

Semaphorin 7A knockdown improves injury and prevents endothelial-to-mesenchymal transition in ox-LDL-induced HUVECs by regulating β 1 integrin expression

XIAOYING SONG¹, JING MENG¹, GUOLIANG YAN², HAIHUI WANG², HAITAO LI² and DANFEI LOU¹

¹Department of Geriatrics; ²Emergency Department, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, Shanghai 200071, P.R. China

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Abstract. Atherosclerosis is the most common cause of cardiovascular disease and is accompanied by high mortality rates and a poor prognosis. Semaphorin 7A (Sema7A) and its receptor β 1 integrin have been reported to participate in the development of atherosclerosis. However, the role of Sema7A and β 1 integrin in endothelial cell injury and endothelial-to-mesenchymal transition (EMT) in atherosclerosis remains undetermined, to the best of our knowledge. The mRNA and protein expression levels of Sema7A and β 1 integrin in HUVECs were analyzed using reverse transcription-quantitative PCR (RT-qPCR) and western blot analyses, respectively. HUVECs were induced with 50 μ g/ml oxidized low-density lipoprotein (ox-LDL) to establish an atherosclerosis cell model. Cell viability was measured using Cell Counting Kit-8 assay and the production of IL-1 β , IL-6 and C-C motif chemokine ligand 2 was determined using ELISA. The expression levels of cell adhesion factors, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 were analyzed using RT-qPCR and western blot analyses. Cell apoptosis was detected using flow cytometry and western blotting. The levels of EMT-related markers were evaluated using RT-qPCR, western blotting and immunofluorescence staining. The results of the present study revealed that the expression levels of Sema7A and β 1 integrin were significantly upregulated in ox-LDL-treated HUVECs. Treatment with ox-LDL significantly decreased cell viability, and increased the levels of inflammatory and adhesion factors, the cell apoptotic rate and the expression levels of EMT-related proteins. Knockdown of Sema7A reversed the ox-LDL-induced

inflammatory responses and EMT, while the overexpression of β 1 integrin reversed the Sema7A-mediated inhibitory effects on ox-LDL-treated HUVECs. In conclusion, the findings of the present study indicated that Sema7A and β 1 integrin may play significant roles in atherosclerosis by mediating endothelial cell injury and EMT progression.

Introduction

Atherosclerosis is a chronic inflammatory disease of the large and medium-sized arteries, and is one of the most prominent causes of mortality worldwide (1-3). Atherosclerosis has been identified as the most common pathological process underlying diseases of the coronary, carotid and peripheral arteries (4). Several types of cells play crucial roles during the development of atherosclerosis, including endothelial cells, intimal smooth muscle cells and leukocytes (5,6). In normal vascular tissue, the normal morphology and functional integrity of endothelial cells permits them to engulf bacteria, as well as senescent and necrotic tissue. However, the lack of morphological integrity and dysfunction of vascular endothelial cells promotes inflammation and arterial thrombosis (7). In addition, damaged endothelial cells may undergo phenotypic changes in a process known as endothelial-to-mesenchymal transition (EMT), which allows endothelial cells to transform into mesenchymal- or myofibroblast-like cells (8). EMT is a complicated process characterized by the loss of specific endothelial markers, such as platelet endothelial cell adhesion molecule-1 and vascular epithelial calcitonin, and the acquisition of mesenchymal markers (9). Accumulating evidence has demonstrated that EMT is closely associated with the pathological processes of atherosclerosis and increases plaque calcification and plaque instability, leading to vascular stenosis (narrowing of the lumen) and ischemia in atherosclerotic tissues (10,11).

Semaphorins are a large class of secreted or transmembrane proteins, which were originally identified as regulators of neuronal growth (12). Previous studies have revealed that semaphorins serve important roles in several physiological processes, including angiogenesis, vascular development and immune responses (13,14). Semaphorin 7A (Sema7A) is a class 7 semaphorin that has a glycosylphosphatidylinositol-anchored

Correspondence to: Dr Danfei Lou, Department of Geriatrics, Shanghai Municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine, 274 Zhijiang Middle Road, Jing'an, Shanghai 200071, P.R. China
E-mail: ldf_shtcm062@163.com

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domain (15). Numerous studies have shown that Sema7A and its membrane receptor, β 1 integrin, exerted regulatory effects on neuronal growth, numerous cancer types and the inflammatory response (16-18). Furthermore, Sema7A and β 1 integrin have been reported to be associated with atherosclerosis progression in previous studies (19,20). However, to the best of our knowledge, research on the biological role of Sema7A in endothelial cell injury and EMT-associated atherosclerosis is currently limited.

The present study was undertaken to investigate the expression of Sema7A and β 1 integrin and their biological functions in atherosclerosis, and examine the effects of Sema7A/ β 1 integrin on the biological functions of oxidized low-density lipoprotein (ox-LDL)-treated HUVECs. The aim was to determine whether Sema7A can regulate cellular injury, apoptosis and EMT in ox-LDL-treated HUVECs by binding to β 1 integrin, and whether the Sema7A/ β 1 integrin axis may serve as a novel target for the treatment of endothelial cell injury and dysfunction in atherosclerosis.

Materials and methods

Cell culture and treatment. HUVECs were obtained from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% streptomycin/penicillin, and maintained with 5% CO₂ at 37°C. To induce inflammatory injury and EMT, HUVECs were pretreated with 50 μ g/ml ox-LDL (Peking Union-Biology Co. Ltd) for 24 h at 37°C.

Plasmid construction and cell transfection. To knock down Sema7A expression, specific short hairpin (sh)RNA targeting Sema7A (shRNA-Sema7A-1/2) and corresponding negative control (NC) shRNA (shRNA-NC) were synthesized by Shanghai Integrated Biotech Solutions Co., Ltd. To overexpress β 1 integrin, a pcDNA3.1 expression vector containing the entire length of β 1 integrin (Ov- β 1 integrin) and empty vector (Ov-NC) were constructed by Shanghai GenePharma Co., Ltd. These recombinants were transfected into HUVECs at 37°C, using Lipofectamine[®] 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. After transfection for 48 h, cells were harvested for subsequent experiments. In addition, transfected HUVECs were treated with 50 μ g/ml ox-LDL for 24 h at 37°C, and harvested as aforementioned.

Cell Counting Kit-8 (CCK-8) assay. The effects of Sema7A and β 1 integrin on HUVEC viability were detected using a CCK-8 assay. Briefly, HUVECs were seeded into 96-well plates (5x10³ cells/well) and transfected with shRNA-Sema7A with or without Ov- β 1 integrin and corresponding NCs for 48 h prior to treatment with 50 μ g/ml ox-LDL for 24 h. Following the incubation, 10 μ l CCK-8 reagent (MedChemExpress) was added to each well and incubated for a further 2 h at 37°C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from HUVECs from different treatment groups using TRIzol[®] reagent (Invitrogen; Thermo

Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA using a HiScriptQ RT SuperMix for qPCR (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. qPCR was subsequently performed using a SYBR Premix ExTaq kit (Takara Bio, Inc.) on an ABI Prism 7900 Real-Time PCR detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR thermocycling conditions were as follows: 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. A final extension step at 72°C for 7 min was performed for each assay. Each treatment group was ran five times. GAPDH was used as the endogenous control. The relative expression of the target genes was quantified using the 2^{- $\Delta\Delta$ C_q} method (21). The primer sequences used are as follows: Sema7A: Forward, 5'-TTCAGCCCGGACGAGA-3' and reverse, 5'-GAACCGAGGGATCTTCCCAT-3'; β 1 integrin: Forward, 5'-AGGAATGTTACACGGCTGCT-3' and reverse, 5'-ACCAAGTTTCCCATCTCCAG-3'; and GAPDH: Forward, 5'-GAAAGCCTGCCGGTGACTAA-3' and reverse, 5'-GCATCACCCGGAGGAGAAAT-3'.

ELISA. HUVECs were transfected with shRNA-Sema7A or shRNA-NC and Ov- β 1 integrin or Ov-NC, followed by treatment with ox-LDL for 24 h at 37°C. Then, the levels of inflammatory factors, including IL-1 β , IL-6 and C-C motif chemokine ligand 2 (CCL2), in the culture supernatant were measured using ELISA kits (cat. no. H002, H007-1-1 and H318-1, respectively; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

Western blotting. Total protein was extracted from ox-LDL-induced HUVECs transfected with shRNA-Sema7A with or without Ov- β 1 integrin and corresponding NCs using RIPA lysis buffer (CoWin Biosciences). Total protein was quantified using a Detergent Compatible Bradford Protein assay kit (Beyotime Institute of Biotechnology) and the proteins were separated via 10% SDS-PAGE. The separated proteins were subsequently transferred onto PVDF membranes and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were then incubated with the following primary antibodies overnight at 4°C: Anti-Sema7A (1:1,000; cat. no. ab255602), anti- β 1 integrin (1:1,000; cat. no. ab52971), anti-intracellular adhesion molecule (ICAM)-1 (1:1,000; cat. no. ab282575), anti-vascular cell adhesion molecule (VCAM)-1 (1:2,000; cat. no. ab134047), anti-Bax (1:2,000; cat. no. ab182733), anti-cleaved caspase-3 (1:500; cat. no. ab32042), anti-caspase-3 (1:5,000; cat. no. ab32351), anti-CD31 (1:1,000; cat. no. ab9498), anti-von Willebrand factor (vWF) (1:1,000; cat. no. ab154193), anti- α -smooth muscle actin (α -SMA) (1:1,000; cat. no. ab7817), anti-Snail family transcriptional repressor 2 (Slug) (1:1,000; cat. no. ab51772), anti-zinc finger E-box binding homeobox 1 (ZEB1) (1:500, ab203829) and anti-GAPDH (1:1,000; cat. no. ab8245; all from Abcam). Following primary antibody incubation, the membranes were washed four times with 0.05% PBS-Tween-20 and incubated with secondary antibodies [goat anti-rabbit IgG, HRP, cat. no. 7074; or goat anti-mouse IgG, HRP, cat. no. 7076; Cell Signaling Technology (1:2,000)] for 1 h at room temperature. Protein bands were visualized using an ECL detection system

(Beyotime Institute of Biotechnology) and semi-quantified using ImageJ software (version 1.49; National Institutes of Health).

Flow cytometry. The effects of the knockdown of *Sema7A* and *Ov-β1* integrin on cell apoptosis were evaluated using flow cytometry. Briefly, HUVECs were transfected with shRNA-*Sema7A* with or without *Ov-β1* integrin for 48 h, then induced for 24 h at 37°C with ox-LDL. The cells were harvested, washed and resuspended in binding buffer, then incubated with 5 μl Annexin V-FITC for 15 min and 10 μl PI (10 mg/ml) for 5 min in the dark. Apoptotic cells were detected by a flow cytometry (Accuri C6; BD Biosciences) and analyzed using FlowJo software (version 10.2; FlowJo LLC).

Immunofluorescence staining. Transfected or control HUVECs were plated into six-well plates with coverslips at a density of 4x10⁵ cells/well and allowed to reach 70-80% confluence. Subsequently, the cells were fixed with 4% paraformaldehyde for 30 min and incubated with 0.1% Triton X-100 for permeabilization for 7 min, both at room temperature. The slides were then blocked with 5% skimmed milk diluted in 0.01 M PBS for 1 h at room temperature, and incubated with an anti-α-SMA primary antibody (1:100; cat. no. ab184675; Abcam) overnight at 4°C. Following incubation, the slides were washed four times with PBS and incubated with a goat anti-mouse IgG H&L secondary antibody (1:200; cat. no. ab150113; Abcam) for 2 h at room temperature. Nuclei were counterstained with DAPI (Vector Laboratories, Inc.) for 5 min at room temperature. Finally, the slides were observed using a fluorescence microscope (magnification, x200; Olympus Corporation).

Statistical analysis. Statistical analysis was performed using SPSS version 19.0 software (IBM Corp.) and GraphPad Prism version 5.0 (GraphPad Software, Inc.) was used to draw the graphs. Data are presented as the mean ± SD from at least three independent experiments. Statistical differences between multiple groups were determined using one-way ANOVA followed by Tukey's multiple comparisons test, while statistical differences between two groups were determined using an unpaired, two-tailed Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

***Sema7A* and *β1* integrin expression is upregulated in ox-LDL-induced HUVECs.** ox-LDL plays a key role in endothelial cell injury during the progression of atherosclerosis (22). To simulate lipid accumulation-induced dysfunction of the endothelium in atherosclerosis, HUVECs were treated with 50 μg/ml ox-LDL for 24 h. As shown in Fig. 1A, the mRNA expression levels of *Sema7A* and *β1* integrin were significantly in HUVECs upregulated following ox-LDL treatment. Similarly, ox-LDL upregulated the protein expression levels of *Sema7A* and *β1* integrin in HUVECs compared with the control group (Fig. 1B). These data suggested that the aberrant expression of *Sema7A* and *β1* integrin in HUVECs was associated with ox-LDL stimulation.

***Sema7A* knockdown and *Ov-β1* integrin ameliorate ox-LDL-induced impaired HUVEC viability.** To determine the role of *Sema7A* and *β1* integrin in atherosclerosis, the effects of *Sema7A* and *β1* integrin on the activities of HUVECs were investigated. As presented in Fig. 2A and B, *Sema7A*-knockdown vectors were transfected into HUVECs and the transfection efficiency was evaluated using RT-qPCR analysis and western blotting. According to the results, shRNA-*Sema7A*-1 exhibited the best interference efficiency, and was thus selected for the following experiments. Subsequently, the effect of *Sema7A* knockdown on *β1* integrin expression was determined. The results revealed that ox-LDL treatment notably upregulated the mRNA and protein expression level of *β1* integrin compared with the control group, while transfection with shRNA-*Sema7A*-1 inhibited the ox-LDL-induced upregulated expression of *β1* integrin (Fig. 2C and D). Furthermore, *β1* integrin overexpression vectors were transfected into *Sema7A*-knockdown cells and the transfection efficiency was investigated (Fig. 2E-G). The results of the CCK-8 assay revealed that cell viability was markedly repressed following ox-LDL induction, while the knockdown of *Sema7A* alleviated the suppressive effects of ox-LDL on cell viability. However, the overexpression of *β1* integrin reversed the *Sema7A* knockdown-induced increase in HUVEC viability (Fig. 2H).

***Sema7A* knockdown and *Ov-β1* integrin inhibit the ox-LDL-induced release of inflammatory cytokines and adhesion factors in HUVECs.** Inflammation and adhesion factors are closely associated with the loss of morphological and functional integrity of vascular endothelial cells in atherosclerosis (10). As shown in Fig. 3A, the production of IL-1β, IL-6 and CCL2 was significantly increased in HUVECs induced with ox-LDL compared with control cells. The knockdown of *Sema7A* abrogated the secretion of IL-1β, IL-6 and CCL2, while the overexpression of *β1* integrin enhanced the *Sema7A* knockdown-mediated decrease in the levels of IL-1β, IL-6 and CCL2. Moreover, the results from RT-qPCR and western blot analyses demonstrated that the stimulation with ox-LDL markedly upregulated the expression levels of ICAM-1 and VCAM-1 in HUVECs. The knockdown of *Sema7A* repressed the production of these adhesion factors, while these effects were blocked by *Ov-β1* integrin (Fig. 3B and C).

***Sema7A* knockdown and *Ov-β1* integrin prevent the apoptosis of HUVECs in response to ox-LDL.** Next, the effects of *Sema7A* knockdown and *Ov-β1* integrin transfection on the apoptosis of HUVECs were analyzed. As illustrated in Fig. 4A and B, the apoptotic rate of HUVECs induced with ox-LDL was markedly increased compared with the control cells. The knockdown of *Sema7A* reduced ox-LDL-induced cell apoptosis, while the overexpression of *β1* integrin reversed the suppressive effect of *Sema7A* knockdown on the apoptosis of HUVECs treated with ox-LDL. Furthermore, the incubation of HUVECs with ox-LDL downregulated Bcl-2 expression levels and upregulated the expression levels of Bax and cleaved caspase-3. The knockdown of *Sema7A* reversed the effects of ox-LDL, while transfection with *Ov-β1* integrin reversed the *Sema7A* knockdown-induced upregulation of

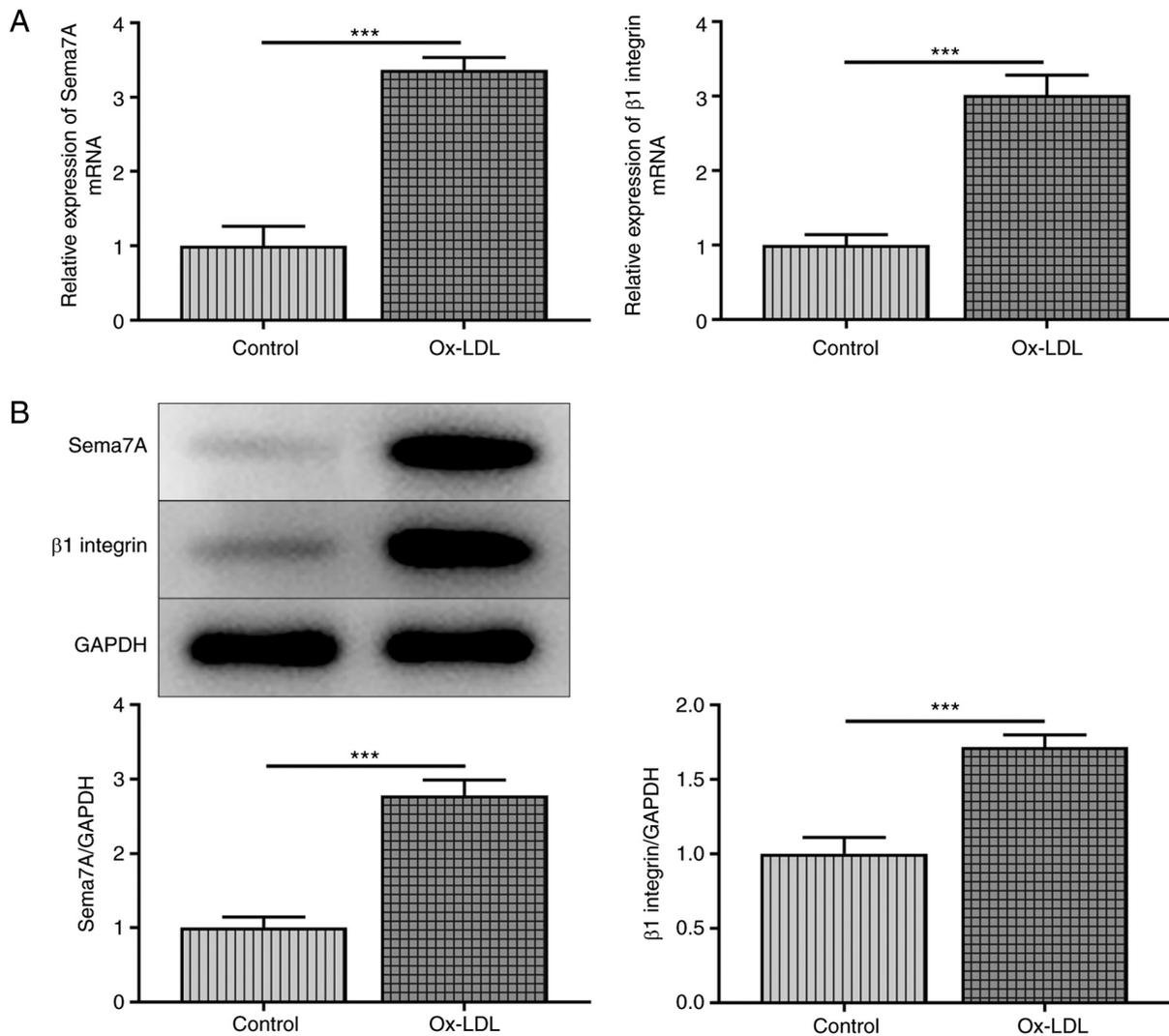


Figure 1. Sema7A and β 1 integrin expression is upregulated in HUVECs in response to ox-LDL. (A) Sema7A and β 1 integrin mRNA expression in untreated or ox-LDL-induced HUVECs was detected by reverse transcription-quantitative PCR analysis. (B) Western blot assay was performed to measure the protein levels of Sema7A and β 1 integrin in untreated or ox-LDL-induced HUVECs. Data are expressed as mean \pm SD. *** P <0.001 vs. control. Sema7A, semaphorin 7A; ox-LDL, oxidized low-density lipoprotein.

Bcl-2 protein expression levels and downregulation of Bax and cleaved caspase-3 expression levels (Fig. 4C).

Sema7A knockdown and Ov- β 1 integrin attenuate ox-LDL-induced EMT in HUVECs. To further determine the functional role of Sema7A and β 1 integrin in the EMT of HUVECs treated with ox-LDL, the protein and mRNA expression levels of EMT-related proteins were analyzed using western blotting and RT-qPCR, respectively. As shown in Fig. 5A and B, the expression levels of α -SMA, Slug and ZEB1 were upregulated, while the expression levels of CD31 and vWF were downregulated in HUVECs following induction with ox-LDL, and these effects were reversed by the knockdown of Sema7A and overexpression of β 1 integrin. In addition, the results of the immunofluorescence assay revealed that α -SMA expression levels were significantly upregulated after HUVECs were induced with ox-LDL, whereas they were downregulated by the knockdown of Sema7A expression. However, the overexpression of β 1 integrin inhibited the effects of Sema7A knockdown on ox-LDL-induced EMT in HUVECs (Fig. 5C).

Discussion

Atherosclerosis is the main cause of morbidity and mortality in cardiovascular diseases, and is responsible for more lives than all cancer types combined (23). The results of the present study revealed that the expression of Sema7A and β 1 integrin was dysregulated ox-LDL-induced HUVECs. In addition, Sema7A and β 1 integrin were serve key roles in ox-LDL-induced HUVECs by regulating cell viability, inflammatory responses, apoptosis and EMT. The knockdown of Sema7A expression increased cell viability, inhibited the release of inflammatory factors and adhesion molecules, suppressed cell apoptosis and inhibited the EMT process, whereas the overexpression of β 1 integrin reversed the effects of Sema7A silencing in HUVECs induced with ox-LDL.

Endothelial cells exist in the walls of blood and lymphatic vessels and have unique functions in vascular biology (24). Vascular endothelial cell injury is a common pathological basis of various cardiovascular diseases and represents the early stage of atherosclerosis (25). Hyperlipidemia, a

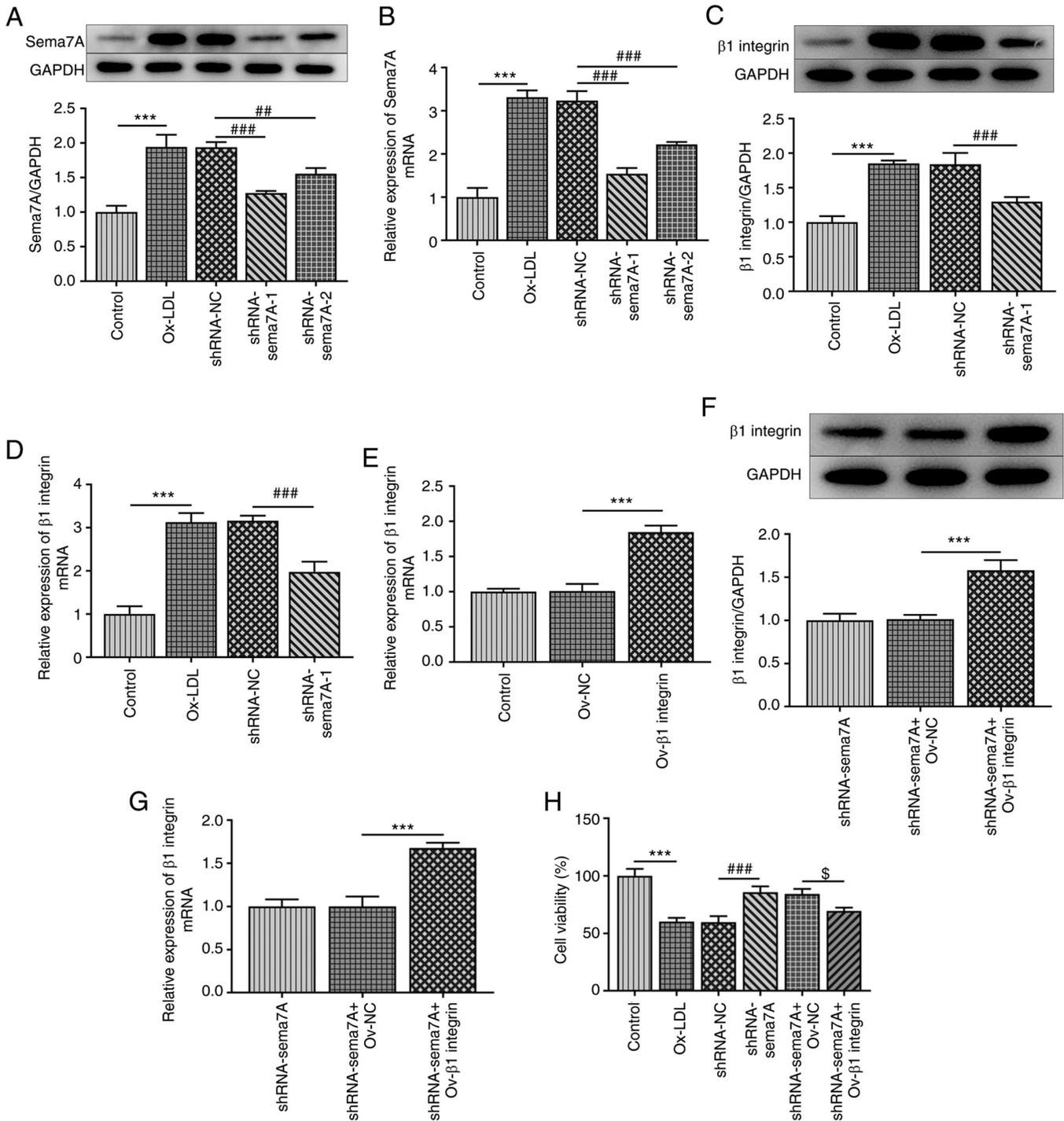


Figure 2. Effects of Sema7A and $\beta 1$ integrin on HUVEC viability. (A) Western blot assay was performed to measure the protein expression of Sema7A in ox-LDL-induced HUVECs transfected with shRNA-Sema7A. (B) RT-qPCR analysis was performed to measure the mRNA expression of Sema7A in ox-LDL induced HUVECs transfected with shRNA-Sema7A. (C) Western blot assay was performed to measure the protein levels of $\beta 1$ integrin in ox-LDL induced HUVECs transfected with shRNA-Sema7A. (D) RT-qPCR analysis was performed to measure the mRNA expression of $\beta 1$ integrin in ox-LDL-induced HUVECs transfected with shRNA-Sema7A. (E) RT-qPCR analysis was performed to measure the mRNA expression of $\beta 1$ integrin in HUVECs transfected with Ov- $\beta 1$ integrin. (F) Western blot assay was performed to measure the protein level of $\beta 1$ integrin in Sema7A-silenced HUVECs transfected with Ov- $\beta 1$ integrin. (G) RT-qPCR analysis was performed to measure the mRNA expression of $\beta 1$ integrin in Sema7A-silenced HUVECs transfected with Ov- $\beta 1$ integrin. (H) HUVEC viability was measured using a Cell Counting Kit-8 assay. Data are expressed as mean \pm SD. *** $P < 0.001$, ** $P < 0.01$ and # $P < 0.05$; Sema7A, semaphorin 7A; ox-LDL, oxidized low-density lipoprotein; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; Ov-, overexpression vector; NC, negative control.

cardiovascular risk factor, promotes the accumulation of plasma LDL in the arterial wall, which drives the formation of atherosclerotic plaques, and induces endothelial cell injury and apoptosis during the occurrence and development

of atherosclerosis (26,27). ox-LDL is a causal risk factor for atherosclerosis and binds to the scavenger receptor in endothelial cells to promote the secretion of inflammatory cytokines, inducing apoptosis and further promoting EMT (28,29).

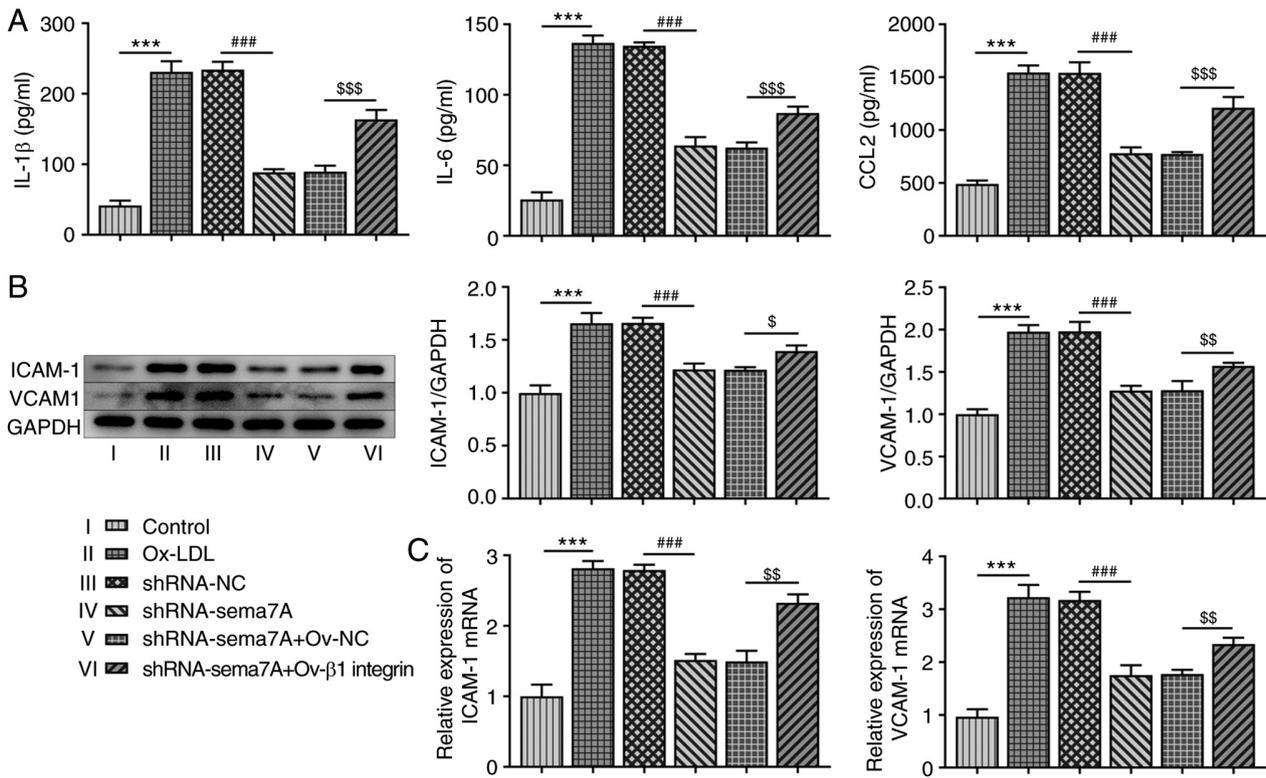


Figure 3. Effects of Sema7A and β 1 integrin on the levels of inflammatory cytokines and adhesion factors in HUVECs. (A) IL-1 β , IL-6 and CCL2 levels in ox-LDL induced HUVECs were examined after transfection with shRNA-Sema7A in the presence and absence of Ov- β 1 integrin. Western blot assay and reverse transcription-quantitative PCR were carried out to detect the (B) protein levels and (C) mRNA expressions of ICAM-1 and VCAM-1 in ox-LDL induced HUVECs transfected with shRNA-Sema7A in the presence and absence of Ov- β 1 integrin. Data are expressed as mean \pm SD. ***P<0.001 vs. control; ###P<0.001 vs. shRNA-NC; $^{\$}$ P<0.05, $^{5\$}$ P<0.01 and $^{55\$}$ P<0.001 vs. shRNA-Sema7A + Ov-NC. Sema7A, semaphorin 7A; ox-LDL, oxidized low-density lipoprotein; CCL2, C-C motif chemokine ligand 2; shRNA, short hairpin RNA; Ov-, overexpression vector; NC, negative control; ICAM-1, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule-1.

Vascular endothelial cell inflammation plays a central role in all stages of atherosclerosis, from lesion initiation to progression and destabilization (30). A previous study reported that ox-LDL induced a local inflammatory response, which inflamed the endothelium and upregulated the expression of adhesion molecules, leading to the adhesion of circulating leukocytes to the endothelium (31). Thus, in the present study, ox-LDL was used to stimulate HUVECs to mimic hyperlipidemia-induced endothelial cell injury. The results demonstrated that ox-LDL treatment significantly reduced HUVEC viability and induced the production of inflammatory factors, including IL-1 β , IL-6 and CCL2. In addition, the expression levels of ICAM-1 and VCAM-1 were markedly upregulated after HUVECs were exposed to 50 μ g/ml ox-LDL for 24 h. The present study also revealed that the knockdown of Sema7A abrogated the generation of ox-LDL-induced inflammatory factors, IL-1 β , IL-6 and CCL2, while the overexpression of β 1 integrin reversed these effects of Sema7A-knockdown. As expected, the upregulated expression levels of ox-LDL-induced ICAM-1 and VCAM-1 were downregulated by the knockdown of Sema7A and recovered by Ov- β 1 integrin transfection. These data indicated that silencing of Sema7A may protect against ox-LDL-induced inflammatory injury in endothelial cells by regulating β 1 integrin.

Inflammatory responses induced by injury stress signals, such as ox-LDL, mediate further interactions between monocytes and endothelial cells, resulting in increased apoptosis of

endothelial cells, which may initiate atherosclerosis (32-34). Wu *et al* (35) found that ox-LDL significantly induced HUVEC apoptosis, while the knockdown of proprotein convertase subtilisin/kexin type 9 suppressed apoptosis via the Bcl/Bax/caspase-9/caspase-3 signaling pathway. In addition, Zhong *et al* (36) demonstrated that myocardial infarction-associated transcript promoted cell proliferation and inhibited apoptosis in ox-LDL-induced atherosclerosis cell models by modulating the microRNA-181b/STAT3 signaling axis. Thus, the suppression of vascular endothelial cell apoptosis may promote pathological remission of atherosclerosis (37). In the present study, the apoptotic rate was increased in HUVECs exposed to ox-LDL, which was accompanied with downregulated expression levels of Bcl-2 and upregulated expression levels of Bax and cleaved caspase-3. However, these changes were mitigated by the knockdown of Sema7A, while β 1 integrin-overexpression abolished the inhibitory effects of Sema7A silencing, which is consistent with previous reports.

EMT serves key roles in plaque instability and atherosclerosis progression (38,39). Previous studies have reported that the formation and instability of atherosclerotic plaques are associated with endothelial cells acquiring morphological and phenotypic properties of mesenchymal cells, such as increased proliferation and migration, and the secretion of leukocyte adhesion molecules, extracellular matrix and matrix metalloproteinases (40). An accumulating number of studies have found that Sema7A and its receptor, β 1 integrin, contributed to vascular

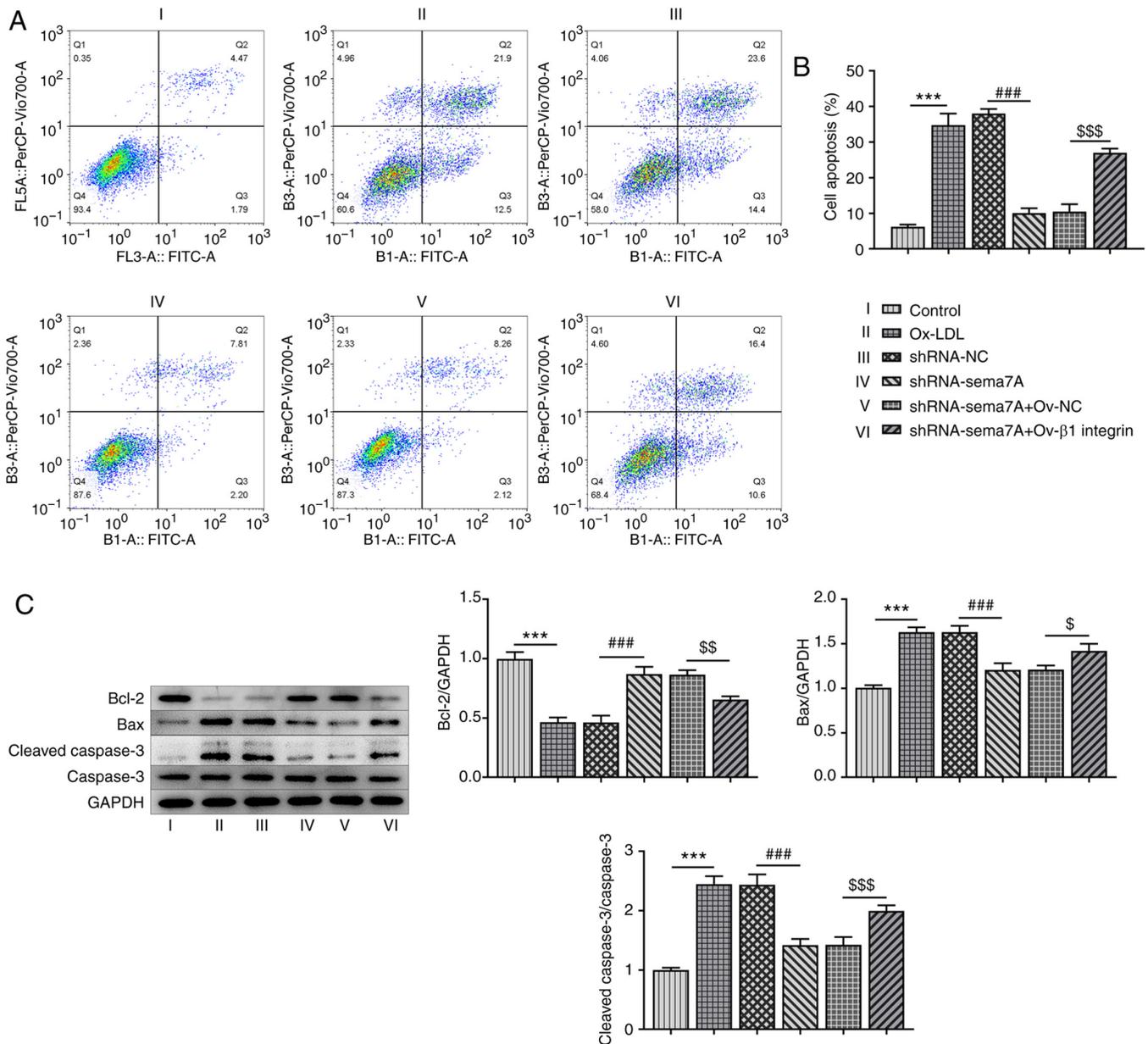


Figure 4. Effects of Sema7A and β 1 integrin on the apoptosis in ox-LDL-induced HUVECs. (A and B) Cell apoptosis was evaluated by flow cytometry in HUVECs transfected with shRNA-Sema7A in the presence and absence of Ov- β 1 integrin. (C) Bcl-2, Bax, caspase-3 and cleaved caspase-3 protein levels in HUVECs were determined using western blot assay after transfection with shRNA-Sema7A in the presence and absence of Ov- β 1 integrin. Data are expressed as mean \pm SD. ***P<0.001 vs. control; ###P<0.001 vs. shRNA-NC; \$P<0.05, \$\$P<0.01, \$\$\$P<0.001 vs. shRNA-Sema7A + Ov-NC. Sema7A, semaphorin 7A; ox-LDL, oxidized low-density lipoprotein; shRNA, short hairpin RNA; Ov-, overexpression vector; NC, negative control.

endothelial cell injury and the pathophysiology of atherosclerosis (19,20,41). In the present study, the expression levels of α -SMA, Slug and ZEB1 were found to be upregulated, while the expression levels of CD31 and vWF were downregulated under the induction of ox-LDL. The knockdown of Sema7A rescued the expression levels of EMT-related proteins, while β 1 integrin-overexpression counteracted the effects of Sema7A silencing on EMT in HUVECs. These findings indicated that the knockdown of Sema7A or overexpression of β 1 integrin may protect HUVECs against ox-LDL-induced EMT. In addition, the present study focused on the regulatory role of Sema7A silencing in ox-LDL HUVECs through modulating β 1 integrin expression, but did not explore whether Sema7A is involved in the regulation of β 1 integrin expression in ox-LDL HUVECs.

Furthermore, the results of the present study were not verified in animal experiments. Thus, the relationship between Sema7A and β 1 integrin and the underlying mechanisms will be further investigated *in vitro* and *in vivo* in future studies.

In conclusion, the findings of the present study suggested that the knockdown of Sema7A/ β 1 integrin may protect HUVECs from ox-LDL-induced injury by inhibiting the stimulation of inflammatory responses, cell apoptosis and EMT. Therefore, Sema7A and β 1 integrin may represent novel targets for the treatment of endothelial cell injury and dysfunction in atherosclerosis. Collectively, the findings of the present study may provide potential targets and therapeutic strategies against vascular endothelial cell inflammation and EMT to promote the pathological remission of atherosclerosis.

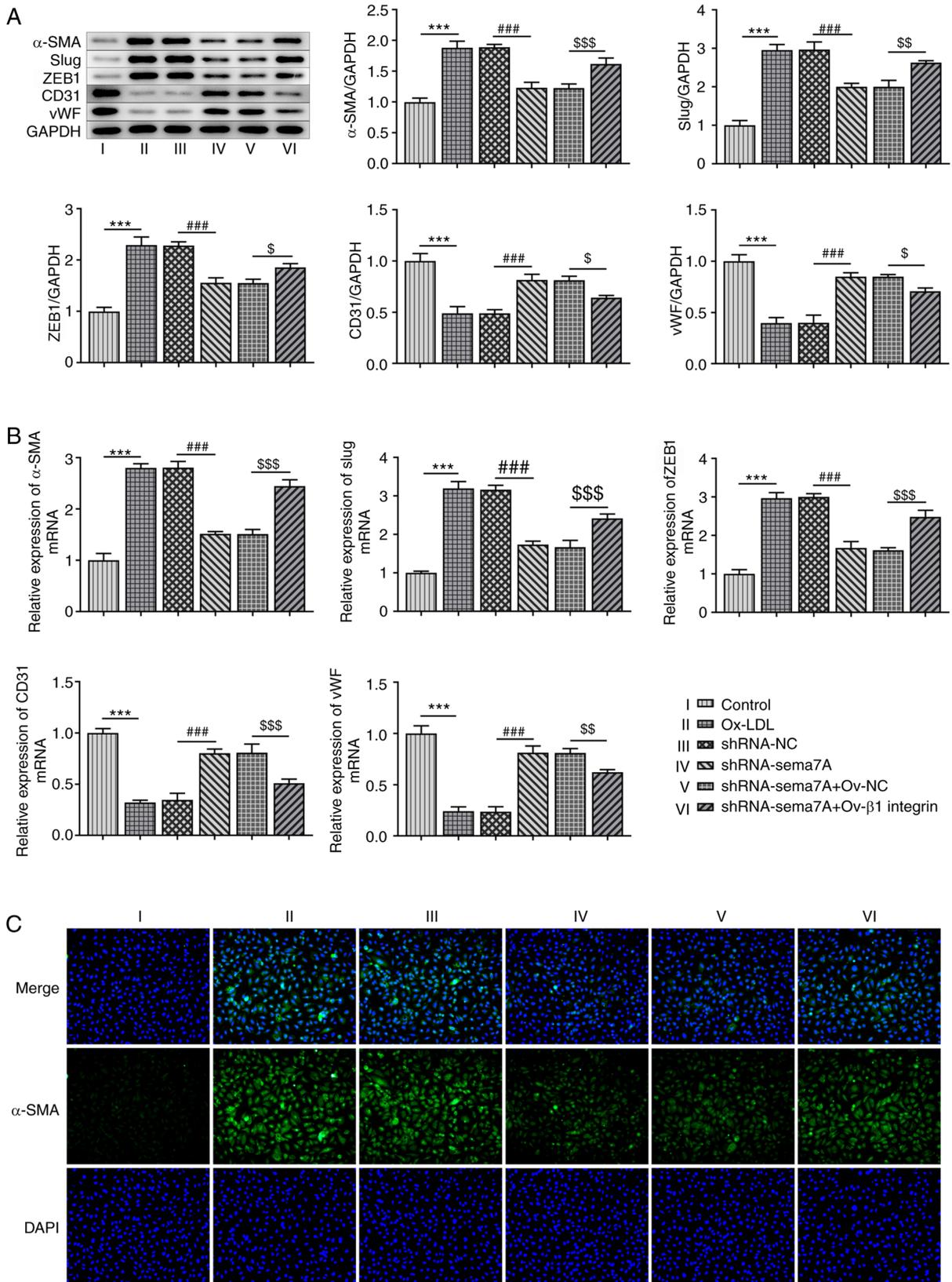


Figure 5. Effects of Sema7A and β 1 integrin on ox-LDL-induced epithelial-to-mesenchymal transition in HUVECs. (A) Western blot assay was used to determine the protein levels of α -SMA, Slug, ZEB1, CD31 and vWF in ox-LDL-induced HUVECs transfected with shRNA-Sema7A in the presence and absence of Ov- β 1 integrin. (B) Reverse transcription-quantitative PCR analysis was used to measure mRNA expression of α -SMA, Slug, ZEB1, CD31 and vWF were estimated by in ox-LDL-induced HUVECs transfected with shRNA-Sema7A in the presence and absence of Ov- β 1 integrin. (C) Immunofluorescence assay was performed to assess α -SMA protein levels in ox-LDL-induced HUVECs transfected with shRNA-Sema7A in the presence or absence of Ov- β 1 integrin. Data are expressed as mean \pm SD. *** P <0.001 vs. control; ### P <0.001 vs. shRNA-NC group; \$ P <0.05, \$\$ P <0.01, \$\$\$ P <0.001 vs. shRNA-Sema7A + Ov-NC group. Sema7A, semaphorin 7A; ox-LDL, oxidized low-density lipoprotein; α -SMA, α -smooth muscle actin; vWF, von Willebrand factor; Slug, Snail family transcriptional repressor 2; ZEB1, zinc finger E-box binding homeobox 1; shRNA, short hairpin RNA; Ov-, overexpression vector; NC, negative control.

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Availability of data and materials

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

Authors' contributions

XS and DL designed and supervised the experiments. XS, JM, GY, HW and HL performed the experiments. HW analyzed the data and HL searched the literature. XS, JM and DL wrote and revised the manuscript. XS and DL have seen and can confirm the authenticity of all the raw data. All the 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.

References

- Abdolmaleki F, Gheibi Hayat SM, Bianconi V, Johnston TP and Sahebkar A: Atherosclerosis and immunity: A perspective. *Trends Cardiovasc Med* 29: 363-371, 2019.
- Taleb S: Inflammation in atherosclerosis. *Arch Cardiovasc Dis* 109: 708-715, 2016.
- Viola J and Soehnlein O: Atherosclerosis - A matter of unresolved inflammation. *Semin Immunol* 27: 184-193, 2015.
- Falk E: Pathogenesis of atherosclerosis. *J Am Coll Cardiol* 47 (Suppl 1): C7-C12, 2006.
- Davies MJ, Woolf N, Rowles PM and Pepper J: Morphology of the endothelium over atherosclerotic plaques in human coronary arteries. *Br Heart J* 60: 459-464, 1988.
- Wolf D and Ley K: Immunity and inflammation in atherosclerosis. *Circ Res* 124: 315-327, 2019.
- Zhu Y, Xian X, Wang Z, Bi Y, Chen Q, Han X, Tang D and Chen R: research progress on the relationship between atherosclerosis and inflammation. *Biomolecules* 8: E80, 2018.
- Correia AC, Moonen JR, Brinker MG and Krenning G: FGF2 inhibits endothelial-mesenchymal transition through microRNA-20a-mediated repression of canonical TGF- β signaling. *J Cell Sci* 129: 569-579, 2016.
- Kovacic JC, Mercader N, Torres M, Boehm M and Fuster V: Epithelial-to-mesenchymal and endothelial-to-mesenchymal transition: From cardiovascular development to disease. *Circulation* 125: 1795-1808, 2012.
- Chen PY, Qin L, Baeyens N, Li G, Afolabi T, Budatha M, Tellides G, Schwartz MA and Simons M: Endothelial-to-mesenchymal transition drives atherosclerosis progression. *J Clin Invest* 125: 4514-4528, 2015.
- Hansson GK and Hermansson A: The immune system in atherosclerosis. *Nat Immunol* 12: 204-212, 2011.
- Simpson KJ, Henderson NC, Bone-Larson CL, Lukacs NW, Hogaboam CM and Kunkel SL: Chemokines in the pathogenesis of liver disease: So many players with poorly defined roles. *Clin Sci (Lond)* 104: 47-63, 2003.
- Wannemacher KM, Wang L, Zhu L and Brass LF: The role of semaphorins and their receptors in platelets: Lessons learned from neuronal and immune synapses. *Platelets* 22: 461-465, 2011.
- Suzuki K, Kumanogoh A and Kikutani H: Semaphorins and their receptors in immune cell interactions. *Nat Immunol* 9: 17-23, 2008.
- Gutiérrez-Franco A, Eixarch H, Costa C, Gil V, Castillo M, Calvo-Barreiro L, Montalban X, Del Río JA and Espejo C: Semaphorin 7A as a potential therapeutic target for multiple sclerosis. *Mol Neurobiol* 54: 4820-4831, 2017.
- Pasterkamp RJ, Peschon JJ, Spriggs MK and Kolodkin AL: Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. *Nature* 424: 398-405, 2003.
- Ma B, Herzog EL, Lee CG, Peng X, Lee CM, Chen X, Rockwell S, Koo JS, Kluger H, Herbst RS, *et al*: Role of chitinase 3-like-1 and semaphorin 7a in pulmonary melanoma metastasis. *Cancer Res* 75: 487-496, 2015.
- Suzuki K, Okuno T, Yamamoto M, Pasterkamp RJ, Takegahara N, Takamatsu H, Kitao T, Takagi J, Rennert PD, Kolodkin AL, *et al*: Semaphorin 7A initiates T-cell-mediated inflammatory responses through α 5 β 1 integrin. *Nature* 446: 680-684, 2007.
- Hu S, Liu Y, You T and Zhu L: Semaphorin 7A promotes VEGFA/VEGFR2-mediated angiogenesis and intraplaque neovascularization in ApoE^{-/-} mice. *Front Physiol* 9: 1718, 2018.
- You T, Zhu Z, Zheng X, Zeng N, Hu S, Liu Y, Ren L, Lu Q, Tang C, Ruan C, *et al*: Serum semaphorin 7A is associated with the risk of acute atherothrombotic stroke. *J Cell Mol Med* 23: 2901-2906, 2019.
- Wang GL, Xia XL, Li XL, He FH and Li JL: Identification and expression analysis of the MSP130-related-2 gene from *Hyriopsis cumingii*. *Genet Mol Res* 14: 4903-4913, 2015.
- Fang F, Yang Y, Yuan Z, Gao Y, Zhou J, Chen Q and Xu Y: Myocardin-related transcription factor A mediates OxLDL-induced endothelial injury. *Circ Res* 108: 797-807, 2011.
- Stocker R and Keaney JF Jr: Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84: 1381-1478, 2004.
- Sun HJ, Hou B, Wang X, Zhu XX, Li KX and Qiu LY: Endothelial dysfunction and cardiometabolic diseases: Role of long non-coding RNAs. *Life Sci* 167: 6-11, 2016.
- Sun HJ, Zhu XX, Cai WW and Qiu LY: Functional roles of exosomes in cardiovascular disorders: A systematic review. *Eur Rev Med Pharmacol Sci* 21: 5197-5206, 2017.
- Grover-Páez F and Zavalza-Gómez AB: Endothelial dysfunction and cardiovascular risk factors. *Diabetes Res Clin Pract* 84: 1-10, 2009.
- Pober JS, Min W and Bradley JR: Mechanisms of endothelial dysfunction, injury, and death. *Annu Rev Pathol* 4: 71-95, 2009.
- Zhang Y, Mu Q, Zhou Z, Song H, Zhang Y, Wu F, Jiang M, Wang F, Zhang W, Li L, *et al*: Protective effect of irisin on atherosclerosis via suppressing oxidized low density lipoprotein induced vascular inflammation and endothelial dysfunction. *PLoS One* 11: e0158038, 2016.
- Lee WJ, Ou HC, Hsu WC, Chou MM, Tseng JJ, Hsu SL, Tsai KL and Sheu WH: Ellagic acid inhibits oxidized LDL-mediated LOX-1 expression, ROS generation, and inflammation in human endothelial cells. *J Vasc Surg* 52: 1290-1300, 2010.
- Onat D, Brillion D, Colombo PC and Schmidt AM: Human vascular endothelial cells: A model system for studying vascular inflammation in diabetes and atherosclerosis. *Curr Diab Rep* 11: 193-202, 2011.
- Maugeri N, Rovere-Querini P, Baldini M, Sabbadini MG and Manfredi AA: Translational mini-review series on immunology of vascular disease: Mechanisms of vascular inflammation and remodelling in systemic vasculitis. *Clin Exp Immunol* 156: 395-404, 2009.

32. Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW and Medford RM: Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 92: 1866-1874, 1993.
33. Tsouknos A, Nash GB and Rainger GE: Monocytes initiate a cycle of leukocyte recruitment when cocultured with endothelial cells. *Atherosclerosis* 170: 49-58, 2003.
34. Lutgens E, de Muinck ED, Kitslaar PJ, Tordoir JH, Wellens HJ and Daemen MJ: Biphasic pattern of cell turnover characterizes the progression from fatty streaks to ruptured human atherosclerotic plaques. *Cardiovasc Res* 41: 473-479, 1999.
35. Wu CY, Tang ZH, Jiang L, Li XF, Jiang ZS and Liu LS: PCSK9 siRNA inhibits HUVEC apoptosis induced by ox-LDL via Bcl/Bax-caspase9-caspase3 pathway. *Mol Cell Biochem* 359: 347-358, 2012.
36. Zhong X, Ma X, Zhang L, Li Y, Li Y and He R: MIAT promotes proliferation and hinders apoptosis by modulating miR-181b/STAT3 axis in ox-LDL-induced atherosclerosis cell models. *Biomed Pharmacother* 97: 1078-1085, 2018.
37. Gong L, Lei Y, Liu Y, Tan F, Li S, Wang X, Xu M, Cai W, Du B, Xu F, *et al*: Vaccarin prevents ox-LDL-induced HUVEC EndMT, inflammation and apoptosis by suppressing ROS/p38 MAPK signaling. *Am J Transl Res* 11: 2140-2154, 2019.
38. Evrard SM, Lecce L, Michelis KC, Nomura-Kitabayashi A, Pandey G, Purushothaman KR, d'Escamard V, Li JR, Hadri L, Fujitani K, *et al*: Endothelial to mesenchymal transition is common in atherosclerotic lesions and is associated with plaque instability. *Nat Commun* 7: 11853, 2016.
39. Boström KI, Yao J, Guihard PJ, Blazquez-Medela AM and Yao Y: Endothelial-mesenchymal transition in atherosclerotic lesion calcification. *Atherosclerosis* 253: 124-127, 2016.
40. Wesseling M, Sakkars TR, de Jager SCA, Pasterkamp G and Goumans MJ: The morphological and molecular mechanisms of epithelial/endothelial-to-mesenchymal transition and its involvement in atherosclerosis. *Vascul Pharmacol* 106: 1-8, 2018.
41. Hu S, Liu Y, You T, Heath J, Xu L, Zheng X, Wang A, Wang Y, Li F, Yang F, *et al*: Vascular Semaphorin 7A upregulation by disturbed flow promotes atherosclerosis through endothelial β 1 integrin. *Arterioscler Thromb Vasc Biol* 38: 335-343, 2018.



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