



# RETRACTED: Platelet-Derived Exosomal MicroRNA-25-3p Inhibits Coronary Vascular Endothelial Cell Inflammation Through Adam10 via the NF- $\kappa$ B Signaling Pathway in ApoE<sup>-/-</sup> Mice

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### Specialty section:

This article was submitted to  
Inflammation,  
a section of the journal  
Frontiers in Immunology

Received: 19 April 2019

Accepted: 30 August 2019

Published: 02 October 2019

### Citation:

Yao Y, Sun W, Sun Q, Jing B, Liu S,  
Liu X, Shen G, Chen R and Wang H  
(2019) Platelet-Derived Exosomal  
MicroRNA-25-3p Inhibits Coronary  
Vascular Endothelial Cell Inflammation  
Through Adam10 via the NF- $\kappa$ B  
Signaling Pathway in ApoE<sup>-/-</sup> Mice.  
Front. Immunol. 10:2205.  
doi: 10.3389/fimmu.2019.02205

**Introduction:** Coronary artery disease originates from the blockage of the inner walls of the coronary arteries due to a plaque buildup. Accumulating studies have highlighted the role of microRNAs (miRs) delivered by exosomes in the progression of coronary artery disease. Thus, the current study was to elucidate the role and mechanism by which miR-25-3p influences oxidized low density lipoprotein (ox-LDL)-induced coronary vascular endothelial cell (CVEC) inflammation.

**Methods:** Primarily isolated CVECs were treated with ox-LDL to induce inflammation. Atherosclerosis models were induced in ApoE<sup>-/-</sup> mice and the peripheral blood platelet exosomes (PLT-Exo) were extracted and induced by thrombin, followed by co-culture with CVECs. The relationship between miR-25-3p and A disintegrin and metalloprotease 10 (Adam10) as well as the involvement of the NF- $\kappa$ B signaling pathway was evaluated. In order to evaluate the effect of PLT-Exo containing miR-25-3p on ox-LDL-induced CVEC inflammation, lipid accumulation and fibrosis, miR-25-3p mimic/inhibitor (*in vitro*), miR-25-3p agomir (*in vivo*), and si-Adam10 were delivered.

**Results:** MiR-25-3p was expressed poorly in ox-LDL-induced CVECs and vascular tissues but exhibited high levels of expression in thrombin-induced PLT-Exo of atherosclerosis models of ApoE<sup>-/-</sup> mice. CVECs endocytosed PLT-Exo upregulated the miR-25-3p expression. Adam10 was identified as a target gene of miR-25-3p. The thrombin-induced activated PLT-Exo carrying miR-25-3p reduced Adam10 expression to inhibit ox-LDL-induced CVEC inflammation and lipid deposition through downregulating levels of  $\alpha$ -smooth muscle actin, Collagen I  $\alpha$ 1, Collagen III  $\alpha$ 1, triglycerides, total cholesterol, interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$ . Furthermore, the NF- $\kappa$ B signaling pathway participated in the inhibitory effect of PLT-Exo carrying miR-25-3p.

**Conclusion:** Collectively, PLT-Exo overexpressing miR-25-3p attenuates ox-LDL-induced CVEC inflammation in ApoE<sup>-/-</sup> mouse models of atherosclerosis.

**Keywords:** microRNA-25-3p, coronary vascular endothelial cell, inflammation, Adam10, NF- $\kappa$ B signaling pathway, platelet, exosomes

## INTRODUCTION

Human coronary vascular endothelial cells (CVECs) are known as an anatomical interface between the vascular smooth muscle and the circulating blood, which are associated with inflammation (1). Endothelial cells play a vital role in inflammatory responses as well as in the immune system, and VECs activated by inflammation have been implicated in the progression of atherosclerosis (2). Atherosclerosis can be defined as a kind of chronic inflammatory disease, whose occurrence is strongly correlated with lipoproteins containing cholesterol, particularly low-density lipoprotein (LDL), which accumulates in the artery wall (3). Oxidized LDL (ox-LDL) acts as a substrate of the necrotic lipid and has been highlighted as a critical enhancer of atherosclerosis (4). Inflammation, the immune system, and macrophages have been implicated in the development and progression of coronary artery disease (5). Inflammation plays a central role in atherosclerosis in various stages of the disease, which when triggered can result in inflammatory alterations in the endothelium (6). The collection of inflammatory responses has been identified as a factor promoting the occurrence and continuation of ventricular and supraventricular arrhythmias in patients with coronary artery disease (7). Therefore, it is necessary to explore novel therapeutic approaches for limiting ox-LDL-induced CVEC inflammation.

Exosomes are derived from the endosomal compartment with a diameter of no more than 10 nm (8). Platelet-derived exosomes (PLT-Exo) account for approximately 70% of the total exosomes in blood serum and have been identified in a wide variety of critical pathophysiologic mechanisms including inflammation and atherogenesis (9). Exosomes are considered to be intercellular communication carriers from a microRNAs (miRs) perspective, providing a crucial method to obtain miR biomarkers in body fluids (10). Moreover, exosome-enclosed miR transfer is a novel communication method identified among cells, while the effect of miRs on various cardiovascular diseases as well as the role of exosomes as conveyors in immune responses have already been demonstrated (11). The significance of secretory miR-25-3p embedded in tumor-derived exosomes on VECs has been recognized (12). MiR-25-3p can be defined as the exudation of liposarcoma cells via extracellular vesicles, which interacts with the surrounding microenvironment to enhance cell metastasis, invasion, and proliferation in liposarcoma (13). Besides, miR-25-3p has recently been revealed to modulate C2C12 cell metabolism through its regulation of AKT1 (14). In addition, it has been noted that many miR-25 target mRNAs are implicated in a variety of biological procedures, including inflammation (15). The data provided by the online prediction

software as well as the dual luciferase reporter gene assay results of our study provided confirmation that A disintegrin and metalloprotease 10 (Adam10) is a target gene of miR-25-3p. Adam10 is a universal transmembrane metalloprotease that splits the extracellular area from more than 40 various target proteins across the membrane, which represents a vital factor in the process of inflammation (16). A recent study revealed the role of Adam10 in lipid accumulation induced by ox-LDL in glomerular podocytes (17). The nuclear factor kappa-B (NF- $\kappa$ B) signaling pathway represents an essential regulator of apoptosis and has been shown to play a role in a large number of cellular events (18). The role of the NF- $\kappa$ B signaling pathway in coronary artery disease has been linked with inflammation (19). Based on the aforementioned literature, we proposed the hypothesis that PLT-Exo carrying miR-25-3p targets Adam10 and influences the development of coronary artery disease by regulating the NF- $\kappa$ B signaling pathway. Hence, the current study was designed to verify this hypothesis and offer an enhanced understanding regarding the underlying molecular mechanisms associated with the progression of ox-LDL-induced CVEC inflammation, which may provide more effective therapeutic strategies for patients with coronary artery disease.

## MATERIALS AND METHODS

### Ethics Statement

All animal experiments were performed with the approval of the Ethics Committee of The First Affiliated Hospital of Harbin Medical University and followed the Guide for the Care and Use of Laboratory Animals. Extensive efforts were made to minimize animal use and suffering.

### Experimental Animals

Specific pathogen-free ApoE<sup>-/-</sup> mice of C57BL/6 inbred strain (Model Animal Research Center of Nanjing University, Nanjing, Jiangsu, China) were housed in laminar flow cabinets with controlled room temperature of 24–26°C and relative humidity of 40–60%. The room was radiated using ultraviolet at regular intervals. Cages, padding, drinking water and feeding stuffs were all sterilized under high pressure. The 8-week-old mice were placed on a high-fat diet (15% fat and 0.25% cholesterol) for 12 weeks. The mice were grouped as follows: ApoE<sup>-/-</sup> normal diet group, ApoE<sup>-/-</sup> high-fat diet group, ApoE<sup>-/-</sup> high-fat diet group, ApoE<sup>-/-</sup> high-fat diet + Exo group, and ApoE<sup>-/-</sup> high-fat diet + miR-25-3p agomir group (100 nM, intravenous injection into caudal veins every other day) ( $n = 7$  in each group).

The mice were euthanized 12 weeks after the experiment via excessive anesthesia with 3% pentobarbital sodium (P3761, Sigma-Aldrich, St. Louis, MO, USA). The mouse extremities were then fixed, after which the medial skin of the thorax abdomen was cut off in order to isolate the muscles and subcutaneous tissues and expose the heart and the aorta. After blood had been collected from the heart using 1 mL syringe, the heart and the aorta were extracted, after which the aorta was dissected along the vertical axis. Next, 2–3 cm samples were collected from the heart to the aortic root, fixed and subsequently sectioned. The sections were stained with hematoxylin-eosin (HE), oil-red O and

**Abbreviations:** miRs, MicroRNAs; ox-LDL, oxidized low density lipoprotein; CVEC, coronary vascular endothelial cell; PLT-Exo, peripheral blood platelet exosomes; TG, triglycerides; TC, total cholesterol; IL, interleukin; TNF, tumor necrosis factor; LDL, low-density lipoprotein; ox-LDL, Oxidized low density lipoprotein; miRs, microRNAs; SPE, Specific pathogen free; HE, hematoxylin-eosin; PBS, phosphate buffer saline; ELISA, Enzyme-linked immunosorbent assay; TEM, transmission electron microscope; BCA, bicinchoninic acid; MUT, mutant type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RIPA, radio-immunoprecipitation assay.

Masson, and subjected to immunohistochemistry. The area of the atherosclerosis lesion in each section was calculated, with the plaque area expressed as the ratio of the plaque to the superficial area of the aorta. Serum and plasma after centrifugation were preserved at  $-20^{\circ}\text{C}$ .

## Primary Isolation of CVECs

C57BL/6 mice (experimental animal center in the second affiliated hospital of Harbin Medical University, Harbin, Heilongjiang, China) and ApoE<sup>-/-</sup> mice of the C57BL/6 inbred strain (Model Animal Research Center of Nanjing University, Nanjing, China) were collected, and the coronary arteries of mice were isolated on a super clean bench. The coronary artery tissues were digested using a mixture of 0.25% trypsin (25200-056, Gibco Company, Grand Island, NY, USA) and collagenase (17101015, Gibco Company, Grand Island, NY, USA) and then dispersed into single-cell suspension, followed by incubation with CD31-labeled Dynal magnetic beads for magnetic separation. The supernatant was subsequently discarded, and beads were re-suspended and cultured in a CVEC special medium (Procell, Wuhan, Hubei, China) at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in saturation humidity. Following CD31 immunofluorescence detection (ab28364, 1: 20, Abcam, Cambridge, UK), the cells at passage 3–5 were utilized for subsequent experimentation.

## Immunofluorescence

After stable growth had been confirmed in the CVECs at passage three, the cells were rinsed with phosphate buffer saline (PBS), fixed by 4% paraformaldehyde, permeabilized, and sealed. The cells were then incubated with primary antibody to p65 (ab16502, 1: 1000, Abcam Inc. Cambridge, UK) at  $4^{\circ}\text{C}$  overnight. After another round of PBS rinsing, the cells were incubated with 2  $\mu\text{g}/\text{mL}$  fluorescence secondary antibody (A-21094, Thermo Fisher Scientific, Shanghai, China) and goat anti-rabbit fluorescence secondary antibody (ab150077, 1: 500, Abcam Inc., Cambridge, MA, USA) under conditions void of light for a 60 min period of incubation and subsequent rinsing under dark conditions. The cells were then covered by mounting medium containing 6-diamidino-2-phenylindole (DAPI) dye liquor (36308ES11, Shanghai Yisheng Biological Technology Co., Ltd., Shanghai, China) and cultured at room temperature for 3–5 min. Finally, the cells were observed and photographed under a fluorescence microscope (DMI8, Leica, Wetzlar, Germany).

## CVEC Treatment

CVECs were treated with ox-LDL (YB-002, Yiyuan Biotech, Guangzhou, Guangdong, China). After the cells had been confirmed to be exhibiting a stable growth state and upon reaching 80–90% confluence, they were synchronized with serum-free medium for 6 h and treated with 0, 25, 50, 75, and 100  $\mu\text{g}/\text{mL}$  ox-LDL for 24 h (20). The CVEC groups were assigned into the control and ox-LDL groups (0, 25, 50, 75, and 100  $\mu\text{g}/\text{mL}$ ).

After the CVECs had reached 80–90% confluence, the cells were transfected in accordance with the instructions of lipofectamine 2000 (11668-019, Invitrogen, Carlsbad, CA, USA).

The CVECs were co-transfected with 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p mimic negative control (NC) and PLT-Exo NC (ox-LDL + NC mimic + PBS group); 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p mimic and PLT-Exo NC (ox-LDL + miR-25-3p mimic + PBS group); 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p mimic NC and PLT-Exo (ox-LDL + NC mimic + PLT-Exo group); 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p mimic and PLT-Exo (ox-LDL + miR-25-3p mimic + PLT-Exo group); or co-transfected with 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p inhibitor NC and PLT-Exo NC (ox-LDL + NC inhibitor + PBS group); 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p inhibitor NC and PLT-Exo (ox-LDL + NC inhibitor + PLT-Exo group); 25  $\mu\text{g}/\text{mL}$  ox-LDL, miR-25-3p inhibitor and PLT-Exo (ox-LDL + miR-25-3p inhibitor + PLT-Exo group); or treated with miR-25-3p mimic NC (NC mimic group) and miR-25-3p mimic (miR-25-3p mimic group); or co-transfected with 25  $\mu\text{g}/\text{mL}$  ox-LDL and miR-25-3p mimic NC (ox-LDL + NC mimic); 25  $\mu\text{g}/\text{mL}$  ox-LDL and miR-25-3p mimic (ox-LDL + miR-25-3p mimic); 25  $\mu\text{g}/\text{mL}$  ox-LDL and Adam10 siRNA NC (ox-LDL + si-NC group); 25  $\mu\text{g}/\text{mL}$  ox-LDL and Adam10 siRNA (ox-LDL + si-Adam10 group); or co-transfected with 25  $\mu\text{g}/\text{mL}$  ox-LDL and PLT-Exo NC (ox-LDL + PBS group); 25  $\mu\text{g}/\text{mL}$  ox-LDL and PLT-Exo (ox-LDL + PLT-Exo group); 25  $\mu\text{g}/\text{mL}$  ox-LDL and Adam10 siRNA NC (ox-LDL + si-NC group); 25  $\mu\text{g}/\text{mL}$  ox-LDL and Adam10 siRNA (ox-LDL + si-Adam10 group). The concentration of miR-25-3p mimic and miR-25-3p inhibitor was 50 nM. Si-Adam10 had multiple uses with a concentration of 50 nM. The above-mentioned plasmids were purchased from (RiboBio Co., Ltd., Guangzhou, Guangdong, China).

## Enzyme-Linked Immunosorbent Assay (ELISA)

The supernatant was collected from the cell culture medium in order to examine the expression of interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) as per the instructions of the ELISA kits (RK00006/RK00008/RK00027, ABclonal, Wuhan, Hubei, China). Next, the levels of mice serum triglycerides (TG) and total cholesterol (TC) were measured based on the instructions of ELISA kits (tw037673/tw035400, Shanghai Tong Wei Biological Technology Co., Ltd., Shanghai, China). The blank, negative and positive wells were set as the controls for the reaction wells. The optical density (OD) value was measured at a wavelength of 450 nm using a microplate reader (BS-1101, Detie Experimental Equipment Co., Ltd., Nanjing, Jiangsu, China) after the blank control well had been zeroed.

## Extraction and Characterization of Mice PLT-Exo

The whole blood was collected from the heart of mice, added into acidic citric acid dextrose solution A at a ratio of 9: 1 and centrifuged at  $150 \times g$  at  $23^{\circ}\text{C}$  for 15 min. After the leukocytes had been filtered, the plasma-free platelet was collected following centrifugation at  $800 \times g$  for 15 min, and re-suspended in 1 mL PBS. Next, 0.1 U/mL thrombin (10602400001, Sigma-Aldrich, St. Louis, USA) was added for incubation at  $37^{\circ}\text{C}$  for 30 min in order to activate the platelet. The activated platelet was centrifuged for supernatant collection, which was incubated with

an ExoQuick-TC solution (EXOQ5A-1, System Biosciences, Palo Alto, CA, USA) at 4°C overnight. After centrifugation at 1,500 × g, the supernatant added with protease and phosphatase inhibitor (P1046, Beyotime Biotechnology Co., Ltd., Shanghai, China) was resuspended with 200 μL PBS and preserved at -80°C for the following experiment (21).

Next, a transmission electron microscope (TEM) was used to identify the exosomes. Initially, 20 μL of exosome was added to the copper wire mesh and permitted to stand for 3 min. After the excess liquid had been blotted using filter paper, the exosome was counterstained with 30 μL phosphotungstic acid solution (pH 6.8) at room temperature for 5 min, dried by baking under an incandescent light, and photographed under a TEM. Next, nanoparticle tracking analysis (Nanosight, Malvern Panalytical, Malvern, Alabama, UK) (22) was employed to analyze particle size. Western blot analysis was performed to identify the surface markers of the exosome. After the exosome suspension was concentrated, the protein level was determined using a bicinchoninic acid (BCA) kit (23227, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared for protein denaturation and electrophoresis. Membrane transfer was subsequently conducted, after which the levels of the exosome specific marker proteins tumor susceptibility gene 101 (TSG101) and CD63 were determined (23).

### Internalization of PKH67-Labeled Exosome by CVECs

After PLT-Exo had been extracted, the exosome was mixed with Diluent C, and promptly mixed with PKH67-Diluent C dye liquor for a 5 min period of incubation in accordance with the instructions of PKH67 (MINI61-KT, Sigma-Aldrich Chemical Company, St. Louis, MO, USA). Next, 0.5% bovine serum albumin (BSA) was applied to terminate the staining process. Afterwards, the exosome extraction kit was used to extract the exosome again, which was regarded as PKH-67-exosome. The CVECs were routinely cultured, settled over the plate, and further cultured with fresh medium after 48 h. Finally, the cells were incubated with PKH67-labeled exosome for 24 h, washed three times with PBS, fixed with 4% paraformaldehyde at room temperature for 30 min, washed three more times with PBS, stained with DAPI dye liquor for 5 min, observed and photographed by fluorescence.

### Dual-Luciferase Reporter Gene Assay

The online website was employed to predict the target relationship between Adam10 and miR-25-3p, which was then verified by dual-luciferase reporter gene assay. The dual-luciferase reporter gene vector of the target gene Adam10 and the mutant type (MUT) vector mutated on the miR-25-3p binding site were constructed respectively, namely, pGL3-Adam10 wild type (WT) and pGL3-Adam10 MUT. Next, the aforementioned two vectors were co-transfected with miR-25-3p plasmid and pRL-TK (internal reference plasmid expressing renilla luciferase) into HEK293 cells. After a 24 h period of transfection, the cells were lysed and the supernatant was collected as per the instructions of the TransDetect dual-luciferase reporter assay kit (FR201-01, Beijing TransGen Biotech Co., Ltd., Beijing, China).

Luciferase activity was detected using the Dual-Luciferase® Reporter Assay System (E1910, Promega Corp., Madison, WI, USA). Next, 100 μL luciferase reaction reagent that had been previously balanced to room temperature was added into the test tube and gently mixed with 20 μL cell lysate. After firefly luciferase was detected, renilla luciferase was detected with the addition of 100 μL luciferase reaction reagent II, which was also balanced to room temperature. The ratio of firefly luciferase to renilla luciferase (FL/RL) was regarded as relative luciferase activity.

### Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Mice PLT-Exo as well as the expression of miR-25-3p in coronary artery vessel tissues and Adam10 in CVECs were initially determined. Next, the total RNA of the cells and tissues together with exosome RNA was extracted using Trizol reagent (15596-018, Beijing Solarbio Life Sciences Co., Ltd., Beijing, China), after which the RNA concentration was measured. The primers used in the experiment were synthesized by Takara (Tokyo, Japan) (Table 1). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were regarded as the internal references. The relative transcription level of the target gene was calculated based on the  $2^{-\Delta\Delta Ct}$  method:  $\Delta\Delta Ct = \Delta Ct_{\text{experimental group}} - \Delta Ct_{\text{control group}}$ ,  $\Delta Ct = Ct_{\text{(target gene)}} - Ct_{\text{(internal reference)}}$  (24).

### Western Blot Analysis

The total protein of the tissues, cells and exosomes were extracted in strict accordance with efficient radio-immunoprecipitation assay (RIPA) lysis buffer (R0010, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The protein was then lysed at 4°C for 15 min and centrifuged at 25,764 × g for 15 min. Then, the supernatant was extracted using a BCA kit (20201ES76, Shanghai Yisheng Biological Technology Co., Ltd., Shanghai, China) in order to determine the protein concentration of each sample and quantified according to different concentrations. The isolation of the cell nucleus and cytoplasm was conducted using a kit (K266-25, Biovision, Milpitas, CA, USA). After being isolated by PAGE, the protein was transferred onto the polyvinylidene fluoride (PVDF) membrane via the wet transfer method and

**TABLE 1 |** Comparison of weight, TC, TG, high-density lipoprotein, and low-density lipoprotein.

Items	Control group	PLT-Exo group	MiR-25-3p agomir group
Weight (g)	35.23 ± 3.23	33.67 ± 0.36	34.66 ± 0.38
TC (mmol/L)	28.34 ± 2.87	11.23 ± 1.34*	9.36 ± 0.98*
TG (mmol/L)	1.76 ± 0.18	0.42 ± 0.15*	0.53 ± 0.12*
High-density lipoprotein (mmol/L)	4.51 ± 0.32	4.86 ± 0.57	4.59 ± 0.48
Low-density lipoprotein (mmol/L)	26.62 ± 0.28	25.79 ± 0.26	24.59 ± 0.25

\* $p < 0.05$  vs. the control group (ApoE<sup>-/-</sup> mice treated with high-fat diet). All data were measurement data and expressed as mean ± standard deviation and data among multiple groups were analyzed using one-way analysis of variance.  $n = 7$ . TC, total cholesterol; TG, triglyceride; miR-25-3p, microRNA-25-3p; PLT-Exo, platelet-exosome.

sealed with 5% BSA at room temperature for 1 h. The membrane was subsequently incubated with diluted primary antibodies rabbit anti-TSG101 (ab30871, 1: 1000), CD63 (ab68418, 1: 1000), IL-6 (ab229381, 1: 1000), TNF- $\alpha$  (ab6671, 1: 1000), IL-1 $\beta$  (ab2105, 1: 1000), p-p65 (ab86299, 1: 5000), Lamin B (ab16048, 1: 1000), alpha-smooth muscle actin ( $\alpha$ -SMA) (ab5694, 1: 1000), Collagen I a1 (Col1a1) (ab34710, 1: 5000), Collagen III a1 (Col3a1) (ab7778, 1: 5000), vascular cell adhesion molecule 1 (Vcam-1) (ab106777, 1: 1000), Adam10 (ab1997, 1: 1000), and mouse anti-intercellular adhesion molecule 1 (Icam-1) (ab171123, 1: 1000) as well as  $\beta$ -actin (ab82426, 1: 5000) on a table concentrator at 4°C overnight. The aforementioned antibodies were procured from Abcam Inc. (Cambridge, MA, USA). Afterwards, the membrane was rinsed by Tris Buffered Saline With Tween (TBST) three times (5 min each time) and incubated with diluted horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) (ab205718, 1: 20000, Abcam Inc., Cambridge, MA, USA) or goat anti-mouse (ab6789, 1: 5000, Abcam Inc., Cambridge, MA, USA) for 1 h at room temperature. After three TBST rinses (5 min each time), proteins were visualized. ImageJ 1.48u software (National Institutes of Health, Bethesda, Maryland, USA) was applied for protein quantification analysis, with reference to the gray value ratio of each protein to internal reference GAPDH.

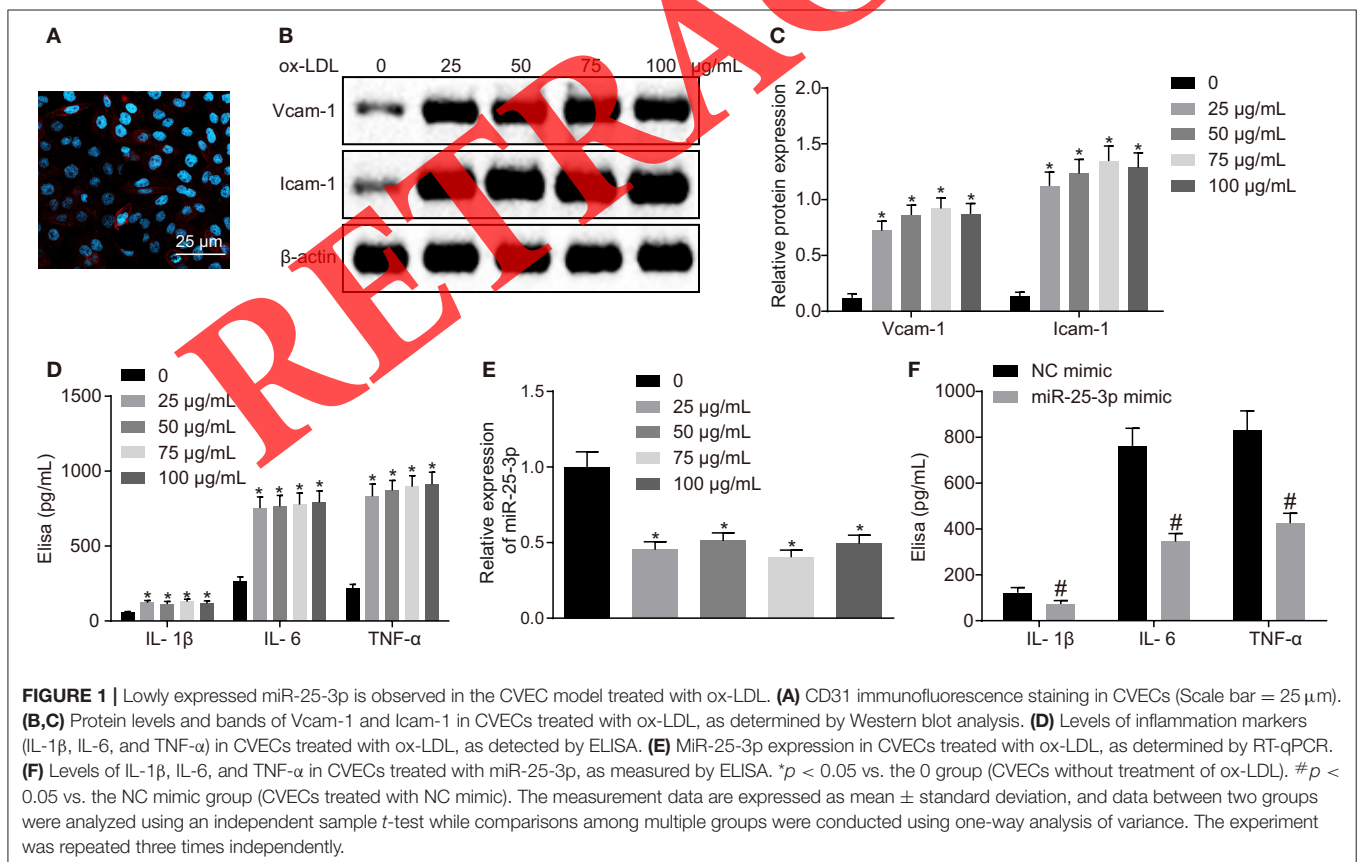
## HE Staining

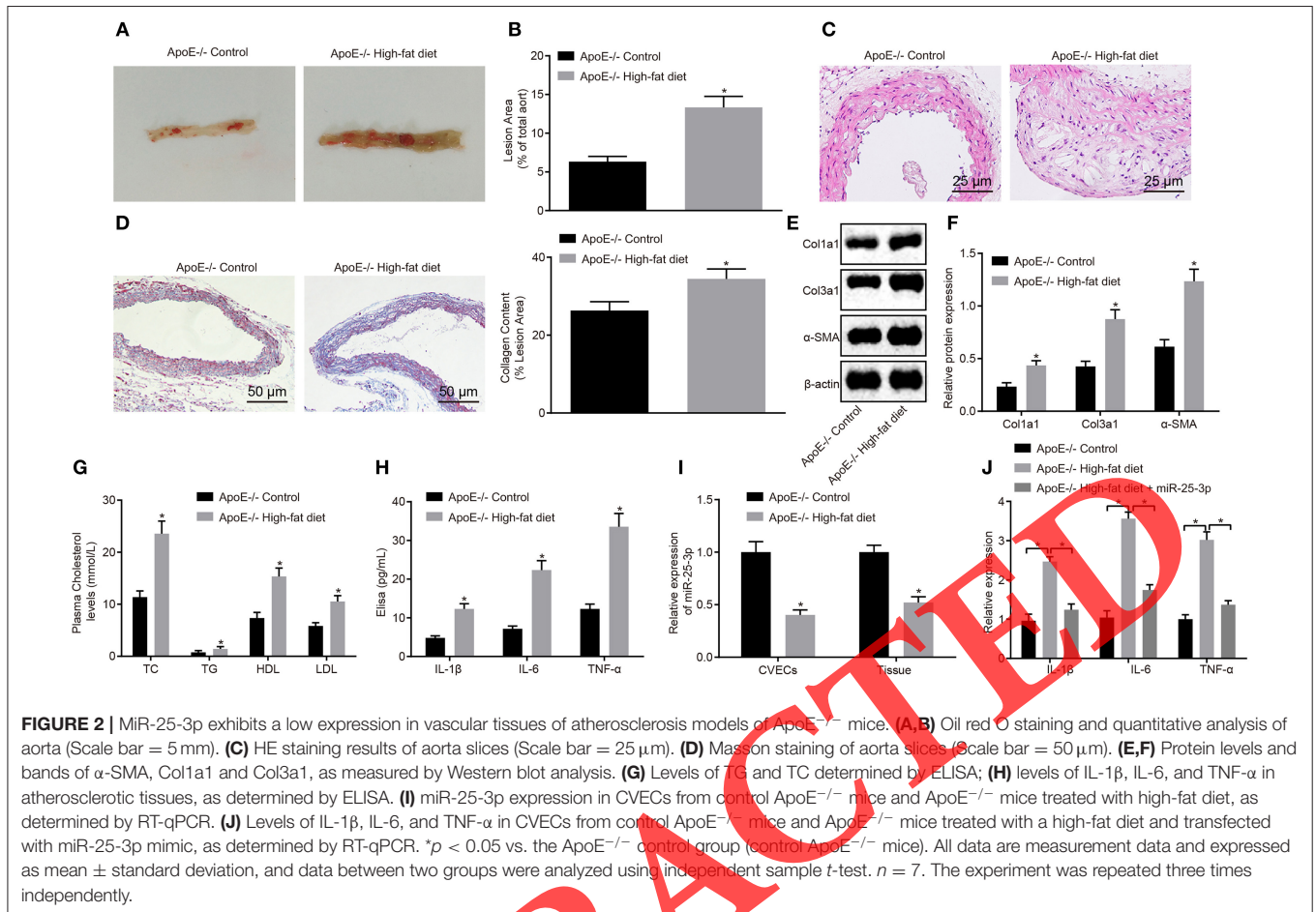
The paraffin slices were dewaxed by xylene I, xylene II, and ethanol with concentrations of 100, 95, 90, and 80%. After

the slices had been immersed under running water for 5 min, hematoxylin was applied to stain the cell nucleus for 5 min and then washed with water. Next, the slices were differentiated by 1% hydrochloric-alcohol solution for 30 s, washed with water and analyzed under a microscope. The slices were then completely immersed with eosin in order to stain the cytoplasm, lightly washed with water, 80 and 95% ethanol I as well as 95% ethanol II after 15 min, and then immersed in 100% ethanol I for 5 min, 100% ethanol II for 10 min and xylene twice (10 min per time). Finally, the slices were sealed using neutral gum, observed and photographed under a light microscope.

## Oil Red O Staining

After the whole aorta was washed under running water for 30 min, and the adipose and muscular tissues surrounding the aorta were carefully stripped. Next, the blood vessels were vertically dissected and immersed in distilled water for 30 min. A total of 60 mL filtered oil red O solution was collected followed by the addition of distilled water into 100 mL to prepare working dye liquor. After uniform mixing for 10 min, the working dye liquor was filtered and utilized for staining for 0.5–1 h. The specimens were subsequently immersed by 70% ethanol for differentiation until plaque was confirmed to have turned red while a non-plaque region was considered to have white transparency. The specimens were then rinsed with distilled water and cautiously spread onto a glass slide and photographed (25).





## Masson's Trichrome Staining

Masson's trichrome staining was performed based on the instructions of the kit (Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, Jiangsu, China). One drop of Masson compound staining solution (Agent A) was added to stain the frozen slice of mouse aorta for 5 min, followed by reaction with one drop of phosphomolybdic acid (Agent C) for 5 min. One drop of aniline blue (Agent D) was subsequently added in order to stain the slice for 5 min, followed by reaction with one drop of dissimulation solution (Agent B) two times (30–60 s per time). Finally, the slice was dehydrated by 95% ethanol and anhydrous ethanol, permeabilized and mounted.

## Immunohistochemistry

The frozen slices were taken out from the -20°C refrigerator, rewarmed at room temperature, and fixed in iced acetone. The acetone was then air-dried, yet the slices could not be dried. The slices were then placed into an antigen retrieval box, rinsed three times with PBS and placed onto a table concentrator. The slices were immersed with 0.3% tritonX-100 at room temperature to fracture the cytomembrane. After the surrounding water drops had been dried, the slices were sealed with 5% BSA. The slices were incubated with diluted primary antibody Adam10 (ab1997, 1: 500, Abcam Inc., Cambridge, MA, USA) on a

humidity chamber at 4°C overnight. HRP-labeled goat anti-rabbit IgG antibody (ab6721, 1: 1000, Abcam Inc., Cambridge, MA, USA) was added to incubate the slice for 30 min. Next, hematoxylin was applied in order to re-stain the nucleus for 1 min, followed by ethanol dehydration, xylene permeabilization and balsam mounting. The brown particles were considered to be representative of the positive expression of the target proteins. Five fields were randomly selected and measured for each slice.

## Statistical Analysis

All data analyses were performed using SPSS 21.0 software (IBM Corp. Armonk, NY, USA). The measurement data were expressed as the mean ± standard deviation. Comparisons between two groups were analyzed by independent sample *t*-test, while comparisons among multiple groups were analyzed by one-way analysis of variance (ANOVA). *p* < 0.05 was considered to be statistically significant.

## RESULTS

### MiR-25-3p Is Poorly Expressed in ox-LDL-Induced CVEC Model

Primarily isolated CVECs displayed irregular cobblestone-like or paving stone-like growth. When cell confluence reached 80–90%, the cells were detached and sub-cultured, and CD31

immunofluorescence was performed in order to identify the CVECs (**Figure 1A**) for subsequent experimentation. The levels of Vcam-1 and Icam-1 in the CVECs were treated with ox-LDL and subsequently determined by Western blot analysis. The results revealed that Vcam-1 and Icam-1 treated with various dosages of ox-LDL exhibited elevated levels, highlighting the influence of ox-LDL ( $p < 0.01$ ) (**Figures 1B,C**). ELISA was performed in order to assess the levels of the inflammatory markers (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) in CVECs treated with ox-LDL, the results of which demonstrated elevated levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  ( $p < 0.05$ ) (**Figure 1D**). The cells were treated with 25  $\mu\text{g}/\text{mL}$  ox-LDL for the subsequent experiment. RNA was extracted from the CVECs in order to assess the expression of miR-25-3p. The results indicated that the treatment of ox-LDL diminished the expression of miR-25-3p ( $p < 0.05$ ) (**Figure 1E**), while CVEC transfection with miR-25-3p resulted in suppressed levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  ( $p < 0.05$ ) (**Figure 1F**). The above results demonstrated that miR-25-3p expression was downregulated in CVECs treated with ox-LDL.

### MiR-25-3p Is Poorly Expressed in Vascular Tissues of Atherosclerosis Models of ApoE<sup>-/-</sup> Mice

In order to explore miR-25-3p expression in atherosclerosis with inflammatory diseases, atherosclerosis models of ApoE<sup>-/-</sup> mice were constructed to measure miR-25-3p expression in vascular tissues and CVECs. HE staining and Masson staining were performed on the isolated aorta vascular and tissue slices. Western blot analysis was conducted in order to determine the protein levels of  $\alpha$ -SMA, Col1a1, and Col3a1. ELISA was employed to measure TG and TC, the results of which verified the successful establishment of the ApoE<sup>-/-</sup> mouse models of atherosclerosis (**Figures 2A–G**) ( $p < 0.05$ ). ELISA was then performed to measure the levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , which revealed upregulated levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in atherosclerotic tissues (**Figure 2H**) ( $p < 0.05$ ). MiR-25-3p was downregulated in atherosclerotic tissues. Next, RT-qPCR was carried out to measure miR-25-3p expression in primarily

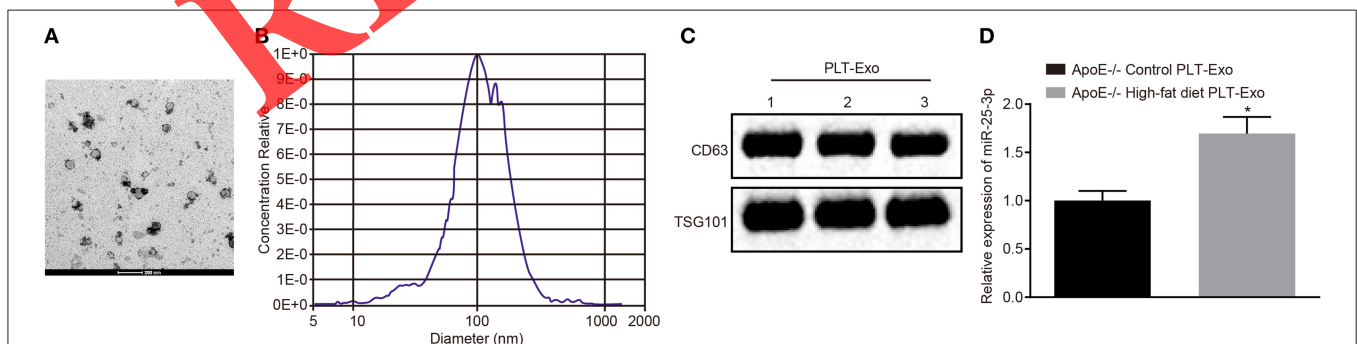
isolated CVECs from control ApoE<sup>-/-</sup> mice and ApoE<sup>-/-</sup> mice treated with a high-fat diet. The results demonstrated the reduced miR-25-3p expression in the CVECs from ApoE<sup>-/-</sup> mice treated with a high-fat diet (**Figure 2I**) ( $p < 0.05$ ), while CVECs from ApoE<sup>-/-</sup> mice treated with a high-fat diet and miR-25-3p mimic exhibited decreased levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , which was consistent with the results observed in the ApoE<sup>-/-</sup> control mice (**Figure 2J**) ( $p < 0.05$ ). Hence, we inferred based on our results that there was a reduction in the expression of miR-25-3p in atherosclerosis models of ApoE<sup>-/-</sup> mice.

### MiR-25-3p Is Highly Expressed in Peripheral Blood PLT-Exo of Thrombin-Induced Atherosclerosis Models of ApoE<sup>-/-</sup> Mice

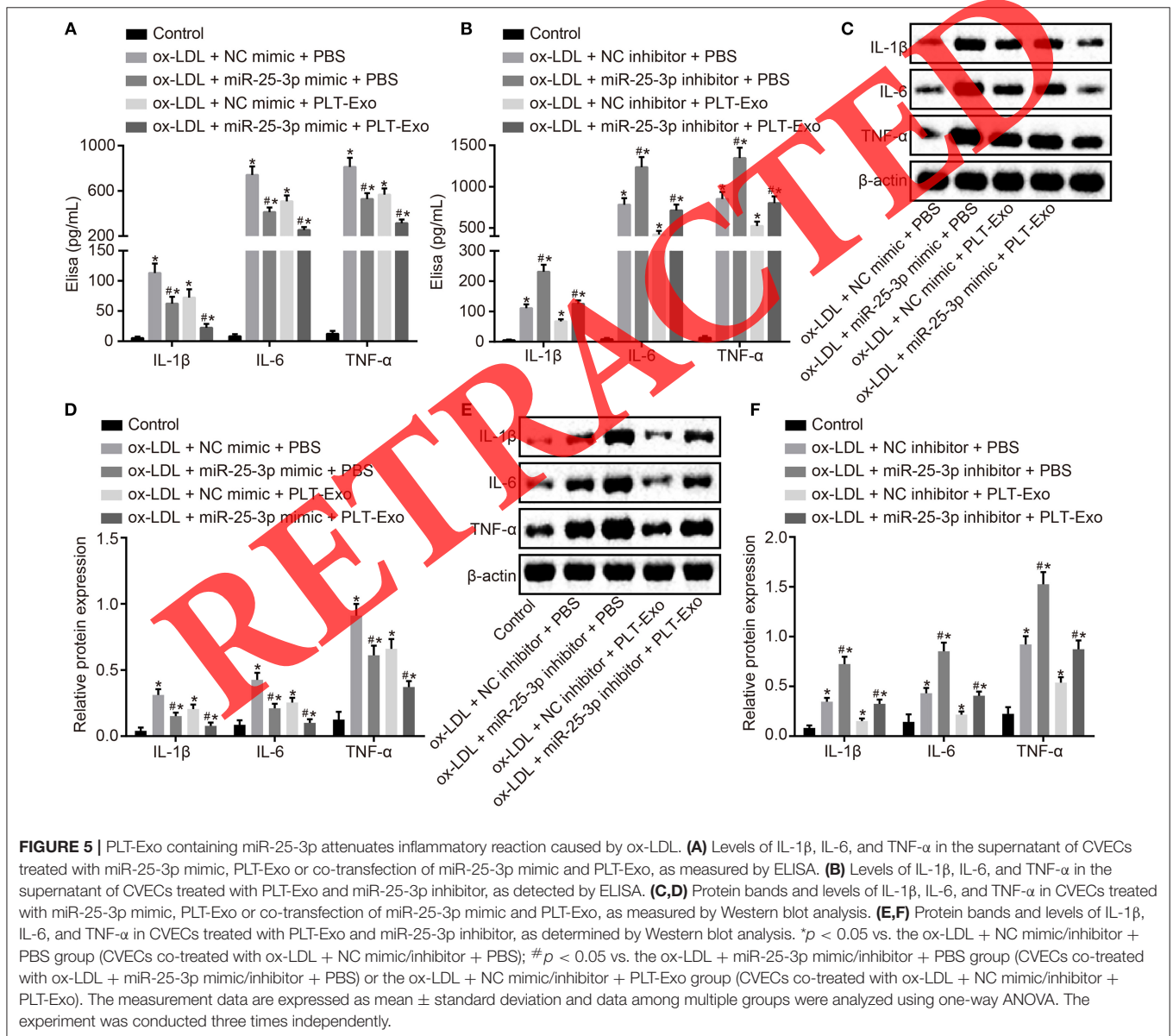
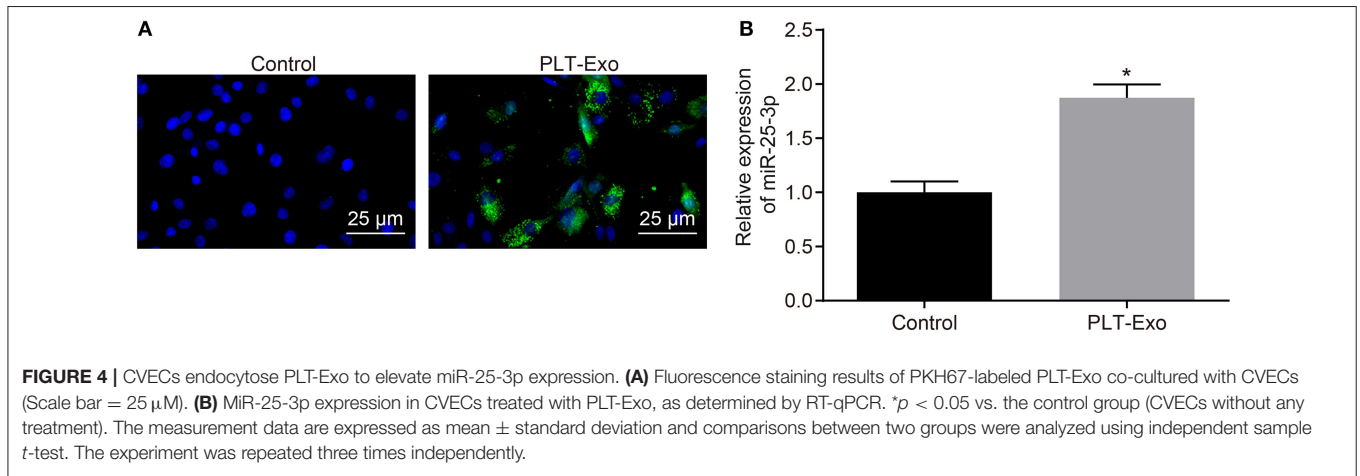
The platelets in the peripheral blood were isolated from mice and activated by thrombin. After the purified mouse PLT-Exo was extracted, TEM observation found that the shape of particles was solid dense bodies with typical double layer membrane structure in disk or cup-mouth shapes, and the average diameter was 119 nm, which was consistent with the characteristics of exosomes (**Figures 3A,B**). Furthermore, Western blot analysis was performed to assess the protein levels of exosome markers (TSG101 and CD63), which demonstrated that TSG101 and CD63 displayed positive expression in PLT-Exo (**Figure 3C**). RT-qPCR was employed to determine the miR-25-3p expression in PLT-Exo. The result showed that PLT-Exo in ApoE<sup>-/-</sup> mice treated with a high-fat diet showed increased miR-25-3p expression ( $p < 0.01$ ) (**Figure 3D**). Therefore, miR-25-3p expression was increased in peripheral blood PLT-Exo of thrombin-induced atherosclerosis models of ApoE<sup>-/-</sup> mice.

### CVECs Endocytose PLT-Exo to Upregulate miR-25-3p

After green fluorescence PKH67-labeled PLT-Exo was co-cultured with CVECs for 48 h, the uptake of exosomes by CVECs was analyzed under an inverted microscope. The results



**FIGURE 3** | MiR-25-3p displays upregulated expression in peripheral blood PLT-Exo of atherosclerosis models of ApoE<sup>-/-</sup> mice. **(A)** Exosome structures identified by TEM. **(B)** Exosome diameter measured by NanoSight analyzer. **(C)** Protein bands of TSG101 and CD63, as determined by Western blot analysis. **(D)** MiR-25-3p expression in PLT-Exo, as determined by RT-qPCR. \* $p < 0.05$  vs. the ApoE<sup>-/-</sup> control PLT-Exo group (PLT-Exo from control ApoE<sup>-/-</sup> mice). All data are measurement data and expressed as mean  $\pm$  standard deviation, and data between two groups were analyzed using independent sample *t*-test.  $n = 7$ . The experiment was repeated three times independently.





demonstrated that CVECs treated with PLT-Exo contained PKH67-labeled exosomes, while CVECs without any treatment only exhibited DAPI blue fluorescence (Figure 4A). In addition, as detected by RT-qPCR, CVECs treated with PLT-Exo displayed elevated miR-25-3p expression ( $p < 0.05$ ) (Figure 4B). Thus, the above-mentioned results suggested that miR-25-3p expression was increased through CVECs-endocytosed PLT-Exo.

### PLT-Exo Containing miR-25-3p Inhibits ox-LDL-Induced Inflammatory Reaction

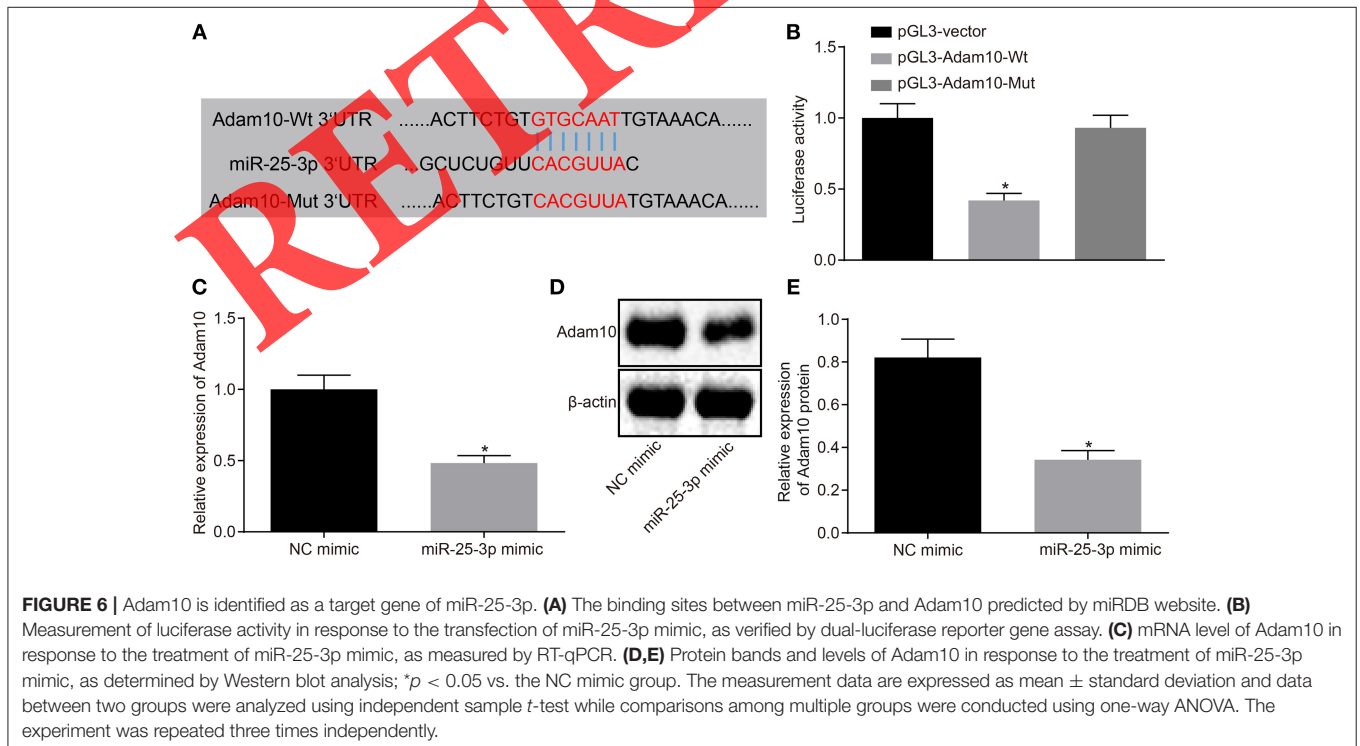
Primarily isolated CVECs of normal C57BL/6 mice were treated with ox-LDL. ELISA was conducted to determine the levels of inflammatory cytokines (IL-1 $\beta$ , IL-6, and TNF- $\alpha$ ) in the supernatant of the culture medium. The results revealed that compared with normal CVECs, levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  were significantly elevated after various treatments. CVECs treated with miR-25-3p mimic alone, PLT-Exo alone or co-transfection of miR-25-3p mimic and PLT-Exo led to suppressed levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in comparison to ox-LDL treatment. The co-transfection of miR-25-3p mimic and PLT-Exo led to lower levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  than transfection of miR-25-3p mimic alone or PLT-Exo alone ( $p < 0.05$ ) (Figure 5A). The treatment of PLT-Exo and miR-25-3p inhibitor resulted in elevated levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  ( $p < 0.05$ ) (Figure 5B). Western blot analysis was employed to determine the protein levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in CVECs treated with ox-LDL, with the same trend detected with ELISA (Figures 5C–F). Thus, it was concluded that inflammatory reaction induced by ox-LDL was inhibited by PLT-Exo containing miR-25-3p.

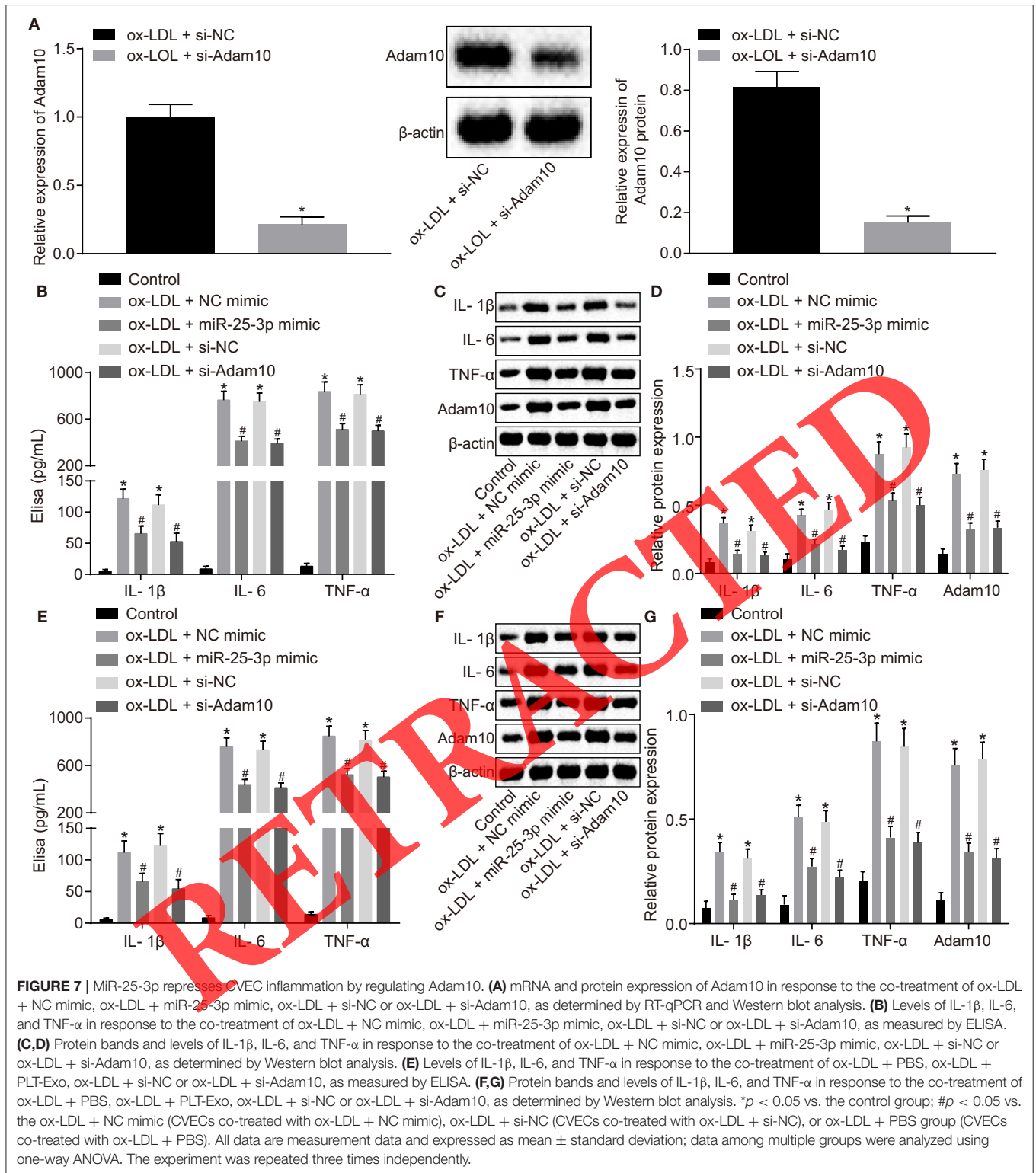
### MiR-25-3p Targets and Negatively Regulates Adam10

Next, miRDB was employed to predict the binding site of miR-25-3p and Adam10, which demonstrated that 490 - 496 fragments of Adam10 might be the binding sites with miR-25-3p 3'untranslated region (3'UTR) (Figure 6A). Dual-luciferase reporter gene assay was used to verify whether Adam10 was a target gene of miR-25-3p. The results showed that luciferase activity of Adam10-WT reduced after the treatment of miR-25-3p mimic ( $p < 0.05$ ), while there was no marked change in the luciferase activity of Adam10-MUT ( $p > 0.01$ ), suggesting that miR-25-3p could target Adam10 (Figure 6B). In order to further verify this finding, RT-qPCR and Western blot analysis were conducted to detect the Adam10 level after the treatment of miR-25-3p mimic. The results demonstrated that CVECs transfected with miR-25-3p mimic displayed decreased Adam10 level ( $p < 0.01$ ) (Figures 6C–E). These results provided evidence verifying that Adam10 was a target gene of miR-25-3p.

### Exosomal MiR-25-3p Inhibits Inflammation Reaction of CVECs by Targeting Adam10

RT-qPCR and Western blot analysis were performed to identify the mRNA level of Adam10. The results showed that the level of Adam10 decreased after the treatment of si-Adam10 (Figure 7A). ELISA was conducted to determine levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , the result of which revealed elevated levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  after different treatments when compared with CVECs without any treatment. Additionally, treatment of miR-25-3p mimic

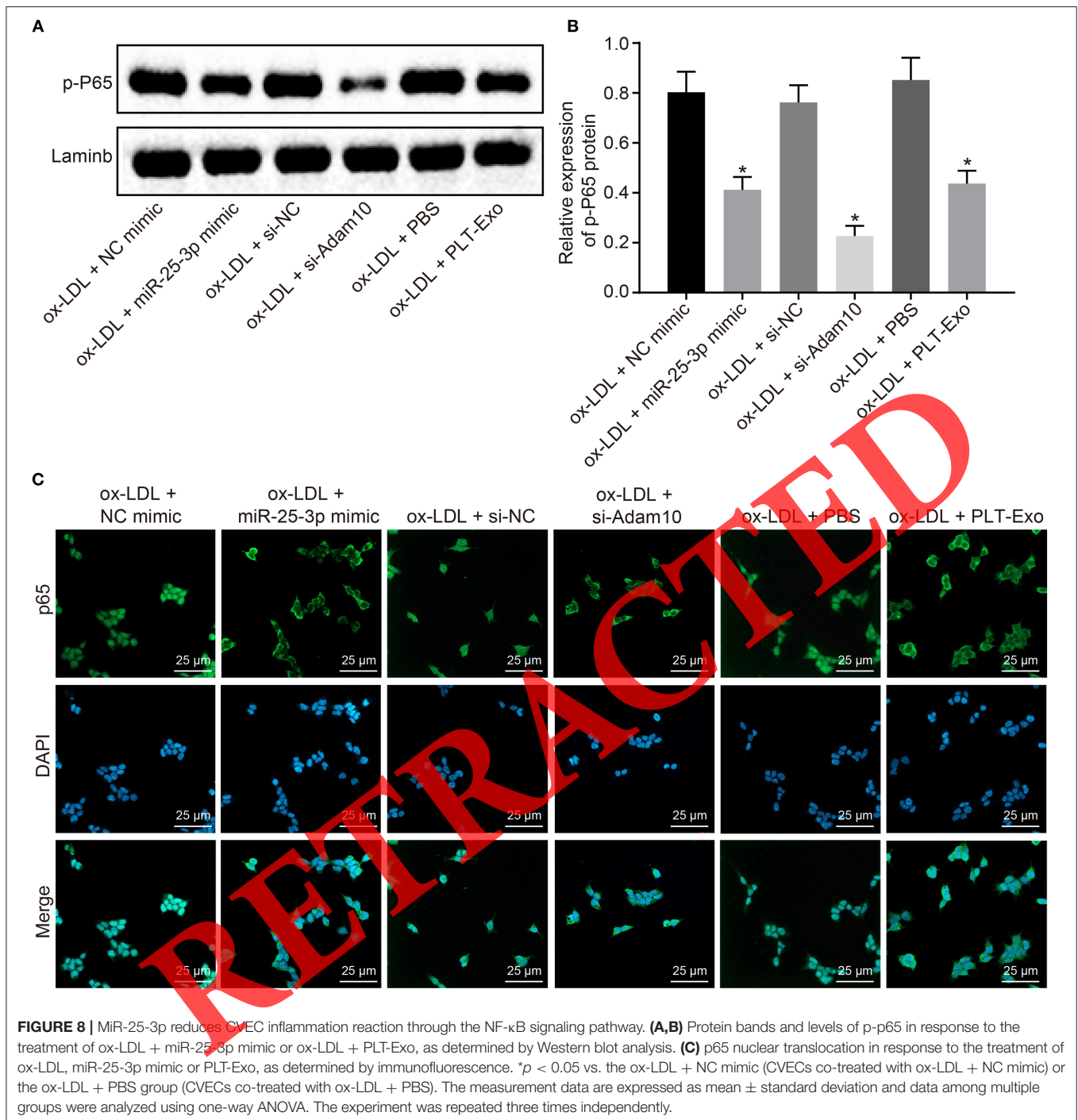




and si-Adam10 produced contrasting results. Western blot analysis also revealed a decline in protein levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (Figures 7B–G). Thus, it was concluded that CVEC inflammation was inhibited by exosomal miR-25-3p by regulating Adam10.

## MiR-25-3p Suppresses CVEC Inflammation Reaction Through Mediating the NF- $\kappa$ B Signaling Pathway

In order to explore the effect of miR-25-3p on inflammation reaction of CVECs, CVECs were transfected with miR-25-3p,



si-Adam10 or PLT-Exo on the basis of ox-LDL treatment. Western blot analysis was subsequently performed in order to detect the extent of p65 phosphorylation, illustrating the effect of transfection of miR-25-3p, si-Adam10, or PLT-Exo induced inhibited extent of p65 phosphorylation ( $p < 0.05$ ) (Figures 8A,B). The result of immunofluorescence detection on p65 nuclear translocation was consistent with the results of the Western blot analysis, which revealed a reduced translocation of p65 into the nuclei of CVECs after the transfection of miR-25-3p,

si-Adam10 or PLT-Exo (Figure 8C). Hence, it was confirmed that CVEC inflammation reaction was suppressed by miR-25-3p through the NF- $\kappa$ B signaling pathway.

### PLT-Exo Containing MiR-25-3p Alleviates Atherosclerosis of Mice

The atherosclerosis mouse models without any treatment exhibited signs of a poor mental state, dull-looking, slow response, reduced appetite, symptoms of ventosity, and diarrhea.

One mouse died due to secondary pulmonary infection. The atherosclerosis mice treated with PLT-Exo and miR-25-3p agomir exhibited signs of being in good spirits, glossy hair color, rapid response, and good appetite. After 12 weeks, the weight of the mice was determined. The mice were euthanized for the following experiment. As shown in **Table 2**, the treatment of PLT-Exo and miR-25-3p agomir induced decreased TG and TC ( $p < 0.05$ ), while no significant difference was detected in weight, high-density lipoprotein and LDL ( $p > 0.05$ ).

In order to detect atherosclerotic plaque formation in ApoE<sup>-/-</sup> mice in response to treatment of normal diet and high-fat diet, co-treatment of high-fat diet and PLT-Exo or high-fat diet combined with miR-25-3p agomir, oil red O, HE, and Masson stainings were performed to determine the size of plaque as well as the degree of fibrosis (**Figures 9A–E**). The treatment of PLT-Exo and miR-25-3p agomir displayed decreased artery plaque area, while the control mice displayed thickened intima of arterial tissues at various degrees, formed fibrous cap, uneven thickness of arterial wall, and distinct atherosclerotic plaque ( $p < 0.01$ ). Western blot analysis was employed to determine the levels of  $\alpha$ -SMA, Col1a1, Col3a1, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and Adam10. The results showed that the treatment of PLT-Exo and miR-25-3p agomir induced decreased  $\alpha$ -SMA (suggesting reduced smooth muscle cells), Col1a1 and Col3a1 as well as suppressed levels of IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . The results of Western blot analysis, RT-qPCR and immunohistochemistry all revealed inhibited Adam10 level (**Figures 9F,G**) and increased miR-25-3p expression (**Figure 9H**) after the treatment of PLT-Exo and miR-25-3p agomir ( $p < 0.05$ ). The above results provided evidence suggesting that PLT-Exo containing miR-25-3p could inhibit artery plaque formation and alleviate artery inflammation in atherosclerosis mouse models.

## DISCUSSION

Atherosclerotic coronary artery disease has been implicated in a wide variety of clinical entities that comprise asymptomatic subclinical atherosclerosis in addition to its clinical complications, such as sudden cardiac death and angina pectoris (26). More recently, exosomes have been revealed to exert important functions in atherosclerosis, which are involved in immune responses and communication among cells (27, 28). The current study elucidated the role of PLT-Exo containing miR-25-3p in ox-LDL-induced CVEC inflammation in ApoE<sup>-/-</sup> mice models of atherosclerosis. Collectively, the obtained data revealed that exosomes derived from platelet over-expressing miR-25-3p were capable of inhibiting ox-LDL-induced CVEC inflammation by suppressing the NF- $\kappa$ B signaling pathway and Adam10 expression.

This study revealed that miR-25-3p is poorly expressed in ox-LDL-induced CVECs and vascular tissues of atherosclerosis models of ApoE<sup>-/-</sup> mice. On the other hand, miR-25-3p exhibited high levels of expression in PLT-Exo of atherosclerosis mice. Exosomes have been reported to be involved in intracellular communication, material transfer on the surface of cell membrane and the modulation of cellular substance during

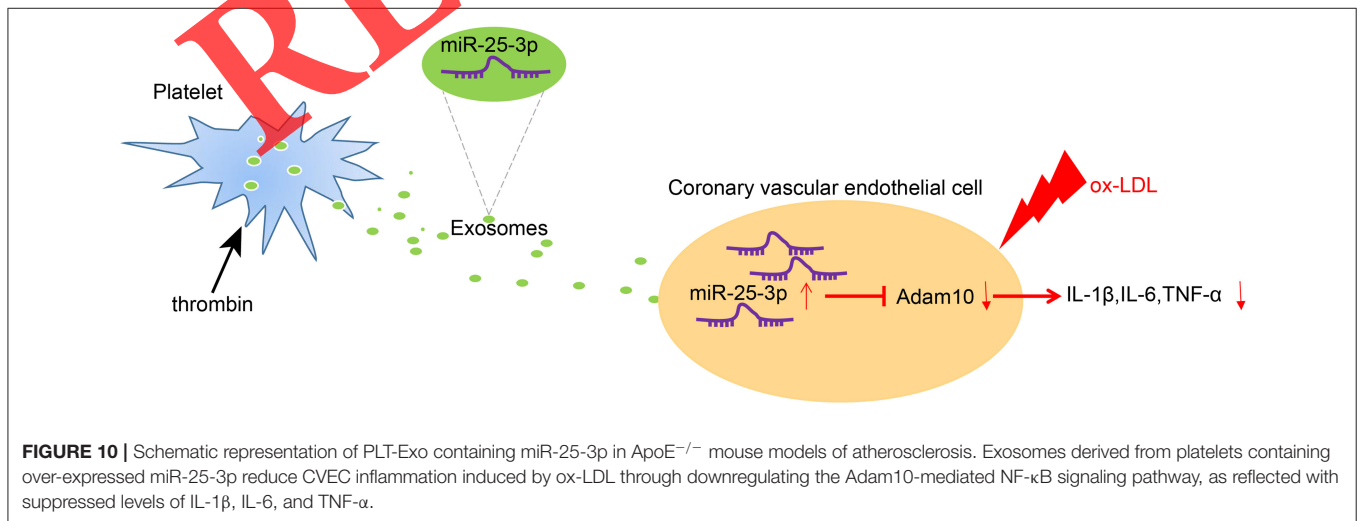
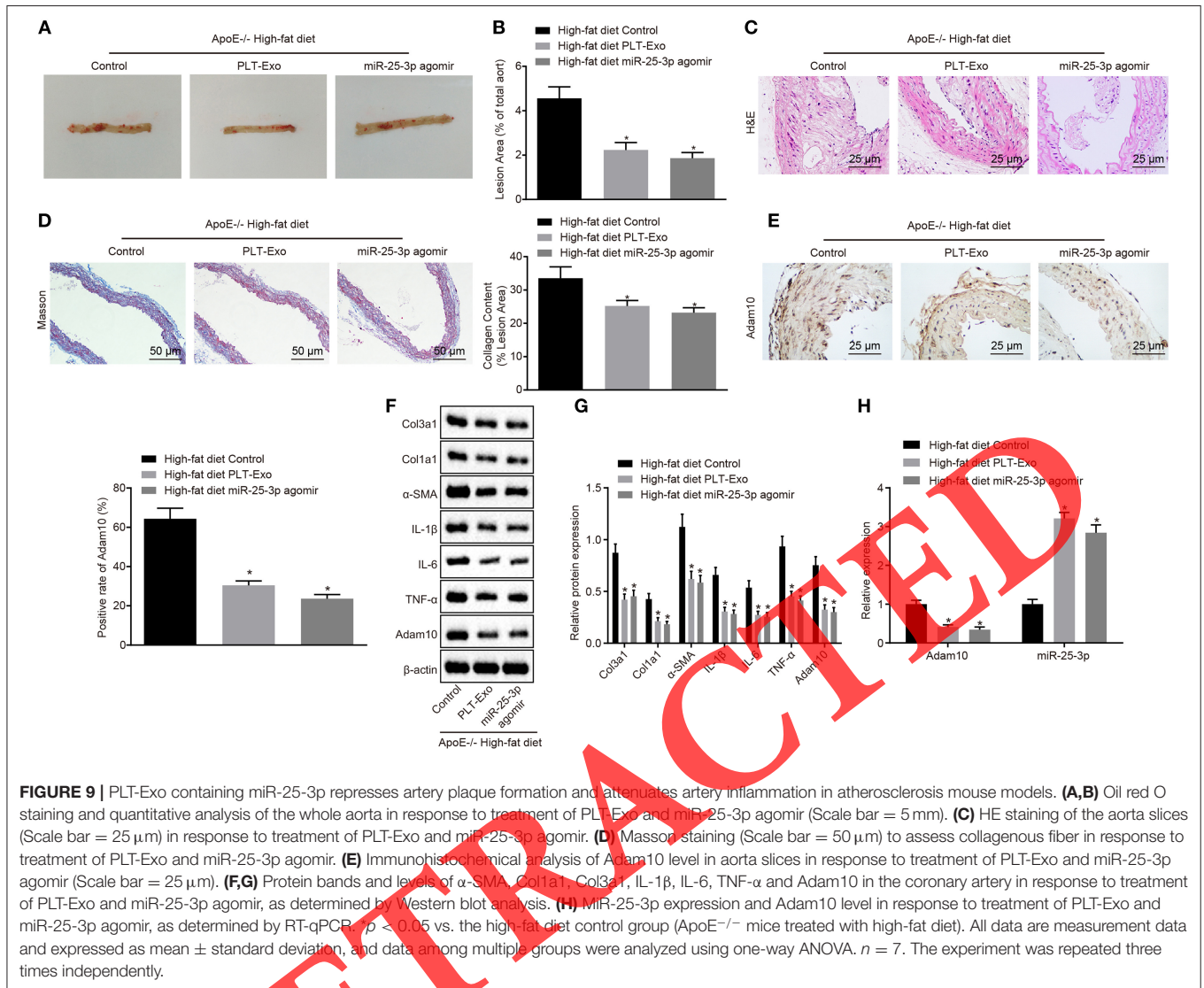
**TABLE 2 |** The primer sequence for RT-qPCR.

Gene	Primer sequence (5'-3')
miR-25-3p	F: GGGGTACCACTGACTTTAGAGGGCGGGT R: CCAAGCTTTGTCAGACCGAGACAAGTGC
Adam10	F: TGAAGTGGAGCGAGAGGGAG R: GTGCATCGATCCTGAGGGAG
IL-1 $\beta$	F: CTCTGTGACTCGTGGGATGA R: AGTTGGGAACTGTGCAGAC
IL-6	F: GGTCTTCTGGAGTTCC GTTTC R: GGGTTGCCGAGTA GACCTCA
TNF- $\alpha$	F: CAAAC CACCAAGTGGAGGAG R: AAG TAGACCTGCCCGGACTC
GAPDH	F: GCTGCCCAGAACATCATCATCC R: GTCAGATCCACGACGGACAC
U6	F: CTCGCTTCGGCAGCAC R: AACGCTTCACGAATTTGCGT

RT-qPCR, reverse transcription quantitative polymerase chain reaction; F, forward; R, reverse; miR-25-3p, microRNA-25-3p; IL, interleukin; TNF, tumor necrosis factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Adam10, A disintegrin and metalloprotease 10.

the process of membrane fusion (29). Activated platelets have been identified in the progression of peripheral artery disease as well as cerebrovascular atherosclerosis (30). A recent study concluded that tumor-derived exosomes carrying secretory miR-25-3p induces an enhancement of vascular endothelial cell invasion as well as capillary formation (15). Multiple miRNAs have been found to exhibit aberrant levels of expression in atherosclerosis, including miR-155, which performs as an anti-inflammatory factor in atherosclerosis by playing a regulatory role in foam cell formation (31). A previous study reported that over-expressed miR-138 could attenuate human CVEC injury and inflammatory reactions (32).

It is worth noting that Adam10 is a target gene of miR-25-3p, which was further confirmed based on the silico prediction and dual luciferase reporter assay. Furthermore, miR-25-3p was found to negatively regulate the expression of Adam10. Adam10 has been identified as a target of multiple miRNAs, including miR-152. A previous study suggested that miR-152 regulates Adam10 expression which results in the suppression of synovial fibroblast proliferation and stimulates apoptosis in rheumatoid arthritis (33). Additionally, our results revealed that CVECs endocytosed PLT-Exos to upregulate the expression of miR-25-3p. Zhao et al. concluded that exosomes derived from bone marrow mesenchymal stem cells overexpressing miR-25 could protect spinal cords against transient ischemia (34). More importantly, platelet extracellular vesicles have been demonstrated to upregulate miR-25-3p in diabetes, metabolic and vascular disease, a finding which was consistent with the observations of our study (35). Other exosomal miRNAs have previously been proven to participate in the development of atherosclerosis. For example, exosomes containing miR-155 have been reported to transfer from smooth muscle cells to the vascular endothelial cells, which leads to endothelial injury and ultimately facilitates atherosclerosis (36). Moreover,



miR-155 ameliorates ox-LDL-induced human umbilical vein endothelial cell inflammation through the repression of the SOCS1-dependent NF- $\kappa$ B signaling pathway (37).

Moreover, our study demonstrated the involvement of the NF- $\kappa$ B signaling pathway in the inhibition of PLT-Exo carrying miR-25-3p on CVEC inflammation by regulating Adam10 in atherosclerosis. The suppressive effects of miR-25 have been previously documented in the macrophage secretion of inflammatory cytokines in sepsis by targeting and regulating HMGB1 (38). Consistent with the findings of the current study, recent evidence has indicated that the upregulation of miR-181b expression reduces NF- $\kappa$ B activation, thus curtailing vascular inflammation as well as atherosclerosis in ApoE<sup>-/-</sup> mice (39). Activated Adam10 induces promotion of cell growth and activation of the TNF- $\alpha$ /NF- $\kappa$ B signaling pathway in mantle cell lymphoma (40). Musumeci et al. asserted that Adam10 could regulate the apoptosis of vascular smooth muscle cells dependent on cleavage of N-cadherin in human atherosclerotic lesions resulting in vulnerable plaque (41).

Furthermore, the results of our study provided evidence indicating that PLT-Exo carrying miR-25-3p suppressed ox-LDL-induced CVEC inflammation by downregulating the levels of  $\alpha$ -SMA, Col1a1, Col3a1, TG, TC, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ .  $\alpha$ -SMA is known as the actin isoform derived from vascular smooth-muscle cells (42). Col1a1 and Col3a1 are two critical collagens responsible for constructing skin connective tissue, whose defects are related to fiber hyperplasia and dermal connective tissue disease (43). A higher risk of coronary artery disease has been strongly correlated with elevated levels of LDL cholesterol and TGs (44). IL-1 $\beta$ , IL-6, and TNF- $\alpha$  are widely considered to be proinflammatory cytokines that attract new cells and are capable of activating dormant cells to accelerate the inflammatory process (45). When these pro-inflammatory cytokines, factors related to lipid accumulation were reduced by PLT-Exo carrying miR-25-3p, the ox-LDL-induced CVEC inflammation could be inhibited in ApoE<sup>-/-</sup> mice models of atherosclerosis.

## CONCLUSIONS

In conclusion, the key findings of the study suggested that PLT-Exo overexpressing miR-25-3p alleviates CVEC inflammation induced by ox-LDL in the ApoE<sup>-/-</sup> mice models of atherosclerosis (Figure 10). Although miR-based

therapeutic strategies are still in their infancy, our findings on miR-25-3p provided evidence for a potential treatment target of ox-LDL-induced CVEC inflammation in atherosclerosis. Thus, it is also recommended in future studies that specimens from atherosclerosis-diagnosed patients be collected for in-depth studies of miR-25-3p in CVECs.

## DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

## ETHICS STATEMENT

All animal experiments were approved by the Ethics Committee of The First Affiliated Hospital of Harbin Medical University and followed the Guide for the Care and Use of Laboratory Animals. Best efforts were made to minimize the suffering of animals.

## AUTHOR CONTRIBUTIONS

YY and HW designed the study. QS and BJ collated the data, designed, and developed the database. SL, XL, and GS carried out data analyses and produced the initial draft of the manuscript. RC and WS contributed to drafting the manuscript. All authors contributed to the revision and approved the final submitted manuscript.

## FUNDING

This study was supported by the National Natural Science Foundation of China—Role of MicroRNA-103a-1 in the Treatment of Abdominal Aortic Aneurysm by Targeting ADAM10 (No. 81570424) and the National Natural Science Foundation of China—Role of MicroRNA-138 in Regulating the Function of Coronary Endothelial Cells and Membrane Particles in the Pathogenesis of Coronary Heart Disease (No. 81770355).

## ACKNOWLEDGMENTS

We would like show sincere appreciation to the reviewers for critical comments on this article.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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