Role and mechanism of the zinc finger protein ZNF580 in foam-cell formation

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Abstract. Coronary atherosclerotic heart disease poses a significant threat to human health. The pathological basis is atherosclerosis and foam-cell formation is the key factor in the initiation of atherosclerosis. In the present study, foam cell models were established using 50 ng/ml oxidized low-density lipoprotein to stimulate *in vitro* cultures of THP-1 cells for 72 h. The expression of zinc finger protein 580 (ZNF580), a Cys2-His2 zinc finger protein containing 172 amino acids that was originally cloned by screening a human aortic cDNA library, was measured in foam cells and its interaction with various regulatory factors during foam-cell formation was investigated. Oil red O staining was used to observe cell morphology and intracellular lipid levels. Lentivirus transfection was employed to either overexpress or silence ZNF580 in THP-1 cells, and an inverted fluorescence microscope was used to observe the distribution of ZNF580 immunofluorescence to determine the transfection rate. RNA and total protein were extracted and the expression levels of ZNF580, CD36, peroxisome proliferator-activated receptor-γ (PPAR-γ), ATP-binding cassette transporter A1 (ABCA1) and apolipoprotein E (ApoE) were measured by reverse transcription-quantitative PCR. The protein levels were examined by western blot analysis to evaluate the interaction between ZNF580 and associated regulatory factors. ZNF580 was able to significantly increase the expression levels of ApoE and ABCA1 and significantly decrease the expression levels of CD36 and PPAR-γ, suggesting that ZNF580-mediated inhibition of foam-cell formation is associated with the PPAR-γ-CD36 signalling pathway. Based on these findings, ZNF580 may be a potential therapeutic candidate for the treatment of coronary atherosclerotic heart disease.

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Introduction

Cardiovascular disease (CVD) poses a significant threat to the health of middle-aged and elderly individuals and leads to a decline in quality of life; coronary atherosclerotic heart disease is an important part of CVD (1). The pathological basis of coronary atherosclerotic heart disease is the development of coronary atherosclerosis, in which the production and accumulation of foam cells are important (2). Foam cells are fatty macrophages that cause artery hardening and may further lead to heart disease. Foam cells are the first visible lesion feature in atherosclerotic diseases (3). Circulating monocytes are recruited to the damaged vascular endothelium from the blood, penetrate the subintima through the endothelial space and differentiate into macrophages. Macrophages oxidize accumulated lipoproteins under the intima. A large amount of oxidized lipoprotein enters macrophages, which form foam cells, and smooth muscle cells also participate in foam cell formation (4). The accumulation of foam cells forms the inner

core and lipid streaks of coronary atherosclerotic plaques. In the late stage of atherosclerosis, foam cells undergo necrosis or apoptosis, resulting in lipid leakage and formation of a necrotic core, which increases plaque instability. The occurrence and development of foam cells contributes to the pathophysiological process of atherosclerosis (5). Therefore, understanding the mechanism by which foam cells form and reducing foam cell formation factors may effectively reduce the formation of atherosclerotic plaques in coronary arteries.

Scavenger receptors (SRs) have an important role in foam cell formation and are part of a significant mechanism by which macrophages identify, ingest and engulf lipoprotein oxide (6). SRs are present on the cell surface in a variety of molecular forms and are mainly divided into five types: A, B, C, D and E. CD36 is a member of the class B SR family and is the main receptor by which macrophages consume lipoprotein oxide. When oxidized low-density lipoprotein (ox-LDL) enters macrophages, which form foam cells, CD36 activation is necessary (7). Since CD36 is not regulated by negative feedback from intracellular cholesterol esters during lipoprotein uptake, the formation of tissue foam cells must be regulated by modulating the regulatory factors upstream of CD36. Macrophage-produced factors, such as interleukin-4 and peroxisome proliferator activated receptor-γ (PPAR-γ), are upstream regulatory factors that may regulate CD36 expression, and PPAR-γ is widely distributed in adipose tissue, vascular smooth muscle and cardiomyopathy tissue. There it participates in lipid metabolism, inflammatory reactions and pathological processes such as those of atherosclerosis (8). Studies have indicated that PPAR-γ activation inhibits foam cell formation by increasing foam cell apoptosis while also regulating the activation of ATP-binding cassette transporter A1 (ABCA1). This occurs by increasing the expression of liver X receptor-α to affect cholesterol outflow and delay the formation of atherosclerotic plaques (9). In addition, studies have indicated that apolipoprotein E (ApoE) has a similar function to that of ABCA1, which transports excess cholesterol esters from within cells to the extracellular space, reducing lipid build-up in cells and delaying foam cell formation (10). Therefore, the aim of the present study was to provide a basis to find drugs that act on these targets to prevent foam cell formation.

Zinc finger protein 580 (ZNF580; GenBank ID, AF184939) is a Cys2-His2 (C2H2) zinc finger protein containing 172 amino acids that was originally cloned by screening a human aortic cDNA library. The protein contains a highly conserved C-terminus, three tandem repeated C2H2 zinc finger domains and a proline-rich N-terminus. The protein structure of ZNF580 is similar to Sp1-like or Krüppel-like transcription factors, and it is also characterized by three tandem repeated C2H2 zinc fingers at the C-terminus (11). Previous studies indicated that ZNF580 was able to protect against CVD through multiple signalling pathways: ZNF580 regulates endothelial nitric oxide synthase expression via the TGF-β1/ALK5/Smad2 pathway and mediates vascular endothelial inflammation through the H_2O_2/NF-κB signalling pathway (12,13). Single-core macrophages or smooth muscle cells are usually used to replicate foam cell models; the available cell types are the human leukaemic monocyte cell line THP-1, rat celiac macrophages and animal aortic smooth muscle cells, while THP-1 cells are the most commonly used (14,15). THP-1 is a human mononucleotic cell line that is induced by phorbol 12-myristate 13-acetate (PMA) to differentiate into macrophages to build a foam cell model. Oil red O (ORO) staining is usually performed to confirm or assess the extent of successful establishment of the foam cell model (16). However, whether ZNF580 affects atherosclerotic plaque formation has remained to be explored. Therefore, in the present study, lentiviruses were used to overexpress or silence ZNF580 to identify genes related to foam cell formation and clarify the role of ZNF580 in foam cell formation. The experimental results provide a new theoretical basis for the treatment of coronary atherosclerotic heart disease.

Materials and methods

Cell culture. THP-1 cells were obtained from the Tianjin Key Laboratory of Hepatopancreatic Fibrosis and Molecular Diagnosis & Treatment and cultured in RPMI-1640 culture medium supplemented with 10% foetal bovine serum (FBS; both from Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin. THP-1 cells were grown in an incubator with 100% humidity containing 95% air and 5% CO_2 at 37˚C. Depending on cell growth, the medium was changed once every 2-3 days and cells were passaged every 3 days. The model was established with third-generation cells.

Cell transfection. THP-1 cells in the logarithmic growth phase were uniformly inoculated at $2x10^5$ cells/well in a 6-well plate and transfected with control small interfering RNA (siRNA). According to the manufacturer's protocol, THP-1 cells were transfected with the non-targeting siRNA but with the green fluorescent protein (negative control; NC; 20 μl/well), a ZNF580 overexpression vector (LV-ZNF580; 20 μl/well) or a ZNF580 silencing vector (Si-ZNF580; 20 μl/well), (all from Shanghai Gene Pharma Co., Ltd.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37˚C for 96 h. FBS-free RPMI-1640 medium was used during transfection. The transfection efficiency was monitored by fluorescence microscopy. As Si-ZNF580 negative control (Si-NC) and LV-ZNF580 negative control (LV-NC) use the same vector, they have the same sequences. In order to avoid the imbalance caused by empty vector, the purpose of LV-NC insertion of non-targeting siRNA is to parallel the gene skeleton of overexpressed vectors (17-19). The sequences for the siRNAs were as follows: Si-NC, 5'-TTCTCCGAACGTGTCACGT-3'; Si-ZNF580, 5'-GGAGCATCATTCCTTCCTTAC-3'; and LV-ZNF580 all from Shanghai GenePharma Co., Ltd.

Cell model. THP-1 cells were inoculated at $1x10^5$ cells/well in a 6-well plate at 37˚C with 5% CO_2 for 12 h. After stimulation with 10 ng/ml PMA (MilliporeSigma), THP-1 cells adhered to the wells and became macrophages. After the RPMI-1640 medium was replaced with fresh medium, 50 ng/ml ox-LDL (Thermo Fisher Scientific, Inc.) was added and the cells were cultured for 24, 48 or 72 h to establish the model (20,21).

Cell grouping. THP-1 cells were grown on 6-well plates and randomly divided into eight groups at a density of $5x10^4$ cells/well. In the control group, THP-1 cells were grown in an incubator containing 95% air and 5% CO_2 at 37˚C after stimulation with 10 ng/ml PMA, and no additional

treatment was performed. In the control + ox-LDL (model) group, after 10 ng/ml PMA was added to control group cells, 50 ng/ml ox-LDL was added and the cells were cultured in an incubator containing 95% air and 5% CO_2 at 37˚C for 72 h. In the LV-NC group, THP-1 cells were transfected with the relevant siRNA lacking the ZNF580 target gene but with the green fluorescent protein, and no additional treatment was performed. In the Si-NC group, THP-1 cells were transfected with the non-targeting siRNA vector but with the green fluorescent protein and no additional treatment was performed. In the LV-ZNF580 group, THP-1 cells were transfected with 20 μl/well LV-ZNF580 lentivirus and no additional treatment was performed. In the LV-ZNF580 + ox-LDL (LV-ZNF580 + model) group, cells treated in the same manner as those in the LV-ZNF580 group were administered 50 ng/ml ox-LDL and cultured in an incubator containing 95% air and 5% CO_2 at 37˚C for 72 h. In the Si-ZNF580 group, THP-1 cells were transfected with 20 μl/well Si-ZNF580 lentivirus and no additional treatment was performed. In the Si-ZNF580 + ox-LDL (Si-ZNF580 + model) group, cells treated in the same manner as those in the Si-ZNF580 group were administered 50 ng/ml ox-LDL and cultured in an incubator containing 95% air and 5% CO_2 at 37˚C for 72 h.

Observation of cell morphology and intracellular lipid levels. ORO staining was used to observe cell morphology and intracellular lipid levels. The cells in the different groups were sequentially washed with PBS and deionized water 2-3 times and incubated with ORO fixative (MilliporeSigma) at 37˚C for 20 min. After the cells were washed 2-3 times, 0.5% ORO stain was added, followed by incubation at 37˚C for 20 min, and the cells were then placed in hematoxylin (cat. no. H3136; MilliporeSigma) solution at 37˚C for 1 min. Finally, the cells were dried in a ventilated place at 37˚C and cell morphology and intracellular lipid levels were observed using an optical microscope (Olympus Corporation). Image-Pro Plus 6.0 software (Media Cybernetics) was used for quantification of lipid levels.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). A total of $1x10^5$ THP-1 cells per well were inoculated into 6-well plates and cultured at 37˚C with 5% CO_2 for 24 h. RT-qPCR was used to measure the expression of ABCA1, CD36, ApoE, PPAR-γ and ZNF580 in each group. TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA and a UV spectrophotometer was used to measure RNA purity. Subsequently, the RNA was reverse-transcribed into cDNA using a HIFIScript cDNA Synthesis Kit (ComWin Biotech) according to the manufacturer's protocol. The cDNA templates were subjected to qPCR with UltraSYBR Mixture Low ROX (CoWin Biosciences) under the following conditions: 40 cycles of 10 sec at 95˚C, 30 sec at 60˚C and 32 sec at 72˚C (stepOnePlus system; Thermo Fisher Scientific, Inc.). The nucleotide sequences of the forward and reverse primers are provided in Table I. The relative expression level of each mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (22).

Protein preparation and western blot analysis. THP-1 cells were washed three times with PBS. Samples from the different groups were lysed in complete RIPA buffer (cat. no. R0020; Beijing Solarbio) at 4˚C for 15 min. The total protein concentrations were determined using a BCA kit (cat. no. A045-4-2; Nanjing Jiancheng Bioengineering Institute). Equal amounts of protein (30 μg/lane) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (EMD Millipore). The membranes were then blocked with Tris-buffered saline plus Tween-20 containing 5% skimmed milk (cat. no. PH1519; Phygene Scientific, Inc.)for 3 h at 25˚C, after which the membranes were incubated with the following primary antibodies overnight at 4˚C: ZNF580 (cat. no. PA5-62904; 1:1,000 dilution; Thermo Fisher Scientific, Inc.), ApoE (cat. no. 18254-1-AP; 1:2,000 dilution; Proteintech Group, Inc.), CD36 (cat. no. 18836-1-AP; 1:2,000 dilution; Proteintech Group, Inc.), ABCA1 (cat. no. AB7360; 1:1,000 dilution; Abcam), PPAR-γ (cat. no. 16643-1-AP; 1:2,000 dilution; Proteintech Group, Inc.) and GAPDH (cat. no. 5174; 1:1,000 dilution; Cell Signalling Technology, Inc.). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (cat. no. 7074 V; 1:5,000 dilution; Cell Signalling Technology, Inc.) for 1 h at 25˚C. Signals were observed using ECL substrate (cat. no. 34579; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Band densities were measured using ImageJ 1.52a software (National Institutes of Health).

Statistical analysis. Values are expressed as the mean ± standard deviation of at least three independent tests. Statistical

Table I. Sequences of the primer pairs used for quantitative PCR.

Primer/direction	Sequence
ABCA1	
Forward	5'-ACCCACCCTATGAACAACATGA-3'
Reverse	5'-GAGTCGGGTAACGGAAACAGG-3'
CD36	
Forward	5'-GGCTGTGACCGGAACTGTG-3'
Reverse	5'-AGGTCTCCAACTGGCATTAGAA-3'
ApoE	
Forward	5'-GTTGCTGGTCACATTCCTGG-3'
Reverse	5'-GCAGGTAATCCCAAAAGCGAC-3'
PPAR-γ	
Forward	5'-GGCCGCAGATTTGAAAGAAG-3'
Reverse	5'-ATTTCGTTAAAGGCTGACTCTCGTT-3'
ZNF580	
Forward	5'-GAGGTTACTGCCTTACCCTGG-3'
Reverse	5'-ACCCAGTTCCGACTGGTTC-3'
β-actin	
Forward	5'-CATGTACGTTGCTATCCAGGC-3'
Reverse	5'-CTCCTTAATGTCACGCACGAT-3'

F, forward; R, reverse; ZNF580, zinc finger protein 580; PPAR-γ, peroxisome proliferator activated receptor-γ; ABCA1, ATP-binding cassette transporter A1; ApoE, apolipoprotein E.

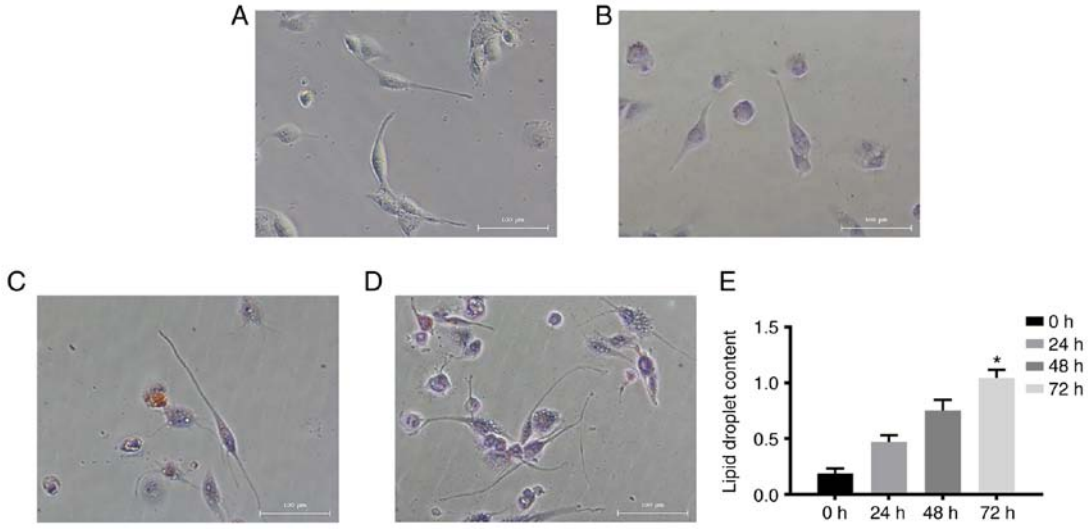

Figure 1. Ox-LDL mediates the transformation of macrophages into foam cells. (A) The cells in the control group had a long spindle-like morphology with full cytoplasm and pseudopodia and grew adherently. After incubation with ox-LDL for (B) 24 h, (C) 48 h, and (D) 72 h, the lipid droplet levels in macrophages gradually increased (Magnification, x100; scale bar, 100 μm). (E) The quantified results indicated that the number of ORO-stained cells increased significantly in the treated groups with increasing incubation time. *P<0.05 compared with the control group. Ox-LDL, oxidized low-density lipoprotein; ORO, oil red O.

comparisons between groups were performed using an unpaired t-test or one-way ANOVA followed by Tukey's post-hoc test with SPSS version 25.0 statistical software (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated at least three times.

Results

Foam cell model verification. Under normal conditions, THP-1 cells grow in suspension and the cells are round or oval in shape. After PMA stimulation, the cells acquired a long spindle-like morphology with pseudopodia, grew adherently and differentiated into macrophages (Fig. 1A). When stimulated by 50 ng/ml ox-LDL, macrophages developed into foam cells, and intracellular lipid droplets were able to be stained with ORO (Fig. 1B-D). With prolonged ox-LDL stimulation, the number of ORO-positive cells increased significantly. Furthermore, the degree of ORO dye aggregation increased significantly and the colour darkened, suggesting that the levels of lipid droplets in macrophages increased and foam cells formed more fully with prolonged ox-LDL incubation time (Fig. 1E).

Selection of the optimal induction time for the foam cell model. ORO staining confirmed that it was possible to successfully establish a foam cell model from ox-LDL-stimulated macrophages. To determine the optimal stimulation time, 50 ng/ml ox-LDL was used to stimulate foam cell formation in macrophages for different durations. The mRNA expression of CD36 increased in the control group with increasing ox-LDL induction time, and CD36 expression was highest at 72 h, suggesting a time-dependent effect on CD36 expression in normal macrophages. In the model group, CD36 mRNA expression levels were consistent with those in the control group, and the growth trends at 24 h were comparable to those at 48 h. However, there were no significant differences in expression levels between 48 and 72 h. At 72 h, the mRNA expression of CD36 in the control group was increased compared with that in the model group and the difference was statistically significant (P<0.05; Fig. 2A). The mRNA expression of PPAR-γ did not differ significantly among different incubation times in the control group, while the mRNA expression of PPAR-γ increased with prolonged ox-LDL induction time in the model group; the mRNA expression of PPAR-γ was highest at 72 h and was significantly higher than that in the control group (P<0.05; Fig. 2B). The mRNA expression of the lipid flow-related genes ABCA1 and ApoE was consistent in the control group and the model group. The mRNA expression levels of ABCA1 and ApoE increased with prolonged ox-LDL induction time; the mRNA expression of ABCA1 and ApoE was the highest at 72 h and the difference between the control and model groups was statistically significant (P<0.05; Fig. 2C and D). Therefore, 72 h was chosen as the optimal time for ox-LDL to induce foam-cell formation in macrophages.

Expression of ZNF580 in the foam cell model. The expression of ZNF580 in each group was analysed by RT-qPCR and western blot analysis to examine whether foam cell formation was associated with ZNF580 expression (Fig. 3). There was no significant difference between the control group and the model group when the ox-LDL induction time was 24 h; however, when the induction time was 48 and 72 h, the mRNA expression level of ZNF580 in the model group was significantly higher than that in the control group (P<0.05; Fig. 3A). The results of the western blot analysis (Fig. 3B) were consistent with those of the RT-qPCR, suggesting an increase in ZNF580 expression upon foam-cell formation at 72 h.

LV-ZNF580 and Si-ZNF580 lentivirus transfection. Fluorescence microscopy confirmed green fluorescence in the cytoplasm and around the nuclei of THP-1 cells after successful transfection, while untransfected cells had no green fluorescence (Fig. 4A). The expression of ZNF580 in each

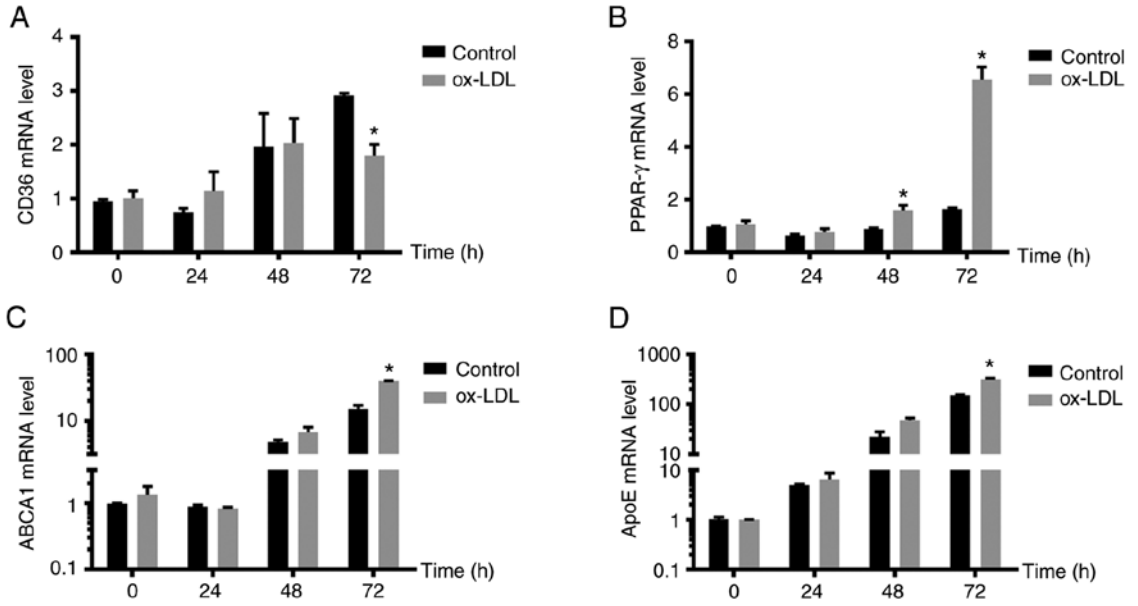

Figure 2. Model foam cells exhibit changes in lipid metabolism-related mRNA expression at different time-points. The mRNA levels of lipid metabolism-related genes, including (A) CD36, (B) PPAR-γ, (C) ABCA1 and (D) ApoE were measured by reverse transcription-quantitative PCR. The mRNA expression levels of CD36, PPAR-γ, ABCA1 and ApoE increased with prolonged ox-LDL induction time compared with those of the control group. At 72 h, the mRNA expression of CD36 in the control group was 1.5 times that of the model group and the mRNA expression of PPAR-γ, ABCA1 and ApoE was highest at 72 h. Therefore, 72 h was chosen as the optimal duration for ox-LDL to induce macrophages to form foam cells. The expression levels in the control group at 0 h, normalized to the housekeeping gene, were set as 1. *P<0.05 compared with the control group at the same time-point. ox-LDL, oxidized low-density lipoprotein; PPAR-γ, peroxisome proliferator activated receptor-γ; ABCA1, ATP-binding cassette transporter A1; ApoE, apolipoprotein E.

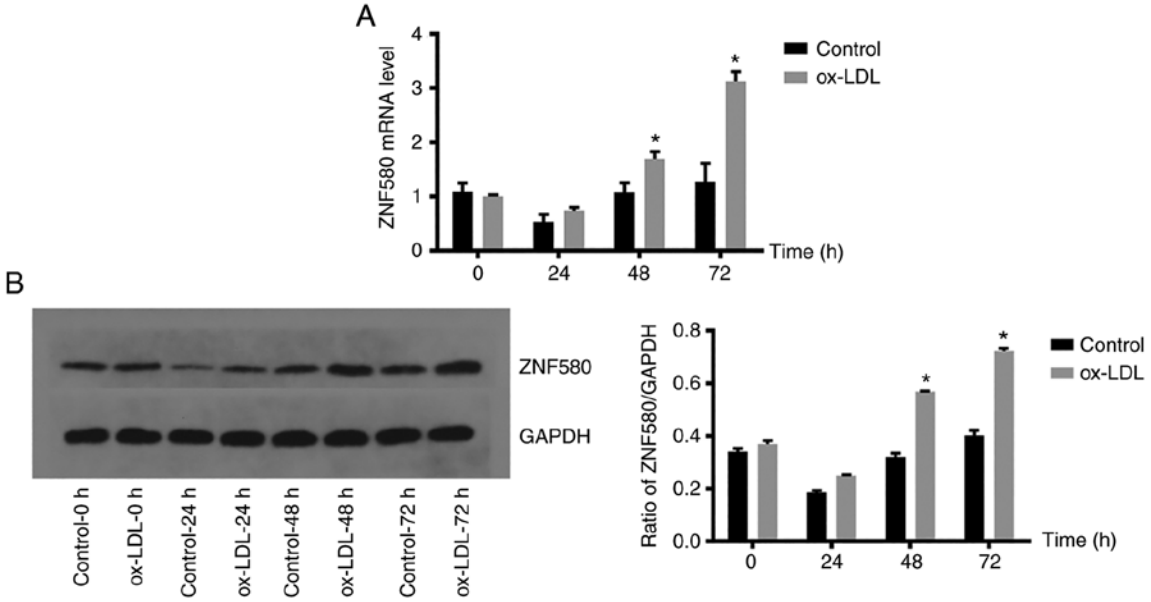

Figure 3. Expression of ZNF580 in foam cells at different time-points. (A) After ox-LDL induction for different durations, the levels of ZNF580 mRNA in each group were measured using RT-qPCR. (B) The protein levels of ZNF580 were measured using western blot analysis. *P<0.05 compared with the control group at the same time-point. ox-LDL, oxidized low-density lipoprotein; ZNF580, zinc finger protein 580; RT-qPCR, reverse transcription-quantitative PCR.

group was quantified by RT-qPCR and western blot analysis to examine whether the cells were successfully transfected (Fig. 4B and C). Compared with that in the LV-NC group, ZNF580 mRNA expression was significantly increased by LV-ZNF580. Compared with that in the Si-NC group, ZNF580 mRNA expression was significantly decreased by Si-ZNF580. No differences were observed between the cells in the control group and the LV-NC group or the Si-NC group, indicating that the empty vector lacking ZNF580 target gene but with the green fluorescent protein had no effect on the cells (P<0.05; Fig. 4B). The protein expression levels of ZNF580 in the different groups were determined by western blot analysis (Fig. 4C) and the results were consistent with those regarding the mRNA levels, indicating that THP-1 cells were successfully transfected with the LV-ZNF580 and Si-ZNF580 lentiviruses.

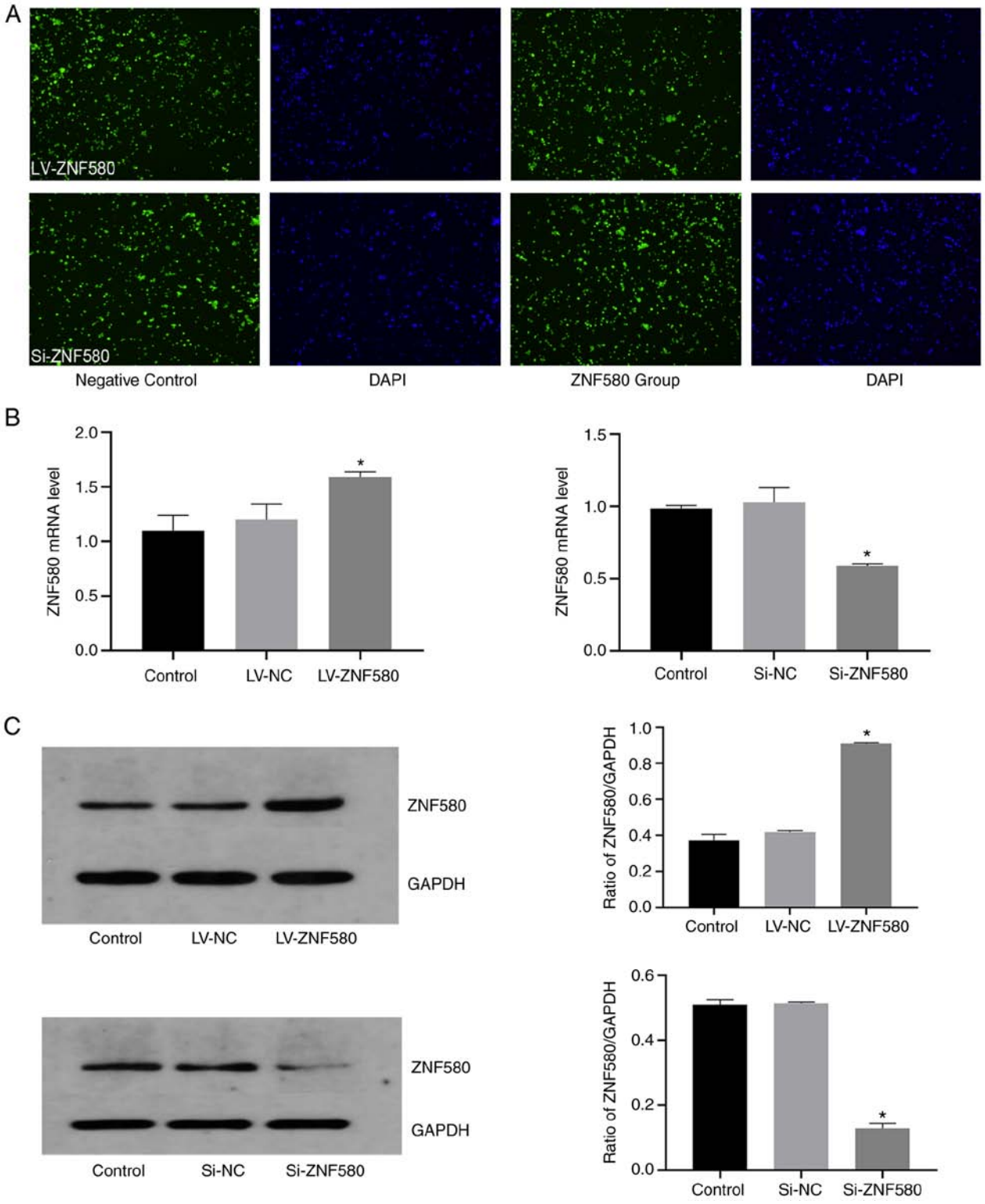

Figure 4. LV-ZNF580 and Si-ZNF580 lentivirus transfection. (A) Fluorescence microscopy was used to examine the transfection efficiency of LV-ZNF580 and Si-ZNF580 lentivirus (magnification, x200). Green fluorescence was visible in the cytoplasm and around the nucleus of transfected THP-1 cells. (B) The level of ZNF580 mRNA expression was measured by reverse transcription-quantitative PCR. (C) The levels of ZNF580 protein in THP-1 cells transfected with lentivirus were measured by western blot analysis. *P<0.05 compared with the NC group. ZNF580, zinc finger protein 580; LV-ZNF580, lentivirus for ZNF580 overexpression; Si-ZNF580, lentivirus expressing small interfering RNA of ZNF580; NC, negative control.

Establishment of foam cells transfected with LV-ZNF580 and Si-ZNF580. As indicated by the ORO staining images (Fig. 5A), the LV-ZNF580 model group had decreased numbers of ORO-stained cells compared to the model group and the lipid droplet area was smaller than that of the model group (Fig. 5B). Compared with those in the model group, the numbers of ORO-stained cells increased in the Si-ZNF580 model group (Fig. 5A) and the lipid droplet area was larger than that of the model group (Fig. 5C), indicating that ZNF580 inhibited foam-cell formation.

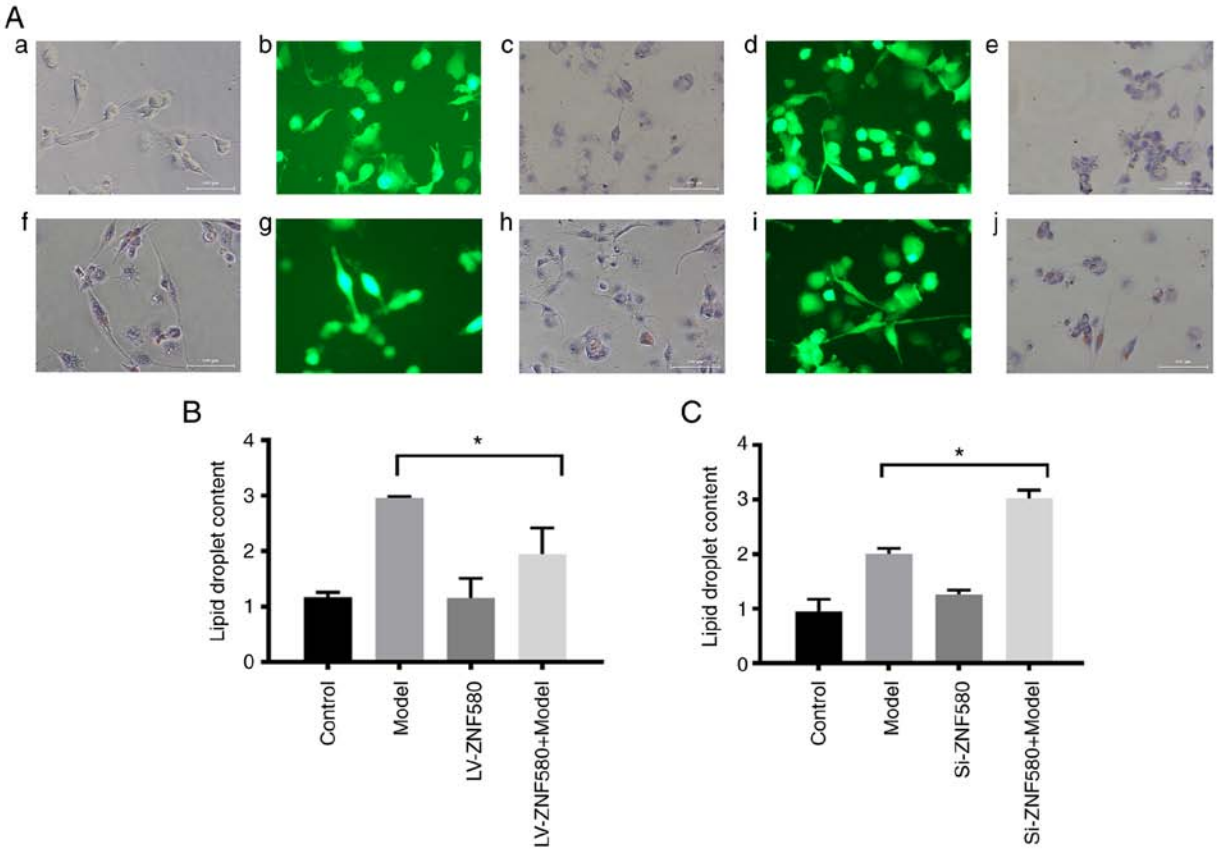

Figure 5. Establishment of foam cells in LV-ZNF580- and Si-ZNF580-transfected cells. (A) Morphological changes of cells in the (a) control, (b) LV-ZNF580 (fluorescence microscopy), (c) LV-ZNF580 (ORO staining), (d) Si-ZNF580 (fluorescence microscopy), (e) Si-ZNF580 (ORO staining), (f) model, (g) LV-ZNF580 + model (fluorescence microscopy), (h) LV-ZNF580 + model (ORO staining), (i) Si-ZNF580 + model (fluorescence microscopy) and (j) Si-ZNF580 + model (ORO staining) groups (magnification, x100; scale bars, 100 μm). (B) Quantification of changes in the lipid droplet area in LV-ZNF580 foam cells, as determined from ORO staining. (C) Quantification of changes in the lipid droplet area in Si-ZNF580 foam cells, as determined from ORO staining. *P<0.05 compared with the model group. ZNF580, zinc finger protein 580; LV-ZNF580, lentivirus for ZNF580 overexpression; Si-ZNF580, lentivirus expressing small interfering RNA of ZNF580; ORO, oil red O.

ZNF580 inhibits foam-cell formation by regulating the expression of lipid-related genes. To determine whether ZNF580 is able to prevent foam-cell formation by regulating ApoE transcription and increasing cholesterol outflow (Fig. 6A), RT-qPCR was performed. The results suggested that the mRNA expression of ApoE in the model group was higher than that in the control group. Compared with that in the model group, the mRNA expression level of ApoE increased significantly in the LV-ZNF580 model group, the mRNA expression level of ApoE decreased significantly in the Si-ZNF580 model group and the difference was statistically significant (P<0.05). The western blot results were consistent with the mRNA levels, indicating that ZNF580-mediated inhibition of foam-cell formation was associated with the regulation of ApoE (P<0.05).

The expression of lipid-related genes, such as CD36, PPAR-γ and ABCA1, in each group was determined using RT-qPCR and western blot analysis to examine the relationship between ZNF580 and lipid-related genes during foam-cell formation (Fig. 6B-D). Compared with those in the model group, the mRNA expression levels of CD36 and PPAR-γ decreased and those of ABCA1 significantly increased in the LV-ZNF580 model group, while the mRNA expression levels of CD36 and PPAR-γ increased and those of ABCA1 significantly decreased in the Si-ZNF580 model group. The western blot results were consistent with the mRNA levels, indicating that ZNF580-mediated inhibition of foam-cell formation was associated with the regulation of ABCA1, CD36 and PPAR-γ.

Discussion

CVD has become the most serious threat to human health among major diseases and is the leading cause of death worldwide; thus, the prevention and treatment of CVD has become a global public health concern. Among CVDs, ischaemic heart disease, mainly coronary heart disease (CHD), threatens human life (23). The main pathological change in CHD is coronary atherosclerosis, the pathological basis of which is atherosclerosis, a chronic inflammatory disease associated with lipid metabolic disorders. Lipids and other substances circulating in the blood are deposited in the endometrium of blood vessels, promoting the growth of local fibrous tissue and causing changes in the endometrial structure, bulging and expansion of the tube cavity and constantly forming lipid stripes and necrotic cores under the influence of persistent chronic inflammation. Lipid stripes and necrosis kernels begin with foam-cell formation and are the structural basis for atherosclerosis (24). Therefore, preventing excessive foam-cell production and accumulation is critical for the treatment

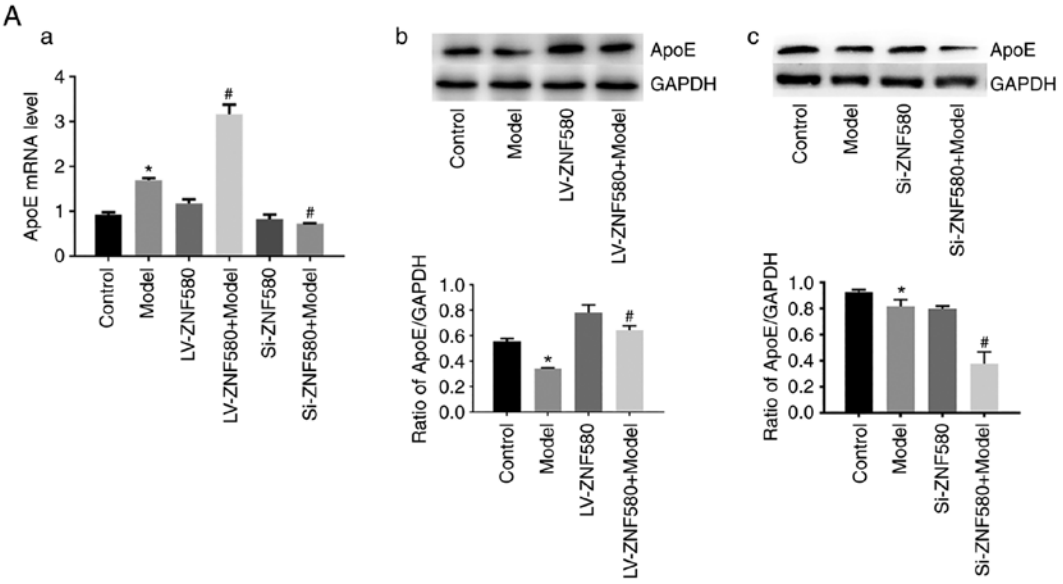

Figure 6. Continued.

of coronary atherosclerosis. Based on lipid metabolism in macrophages, the present study examined the receptors and proteins associated with lipid internalization, fat decomposition and cholesterol and phospholipid efflux, and explored the possible correlation between the zinc finger gene ZNF580 and foam-cell formation.

The class B SR CD36 has an important role in lipid uptake (25). CD36 is a single-stranded transmembrane glycoprotein with a long chain that mostly extends outside the cell; thus, the regulation of non-negative feedback is not limited by recognition and the intake of ox-LDL accelerates foam-cell formation (26). After CD36 gene deletion, the formation of intravascular foam cells was significantly lower than that in mice without CD36 gene deletion, suggesting that reducing the expression of CD36 was able to effectively reduce the risk of developing atherosclerosis (27). In the present study, ox-LDL-stimulated macrophages had increased CD36 expression, confirming that CD36 is involved in the process of foam-cell formation by macrophages. The mRNA expression of CD36 increased with time in ox-LDL-induced macrophages, indicating that CD36 affected foam-cell formation by macrophages in a time-dependent manner. Therefore, it is important to effectively inhibit foam-cell formation to study the regulatory factors that affect CD36 expression.

PPARs are members of the ligand-activated nuclear transcription factor superfamily and are divided into three types (PPAR-α, PPAR-β/δ and PPAR-γ). PPAR-γ is an important regulatory factor that is able to effectively regulate CD36 expression (28). It was reported that inhibiting PPAR-γ activation may effectively reduce the expression of CD36, thereby reducing the accumulation of lipids in high sugar-induced THP-1 cells (29). Therefore, by suppressing the expression of the upstream factor PPAR-γ, the expression of CD36 may be indirectly reduced. In the present experiment, RT-qPCR was used to measure the expression level of PPAR-γ and the results suggested that the trend was consistent with the trend of CD36 mRNA expression, supporting the conclusions of the published studies (28,30). Other studies have also indicated that activating PPAR-γ directly inhibits the migration and proliferation of single-core macrophages and indirectly inhibits foam-cell formation (31). The PPAR-γ agonist pyrethroidone is an important insulin allergen that is widely used to treat insulin resistance caused by elevated blood sugar. It is able to induce the expression of lipoprotein esterase to further promote fat breakdown and reduce plasma cholesterol and triglyceride levels. In addition, PPAR-γ is able to competitively inhibit the expression of inflammatory pathways and inflammatory factors to inhibit the occurrence and expansion of inflammation and reduce inflammation associated with endothelial damage in blood vessels (32). Therefore, it is urgent to find a suitable way to modulate PPAR-γ. PPAR-γ activation is also beneficial in reducing the risk factors associated with atherosclerotic plaque formation (33). Whether activating or suppressing PPAR-γ, it is important to find a suitable regulatory point to treat coronary atherosclerosis.

In the normal state, macrophages remove lipids to reduce cholesterol in and around the microenvironment and reverse the transfer of cholesterol to the liver for excretion. They also use cell surface ABCA1, a transmembrane protein capable of transporting cholesterol and phospholipids to the outside of cells in order to form a high-density lipoprotein precursor to achieve cholesterol reuse and excretion, reduce the accumulation of cholesterol esters in cells and inhibit foam-cell formation (34). This protects vascular walls and cardiovascular health. Furthermore, ABCA1 is able to delay the progression of atherosclerotic plaques and the erosion of plaques by inhibiting inflammatory factors involving Toll-like receptors at the atherosclerotic plaque formation site (35). Therefore, it is critical to explore the regulatory factors that affect ABCA1 to prevent and treat CHD. In the present study, RT-qPCR was used to measure gene expression and ABCA1 mRNA increased with the ox-LDL induction time, indicating that the cellular demand for cholesterol efflux increased. Another study confirmed that PPAR-γ activation promoted the transport efficiency of ABCA1 to facilitate efflux, suggesting that, by regulating PPAR-γ, the expression of ABCA1, a gene

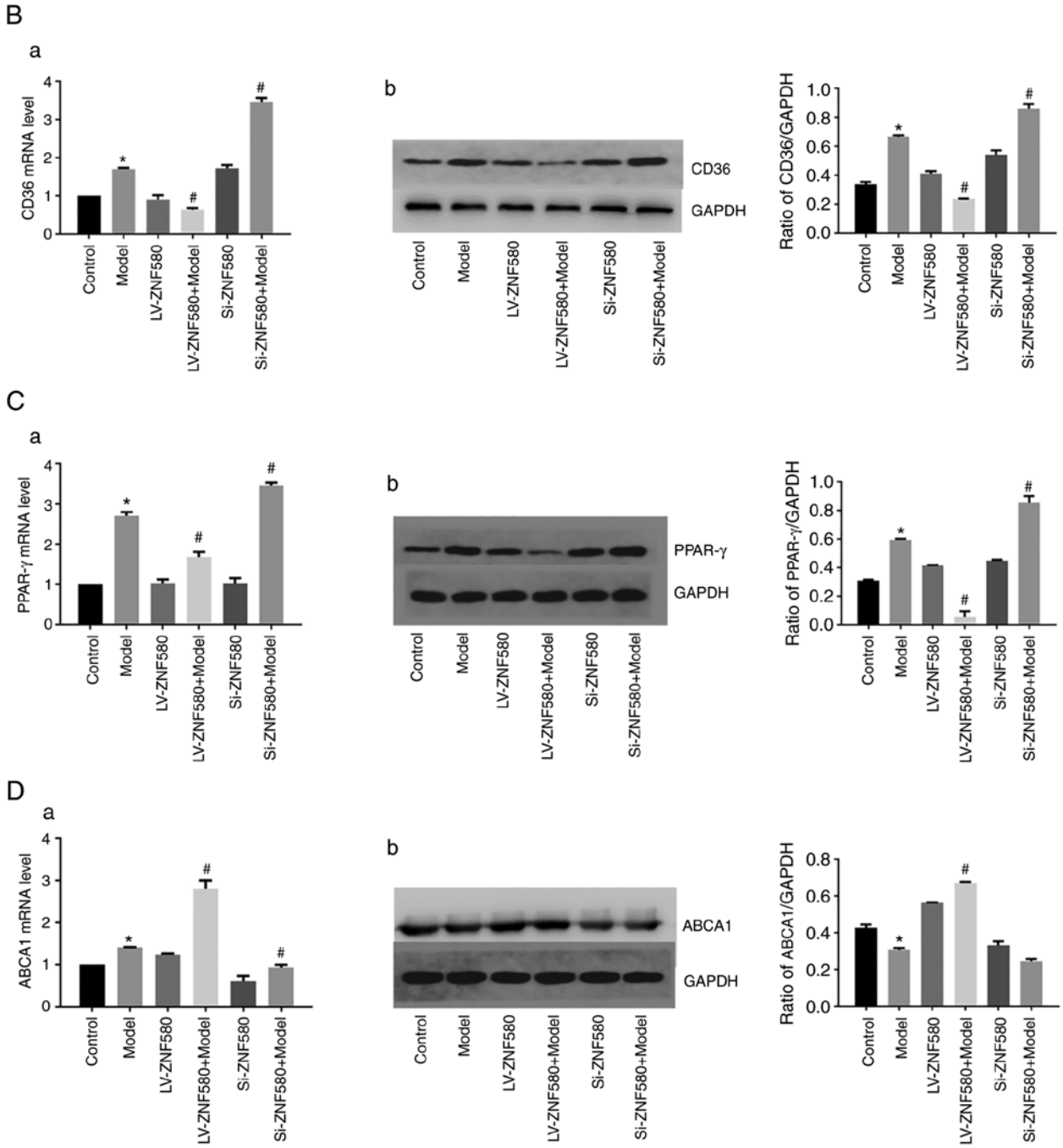

Figure 6. ZNF580 inhibits foam cell formation by regulating the expression of lipid-related genes. (A) The expression of ApoE in LV-ZNF580 and Si-ZNF580 foam cells was measured by (a) RT-qPCR and (b) western blot analysis. The expression of (B) CD36, (C) PPAR-γ and (D) ABCA1 in LV-ZNF580 and Si-ZNF580 foam cells was measured by (a) RT-qPCR and (b) western blot analysis. *P<0.05 compared with the control group; #P<0.05 compared with the model group. ZNF580, zinc finger protein 580; LV-ZNF580, lentivirus for ZNF580 overexpression; Si-ZNF580, lentivirus expressing small interfering RNA of ZNF580; PPAR-γ, peroxisome proliferator activated receptor-γ; ABCA1, ATP-binding cassette transporter A1; ApoE, apolipoprotein E; RT-qPCR, reverse transcription-quantitative PCR.

associated with cholesterol efflux, may be indirectly regulated to reduce atherosclerotic plaque formation (36).

ApoE is an alkaline protein that is rich in arginine; is found in high-density lipoproteins, very low-density lipoproteins and celiac particles; and has an important role in maintaining the structure of the abovementioned lipoproteins (37). Furthermore, ApoE is an independent risk factor for the formation of atherosclerotic heart disease due to its gene polymorphism, which is involved in various lipid metabolic processes, such as cholesterol efflux, transport and storage. Since it was indicated that ApoE deficiency rapidly leads to hyperlipidaemia in lipid metabolism disorders in the body, most experiments have modelled atherosclerotic plaques by knocking out this gene (38). In foam cells, ApoE has a function similar to that of ABCA1, which is able to transfer excess cholesterol esters from the cell to the extracellular space, reduce the accumulation of lipids in foam cells, and reduce and delay the formation and accumulation of foam cells. The results suggested that

ox-LDL stimulated macrophages, leading to the upregulation of CD36, which regulated PPAR-γ-mediated phagocytosis, and an increase in the mRNA expression of ApoE upon increased ox-LDL uptake by macrophages.

LDL is the most common cholesterol lipoprotein and is mainly responsible for the transfer of cholesterol to organs and tissues. As natural LDL cannot induce foam-cell formation in macrophages, acetylation and other modifications may only induce mononuclear cells to form macrophages and achieve lipid uptake, accumulation and eventually foam-cell formation when LDL is oxidized (39). Oxidation or other modifications to LDL have a higher risk of progression to atherosclerotic-like plaques; thus, reducing ox-LDL damage to blood vessel walls, ox-LDL accumulation in mononuclear cells and macrophage uptake of ox-LDL are novel strategies to prevent plaque formation (40).

ZNF580, a nuclear transcription factor that is similar in protein structure to the Sp/KLF family, is widely distributed in human organs and regulates downstream target genes to participate in physiological and pathological processes in the body in a variety of ways. ZNF580 has an important role in the disease-causing mechanism of CHD and a previous study suggested that ZNF580 was able to participate in ischaemic reperfusion damage mediated by umbilical vein endothelial cell growth factor-15 (41). After establishment of the experimental foam-cell model, ZNF580 mRNA levels were measured and the results suggested that the expression of ZNF580 was higher than that in the normal control group, indicating that ox-LDL-stimulated macrophages exhibited changes in ZNF580 expression. To further study the effect of ZNF580 on foam-cell formation, ZNF580 mRNA and protein levels were measured in the control group at 24, 48 and 72 h, and the results confirmed that ZNF580 expression increased with increasing foam-cell formation over time. This trend was largely the same as that of CD36, PPAR-γ, ABCA1 and ApoE, which are associated with lipid uptake and efflux in macrophages. Based on the results of this experiment, it may be assumed that ZNF580 is able to block foam-cell formation by affecting relevant lipid regulatory molecules, possibly in relation to the PPAR-γ-CD36 signalling pathway. However, the more specific relationship will be further explored in future work. Lentivirus transfection was then used to induce high expression or silence the ZNF580 gene in THP-1 cells, after which the foam-cell model was established and the above indicators examined. The results suggested that CD36 and PPAR-γ mRNA expression in the high-expressing ZNF580 group was lower than that in the model group, and CD36 and PPAR-γ mRNA expression in the ZNF580 silencing group was significantly higher than that in the model group. These data indicated that ZNF580 is able to inhibit the expression of PPAR-γ mRNA, thereby reducing the expression of the downstream molecule CD36 and LDL intake, hindering the formation of foam cells. The genes associated with lipid outflow were examined and it was indicated that high expression of ZNF580 was able to induce an increase in ABCA1 and ApoE mRNA expression that was significantly higher than that in the model group. In addition, silencing ZNF580 was able to inhibit the mRNA expression of ABCA1 and ApoE, which was significantly lower than that in the model group, suggesting that ZNF580 is able to hinder foam cell formation by increasing cholesterol efflux. Therefore, it may be hypothesized that ZNF580 may be situated upstream of PPAR-γ, directly modulating PPAR-γ and thereby affecting downstream CD36 expression and reducing lipid build-up.

In conclusion, the present study provided new insight into the utility of ZNF580 in the prevention of atherosclerosis. High expression of ZNF580 was able to decrease lipid uptake by macrophages and increase cholesterol efflux, reduce the possibility of lipid accumulation in foam cells, reduce the proportion of foam-cell formation, reduce the risk of plaque formation and delay the progression of atherosclerotic plaques. However, the present study only carried out cell experiments, which may be considered a limitation, and future *in vivo* studies will be conducted to more fully explore the role of ZNF580 in atherosclerosis. Based on these findings, ZNF580 may be a potential target for preventing atherosclerosis.

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Availability of data and materials

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

Authors' contributions

ZBZ, XTQ and MZ designed the experiments and revised the manuscript. ZBZ, HXC, YCL and MY performed the RT-qPCR assays. JXC, HWX, YT and YCL performed ORO staining. ZBZ, XTQ, HXC, CL and MY performed western blot analysis. ZBZ, CL, JYL, JXC and MZ analysed the datasets and supervised the project. All authors read and approved the final manuscript and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare.

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