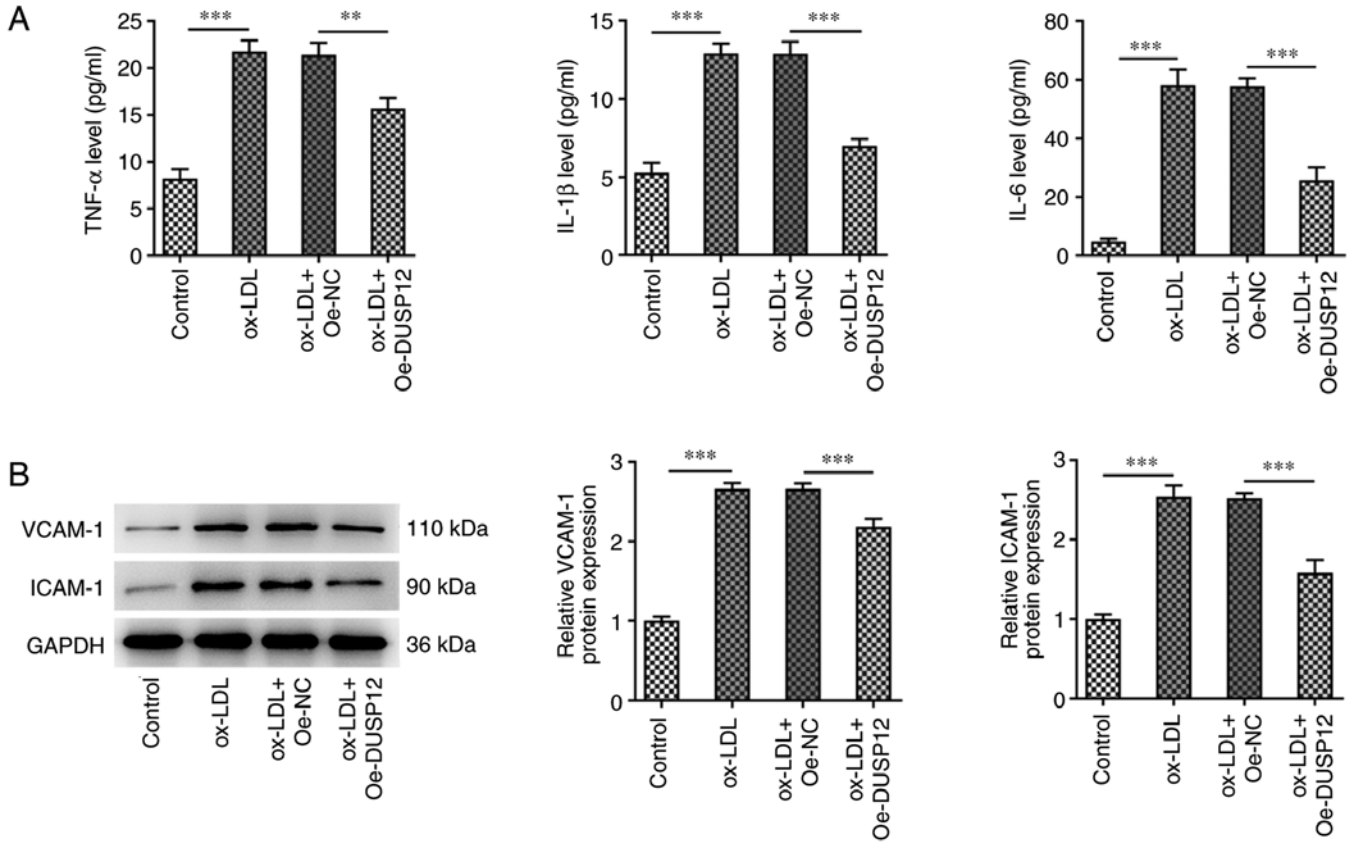

Figure 2. Overexpression of DUSP12 inhibits inflammation in ox-LDL-induced HUVECs. (A) ELISA was used to measure the inflammation-related indicators TNF-α, IL-1β and IL-6, to examine the effect of DUSP12 expression on ox-LDL-induced cellular inflammation. (B) Western blotting was used to measure the levels of inflammatory proteins VCAM-1 and ICAM-1. **P<0.01 and ***P<0.001. DUSP12, dual specificity phosphatase 12; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intracellular cell adhesion molecule 1.

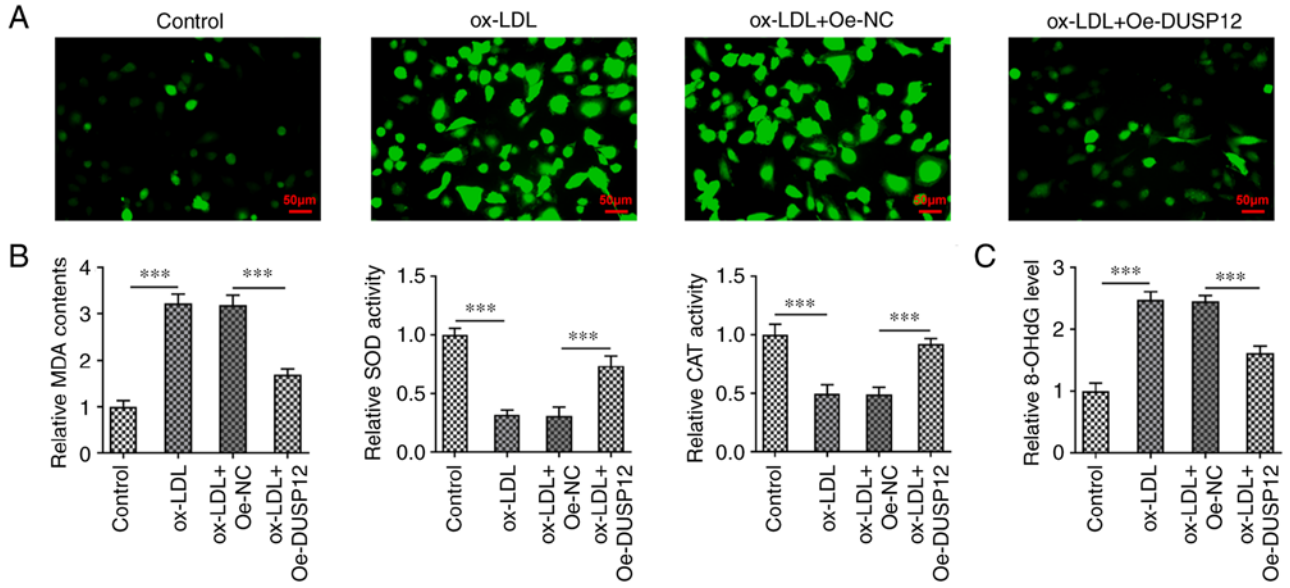

Figure 3. Overexpression of DUSP12 inhibits oxidative stress damage in oxidized low-density lipoprotein-induced HUVECs. (A) The DCFH-DA fluorescent probe was used to measure the levels of reactive oxygen species in cells. (B) Levels of oxidative stress markers MDA, SOD and CAT. (C) Levels of 8-OHdG. ***P<0.001. DUSP12, dual specificity phosphatase 12; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; DCFH-DA, 2',7'-dichlorofluorescein diacetate; 8-OHdG, 8-hydroxy-2-deoxyguanosine.

induced the increase of 8-OhdG in cells, while the level of 8-OHdG in HUVECs was decreased after overexpression of DUSP12 (Fig. 3C).

Overexpression of DUSP12 alleviates endothelial dysfunction in ox-LDL-induced HUVECs. The level of NO in HUVECs was significantly increased following ox-LDL induction.

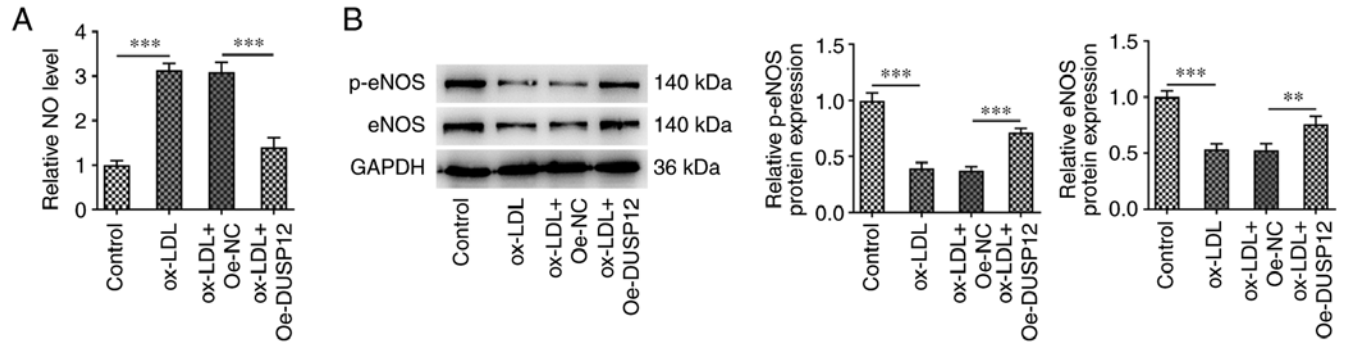

Figure 4. Overexpression of DUSP12 alleviates endothelial dysfunction in ox-LDL-induced HUVECs. (A) NO production was measured using a kit. (B) Western blotting was used to measure the expression of eNO synthase and peNOS. **P<0.01 and ***P<0.001. DUSP12, dual specificity phosphatase 12; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; NO, nitric oxide; p, phosphorylated; eNOS, endothelial NO synthase; Oe, overexpression; NC, negative control.

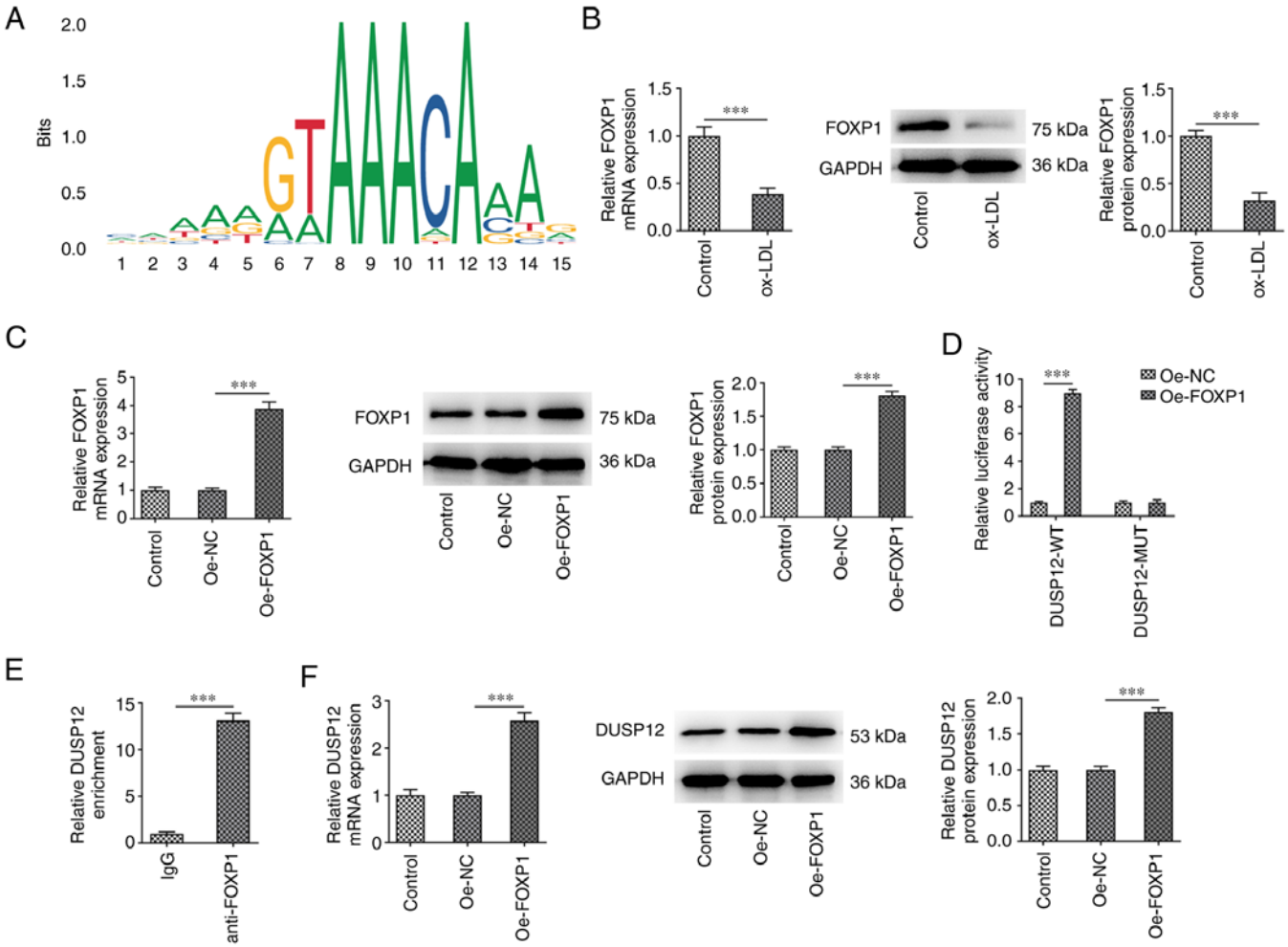

Figure 5. FOXP1 promotes the transcription of DUSP12 in ox-LDL-induced HUVECs. (A) JASPAR database predicted that the promoters of DUSP12 and transcription factors FOXP1 had binding sites. (B) RT-qPCR and western blotting were used to measure FOXP1 expression in ox-LDL-induced HUVECs. (C) FOXP1 was overexpressed in cells and its transfection efficiency was measured using RT-qPCR and western blotting. The binding between DUSP12 and FOXP1 was verified using (D) Dual-luciferase reporter and (E) Chromatin immunoprecipitation assays. (F) The expression of DUSP12 was measured by western blotting in cells after overexpressing FOXP1. ***P<0.001. FOXP1, Forkhead box P1; DUSP12, dual specificity phosphatase 12; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; RT-qPCR, reverse transcription-quantitative PCR; PARP, poly (ADP-ribose) polymerase.

After further overexpression of DUSP12, the level of NO in the cells was decreased (Fig. 4A). Western blotting showed that, compared with those in the control group, the expression levels of endothelial NO synthase (eNOS) and phosphorylated (p)-eNOS and eNOS decreased following ox-LDL induction. Compared with those in the ox-LDL + Oe-NC group, p-eNOS

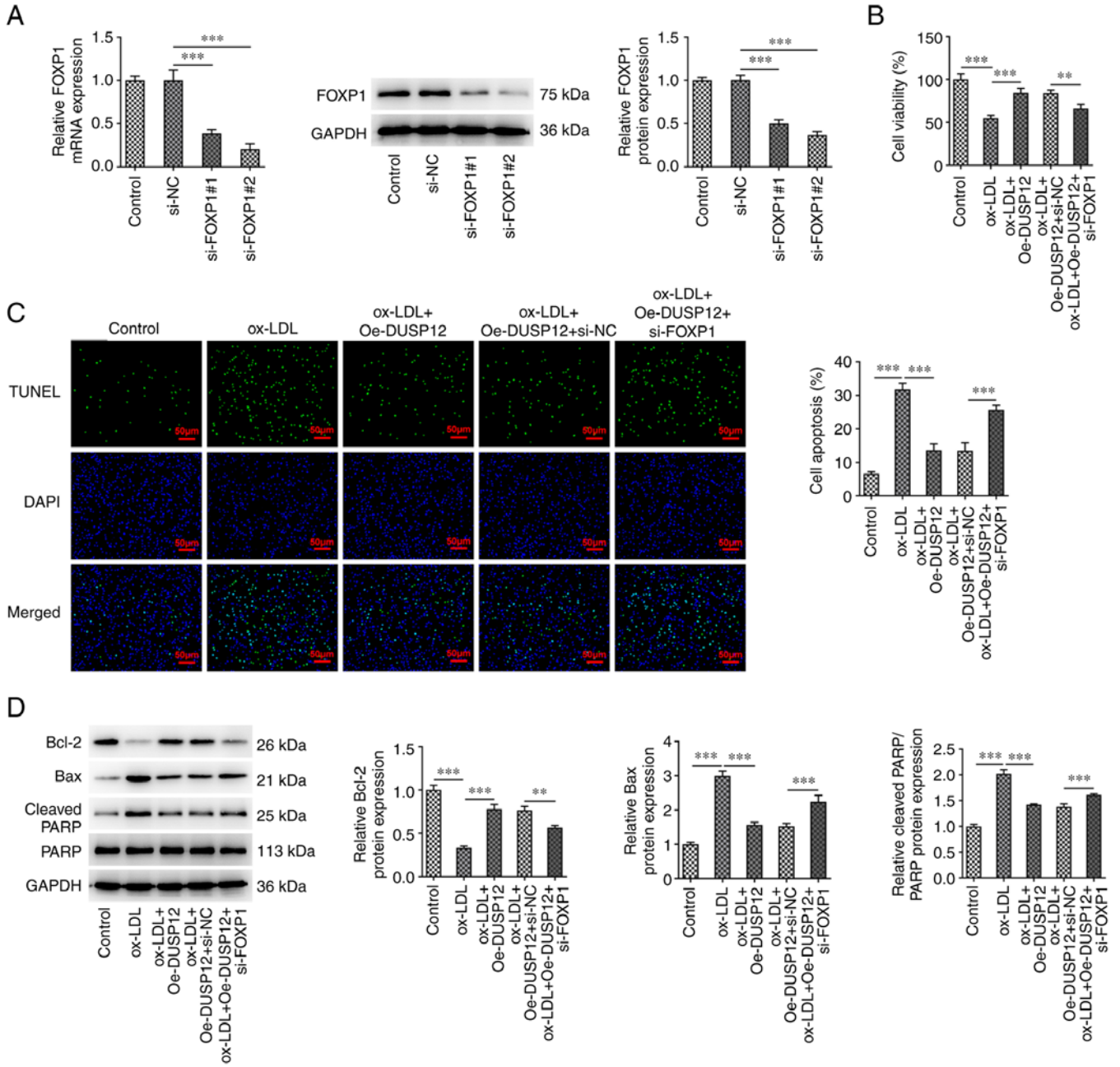

Figure 6. FOXP1 alleviates ox-LDL-induced apoptosis in HUVECs by regulating the expression of DUSP12. (A) The expression of FOXP1 in cells was inhibited using siRNA transfection and the transfection efficiency was measured using RT-qPCR and western blotting. (B) The cells were then divided into control, ox-LDL, ox-LDL + Oe-DUSP12, ox-LDL + Oe-DUSP12 + si-NC and ox-LDL + Oe-DUSP12 + si-FOXP1 groups and Cell Counting Kit-8 was used to measure the cell viability. (C) TUNEL assay was used to measure cell apoptosis. (D) The apoptosis-related proteins Bcl-2, Bax and cleaved PARP were measured by western blotting. **P<0.01 and ***P<0.001. FOXP1, Forkhead box P1; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; DUSP12, dual specificity phosphatase 12; si, short interfering; RT-qPCR, reverse transcription-quantitative PCR; Oe, overexpression; NC, negative control.

and eNOS protein levels were significantly increased in the ox-LDL + Oe-DUSP12 group (Fig. 4B).

FOXP1 promotes the transcription of DUSP12. The JASPAR database predicted that the promoters of DUSP12 and transcription factors FOXP1 had binding sites (Fig. 5A). Therefore, RT-qPCR and western blotting were used to measure FOXP1 mRNA and protein expression in HUVECs. The results showed that FOXP1 expression in cells was significantly decreased after ox-LDL induction (Fig. 5B). Subsequently, FOXP1 was overexpressed in HUVECs and the transfection efficiency was measured (Fig. 5C). The binding between DUSP12 and FOXP1 was verified using duel-luciferase reporter and ChIP assays (Fig. 5D and E). In addition, the expression of DUSP12 in cells was also significantly increased after overexpressing FOXP1 (Fig. 5F).

FOXP1 alleviates ox-LDL-induced apoptosis in HUVECs by regulating the expression of DUSP12. The expression of FOXP1 in cells was inhibited through siRNA transfection and the transfection efficiency was measured using RT-qPCR and western blotting (Fig. 6A). Since si-FOXP1#2 has a

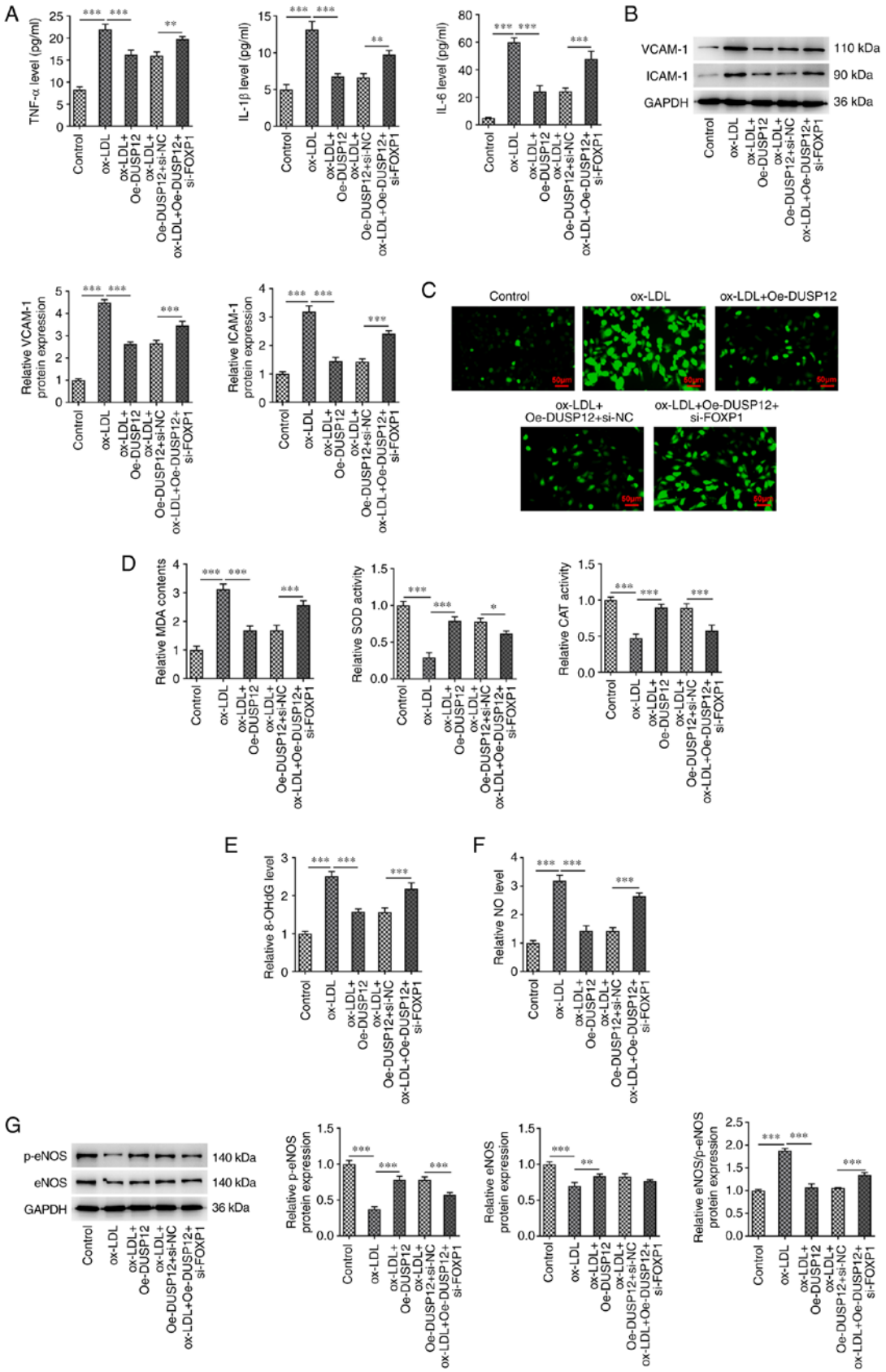

Figure 7. FOXP1 reduces ox-LDL-induced inflammation and oxidative stress injury in HUVECs by regulating the expression of DUSP12. (A) ELISA was used to measure the inflammation-related indicators IL-6, IL-1β and TNF-α to examine the effect of DUSP12 expression on ox-LDL-induced cellular inflammation. (B) Western blotting was used to measure the levels of inflammatory proteins vascular cell adhesion molecule 1 and intracellular cell adhesion molecule 1. (C) DCFH-DA fluorescent probe was used to measure the levels of reactive oxygen species levels. (D) Activities of MDA, SOD and CAT. (E) Levels of OHdG. (F) Levels of NO. (G) Western blotting analysis of the expression of eNOS and p-eNOS. *P<0.05, **P<0.01 and ***P<0.001. FOXP1, Forkhead box P1; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; DUSP12, dual specificity phosphatase 12; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde; DCFH-DA, 2',7'-dichlorofluorescein diacetate; 8-OHdG, 8-hydroxy-2-deoxyguanosine; NO, nitric oxide; eNOS, endothelial NO synthase; p, phosphorylated.

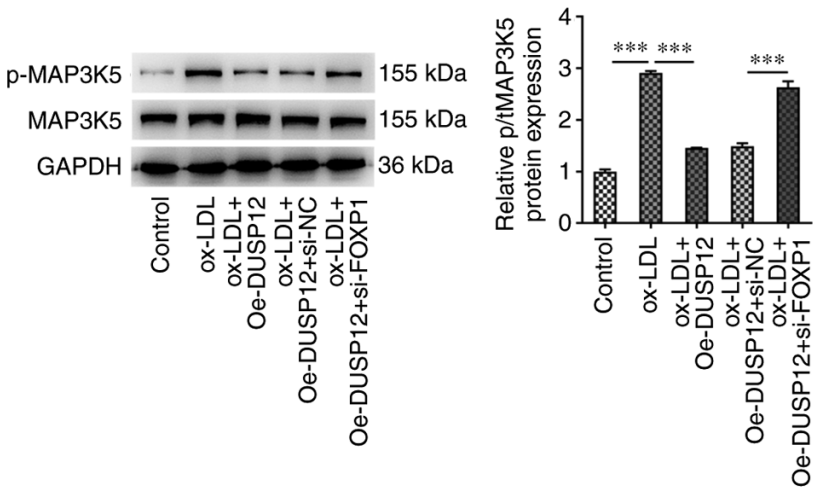

Figure 8. FOXP1 inhibits the MAP3K5 pathway by regulating the expression of DUSP12, thereby reducing ox-LDL-induced inflammation and oxidative stress injury in HUVECs. Western blotting was used to measure the expression of proteins related to the MAP3K5 signaling pathway downstream of DUSP12. ***P<0.001. FOXP1, Forkhead box P1; DUSP12, dual specificity phosphatase 12; ox-LDL, oxidized low-density lipoprotein; HUVECs, human umbilical vein endothelial cells; p, phosphorylated; si, short interfering; Oe, overexpression; NC, negative control.

more significant inhibition efficiency, this was chosen for follow-up experiments. HUVECs were divided into control, ox-LDL, ox-LDL + Oe-DUSP12, ox-LDL + Oe-DUSP12 + si-NC and ox-LDL + Oe-DUSP12 + si-FOXP1 groups. CCK-8 assay showed that cell viability in the ox-LDL + Oe-DUSP12 + si-FOXP1 group was significantly decreased compared with that in the ox-LDL + Oe-DUSP12 + si-NC group (Fig. 6B). TUNEL assay and western blotting showed that, compared with that in the ox-LDL + Oe-DUSP12 + si-NC group, apoptosis was significantly increased in the ox-LDL + Oe-DUSP12 + si-FOXP1 group, along with decreased levels of Bcl-2 and Bax and increased levels of cleaved PARP (Fig. 6C and D).

FOXP1 reduces ox-LDL-induced inflammation and oxidative stress injury in HUVECs by regulating the expression of DUSP12. Compared with those in the ox-LDL + Oe-DUSP12 + si-NC group, the levels of TNF-α, IL-1β and IL-6 were significantly increased (Fig. 7A), VCAM-1 and ICAM-1 protein levels were increased (Fig. 7B) in the ox-LDL + Oe-DUSP12 + si-FOXP1 group. Compared with those in the ox-LDL + Oe-DUSP12 + si-NC, ROS and MDA levels were increased in the ox-LDL + Oe-DUSP12 + si-FOXP1 group, while SOD and CAT activities were decreased (Fig. 7C and D). In addition, compared with that in the ox-LDL + Oe-DUSP12 + si-NC, the expression of 8-OhdG was significantly increased in the ox-LDL + Oe-DUSP12 + si-FOXP1 group (Fig. 7E). The analysis of endothelial dysfunction related indicators showed that the level of NO in the ox-LDL + Oe-DUSP12 + si-FOXP1 group was significantly higher than that in the ox-LDL + Oe-DUSP12 + si-NC group. However, the levels of p-eNOS and eNOS in the ox-LDL + Oe-DUSP12 + si-FOXP1 group decreased (Fig. 7F and G).

FOXP1 inhibits the MAP3K5 pathway by regulating the expression of DUSP12, thereby reducing ox-LDL-induced inflammation and oxidative stress injury in HUVECs. To further explore the regulatory mechanism, western blotting was performed to measure the expression of proteins related to the MAP3K5 signaling pathway downstream to DUSP12. The results showed that the level of p-MAP3K5 was significantly increased after ox-LDL induction compared with that in the control group. After further overexpression of DUSP12, the level of p-MAP3K5 in HUVECs was significantly inhibited. Compared with that in the ox-LDL + Oe-DUSP12 + si-NC group, the level of p-MAP3K5 was significantly increased in the ox-LDL + Oe-DUSP12 + si-FOXP1 group (Fig. 8).

Discussion

LDL and its modifier ox-LDL are the main lipoproteins deposited in atherosclerotic plaques in vascular walls and the level of ox-LDL in patients with AS is significantly higher compared with that of normal people; therefore, the level of ox-LDL in plasma can be used as an early screening indicator for AS (15). *In vitro* experiments show that ox-LDL has a damaging effect on vascular endothelial cells, which can be used as an *in vitro* model to study the mechanism of AS (16,17). In the present study, ox-LDL was used to induce HUVECs to form an *in vitro* endothelial cell injury model and the apoptosis of HUVEC was detected by CCK8 and Tunel assay. Apoptosis, inflammation, oxidative stress related indicators were tested to determine whether the HUVEC damage model had been successfully established.

Inflammation, oxidative stress and apoptosis of vascular endothelial cells are important processes in the development of AS. Once endothelial cells produce a large number of inflammatory cytokines and eventually undergo apoptosis, the vascular endothelium may lose its ability to regulate the equilibrium of the liposome and the regulation of immunity and inflammation, resulting in the deposition and retention of lipoproteins under the endodermis, forming an environment that promotes the formation of arterial plaque (18). Therefore, regulation of vascular endothelial cell inflammation and oxidative stress injury is a potential preventive and therapeutic approach for AS. Therefore, the current study was of

theoretical value and practical significance for the prevention and treatment of AS by seeking the causes of inflammation and oxidative stress of vascular endothelial cells and exploring their pathways.

A previous study showed that DUSP12 expression level is decreased in LPS-induced endothelial cells and mice overexpressing DUSP12 could reduce the inflammation and injury of vascular endothelial cells induced by LPS (19). However, to the best of the authors' knowledge, the role of DUSP12 in ox-LDL-induced HUVECs injury has yet to be reported. The present study showed that DUSP12 expression was significantly decreased in ox-LDL-induced HUVECs. Overexpression of DUSP12 significantly inhibited apoptosis, inflammation and oxidative stress in ox-LDL-induced HUVECs. In a recent study, overexpression of DUSP12 reduced the expression of lactate dehydrogenase, reduced the size of heart infarction in rats and inhibited the apoptosis of myocardial tissue and oxidative stress (20). Overexpression of DUSP12 inhibited the production of pro-inflammatory cytokines and chemokines that activate TLR4, stimulated heat-inactivated *Mycobacterium tuberculosis* and infected intracellular bacteria including *Listeria monomonas* and *Mycobacterium bovis BCG* by specifically inhibiting p38 and JNK (21). The aforementioned studies were consistent with the present results.

The binding between FOXP1 and the DUSP12 promoter was predicted using the JASPAR database. FOXP1 and the DUSP12 promoter had transcriptional regulatory effects in ox-LDL-induced HUVECs and FOXP1 promoted the transcription of DUSP12. FOXP1 is involved in cardiovascular disease (22). A previous study showed that endothelial FOXP1 could inhibit AS by regulating the activation of NLRP3 inflammasome (23). FOXP1 inhibits ox-LDL-induced inflammation and lipid accumulation in macrophages (11). In the current study, FOXP1 expression was significantly decreased in ox-LDL-induced HUVECs. Inhibition of FOXP1 expression in cells significantly reversed the inhibitory effect of overexpressed DUSP12 on ox-LDL-induced apoptosis and oxidative stress damage in HUVECs. These results suggested that, in AS disease, FOXP1 promoted transcription of DUSP12, thereby reducing ox-LDL-induced inflammation and oxidative stress injury in HUVECs.

The present study further explored the mechanism. Through literature review, it was found that DUSP12 reduced lung vascular endothelial cell injury in mouse models of LPS-induced acute lung injury through the MAP3K5-JNK pathway (19). DUSP12 could act as a novel endogenous protective signal against hepatic ischemia-reperfusion injury through the inhibition of MAP3K5 (8). In addition, inhibition of macrophage MAP3K5-JNK signaling may be a useful strategy for antagonizing AS diseases (24). Ox-LDL induced endoplasmic reticulum stress and endothelial cell injury through inflammasome activation mediated by the MAP3K5/NLRP3 signaling pathway (25). Therefore, it was hypothesized that DUSP12 was involved in ox-LDL-induced cellular inflammation and oxidative stress injury by regulating the downstream MAP3K5 signaling pathway. The current study demonstrated that FOXP1-induced DUSP12 alleviated vascular endothelial cell inflammation and oxidative stress injury induced by ox LDL via the MAP3K5 signaling pathway. The present findings provided a solid theoretical basis for the clinical treatment of AS disease.

The present study has some limitations. First, it employed cell experiments, but further verification is needed in animal experiments. Secondly, in terms of experimental grouping design, there was no comparison between the si-FOXP1 + Oe-DUSP12 group and the si-FOXP1 alone group, to confirm whether si-FOXP1 alone could have any effect in the absence of DUSP12 overexpression. This will also be improved in future experiments.

Therefore, the results indicated that FOXP1 inhibited the MAP3K5 pathway by regulating the expression of DUSP12, thereby reducing ox-LDL-induced inflammation and oxidative stress injury in HUVECs.

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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

WZ conceived the study. YL and LG performed the experiments and wrote the manuscript. JZ, CH and WZ processed the experimental data and analyzed the data. YL and WZ confirm the authenticity of all the raw data. All authors have 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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