Atherosclerosis 289 (2019) 143–161

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Krüppel-like factor 14 inhibits atherosclerosis via mir-27a-mediated down-regulation of lipoprotein lipase expression *in vivo*

Wei Xie[a,b,1], Liang Li[a,c,1], Duo Gong[a,1], Min Zhang[a], Yun-Cheng Lv[b], Dong-ming Guo[b], Zhen-Wang Zhao[a], Xi-Long Zheng[d,e], Da-Wei Zhang[f], Xiao-Yan Dai[g], Wei-Dong Yin[a,*], Chao-Ke Tang[a,**]

[a] *Institute of Cardiovascular Research, Key Laboratory for Atherosclerology of Hunan Province, Medical Research Center, Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, University of South China, Hengyang, 421001, Hunan, China*
[b] *Clinical Anatomy & Reproductive Medicine Application Institute, University of South China, Hengyang, 421001, Hunan, China*
[c] *Department of Pathophysiology, University of South China, Hengyang, 421001, Hunan, China*
[d] *Department of Biochemistry and Molecular Biology, The Libin Cardiovascular Institute of Alberta, Cumming School of Medicine, The University of Calgary, Health Sciences Center, 3330 Hospital Dr NW, Calgary, Alberta, Canada, T2N 4N1*
[e] *Key Laboratory of Molecular Targets & Clinical Pharmacology, School of Pharmaceutical Sciences, Guangzhou Medical University, Guangzhou, Guangdong, 511436, China*
[f] *Department of Pediatrics and Group on the Molecular and Cell Biology of Lipids, University of Alberta, Alberta, Canada*
[g] *Guangdong Key Laboratory of Molecular Target & Clinical Pharmacology, School of Pharmaceutical Sciences and the Fifth Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong, 511436, China*

HIGHLIGHTS

- KLF14 inhibits proinflammatory cytokines and lipid accumulation in macrophages via binding to the promoter of miR-27a.
- MiR-27a-mediated LPL downregulation participates in the inhibitory effect of KLF14 on inflammation and lipid accumulation.
- Gypenosides, a KLF14 activator, delays the development of atherosclerosis in *apoE*$^{-/-}$ mice.

ARTICLE INFO

Keywords:
Krüppel-like factor
MicroRNA
Lipoprotein lipase
Inflammation
Macrophage
Atherosclerosis

ABSTRACT

Background and aims: Krüppel-like factor 14 (KLF14) is known to play a role in atherosclerosis, but the underlying mechanisms are still largely unknown. The aim of our study was to explore the effects of KLF14 on lipid metabolism and inflammatory response, providing a potential target for lowering the risk of atherosclerosis-causing disease.

Methods and results: mRNA and protein levels of KLF14 were significantly decreased in oxidized low-density lipoprotein (oxLDL)-treated macrophages and in the atherosclerotic lesion area. Chromatin immunoprecipitation (ChIP) and luciferase reporter gene assays were used to confirm that KLF14 positively regulated miR-27a expression by binding to its promoter. We also found that KLF14 could restored appropriate cellular lipid homeostasis and inflammatory responses via negatively regulating lipoprotein lipase (LPL) expression in THP1-derived macrophages through miR-27a. In addition, gypenosides (GP), a KLF14 activator, delayed the development of atherosclerosis in apolipoprotein E deficient (*apoE*$^{-/-}$) mice.

Conclusions: KLF14 plays an antiatherogenic role via the miR-27a-dependent down-regulation of LPL and subsequent inhibition of proinflammatory cytokine secretion and lipid accumulation.

* Corresponding author. Institute of Cardiovascular Research, University of South China, Hengyang, Hunan, 421001, China.
** Corresponding author. Institute of Cardiovascular Research, University of South China, Hengyang, Hunan, 421001, China.
E-mail addresses: wdy20042004@126.com (W.-D. Yin), tangchaoke@qq.com (C.-K. Tang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.atherosclerosis.2019.08.012
Received 5 October 2018; Received in revised form 15 August 2019; Accepted 22 August 2019
Available online 26 August 2019
0021-9150/

1. Introduction

Atherosclerotic cardiovascular disease (ASCVD) is a major cause of mortality and disability worldwide. Atherosclerosis (AS) is characterized by the transformation of macrophages into foam cells and excessive secretion of inflammatory cytokines in the vascular subendothelial space [1,2]. Thus, it is critical to clarify the mechanisms underlying lipid accumulation and inflammatory response for treatment of ASCVD and subsequent clinical complications [3,4]. Previous studies have indicated that Krüppel-like factor 14 (KLF14), a zinc finger transcription factor, participates in lipid metabolism in *vivo* [5,6], but it remains unclear if and how KLF14 alters composition and content of lipids and the secretion of proinflammatory cytokines in the macrophage, affecting AS.

KLF14, a member of KLF family, can regulate expression of genes involved in many important biological and pathological processes of the organism via binding to the GT/GC-rich *cis*-regulatory sites in target gene promoters or enhancers [7]. It is noteworthy that abnormal expression of KLF14 has been implicated in many atherosclerosis-related risk factors such as diabetes [8,9], metabolic syndrome [10], endothelial dysfunction [11] and inflammation [12] etc. Genome-wide association studies (GWAS) reveal that KLF14 acts as a master *trans*-mediator of adipose gene expression [8,13,14], affecting the concentration or sizes of low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very-low-density lipoprotein (VLDL) in plasma [6]. Another independent study demonstrated that rs4731702 with a C to T substitution in the single nucleotide polymorphism (SNP) of ~14 kb upstream of *KLF14* gene is associated with reduced HDL cholesterol (HDL-C) levels and the ratio of APOA-I to APOB [15], and a *KLF14* SNP is identified as a high risk factor of ASCVD [16]. Furthermore, liver-specific deletion of *klf14* markedly decreases plasma HDL-C levels and cholesterol efflux capacity in apolipoprotein E deficient ($apoE^{-/-}$) mice as a result of the loss of klf14-mediated stimulatory effects on apoA-I promoter, and thus demonstrated that hepatic klf14 reduces atherosclerosis, highlighting the important role of klf14 in the pathogenesis of ASCVD [5]. In addition, recent studies revealed that klf14 inhibited the endothelial inflammatory response, suggesting it serves as an important potential anti-inflammatory regulator [11]. Indeed, numerous studies have shown that KLF family members are involved in the regulation of phenotypes and functions of macrophages. For instance, myeloid-specific *klf2* deletion in $apoe^{-/-}$ mice increases macrophage adhesion and atherosclerosis [17]. Furthermore, klf4 regulates polarization of macrophages from the M1 to M2 phenotype and thereby acts as an anti-inflammatory role [18]. However, it is still unclear whether KLF14 affects macrophage inflammation-mediated atherosclerosis. Therefore, exploration of the role of KLF14 in lipid metabolism and inflammatory response in macrophages may shed new light on the diagnosis and treatment of atherosclerosis.

Lipoprotein lipase (LPL) is an enzyme expressed and secreted by parenchymal cells, such as macrophage, skeletal muscle cell and adipocytes. Increasing attention has been paid to the proatherogenic effect of macrophage-derived LPL [19] although a large number of studies have been focused on its role in the hydrolysis of triglyceride (TG) core of circulating triglyceride-rich lipoproteins (TRL*s*) [20]. For example, macrophage-specific *lpl* knockout mice do not demonstrate any changes in plasma lpl hydrolytic activities, but have a remarkable repression in formation of foam cells and atherosclerotic plaques [21], which can be reversed by specifically expressing lpl in macrophages in $apoE^{-/-}$ mice and rabbits [22,23]. It is well established that macrophage LPL plays an atherogenic mediator through its non-enzymatic molecular bridge between proteoglycans and lipoprotein receptors, inducing the retention of atherogenic lipoproteins for subsequent cellular uptake and gene expression of inflammatory factors in subendothelial spaces. Therefore, it is likely that negative regulation of LPL expression in macrophages may become an important approach for prevention and treatment of AS. MicroRNAs, as an important negative regulator of target gene expression at post-transcriptional level, participate in maintaining lipid homeostasis and regulating inflammation in AS [24–27]. Notably, we have previously demonstrated that miR-27 inhibits lpl expression through targeting the 3′UTR of *lpl* gene, attenuating proinflammatory cytokine secretion and lipid accumulation and thus reducing AS [28]. Interestingly, we identified potential binding sites of KLF14 in the promoter of miR-27 but not other miRNAs using bioinformatics sequence tools (JASPAR). Thus, it is possible that miR-27 acts as a potential target of KLF14 to regulate inflammation and lipid metabolism in AS.

In the present study, we have identified that KLF14 downregulated LPL expression in a miR-27a dependent manner and consequently reduced cellular lipid accumulation in macrophages, decreased proinflammatory cytokine secretion, and inhibited the progress of AS. Upregulation of klf14 by gypenosides (GP), a KLF14 activator, delayed the development of AS in $apoE^{-/-}$ mice. Taken together, our studies reveal one underlying molecular mechanism by which the KLF14/miR-27a pathway down-regulates the expression of LPL.

2. Materials and methods

2.1. Clinical samples

Atherosclerotic arterial samples were collected from aortic dissection patients (thoracic or abdominal aortic aneurism) at The First Affiliated Hospital of University of South China, Hengyang, China. Arteries without macroscopic evidence of atherosclerosis were collected from individuals who died in a road traffic accident. The tissue samples collected were snap frozen in liquid nitrogen and grinded to extract the mRNA and protein before the analyses of this study. All experiments were approved by the Ethics Committee of the University of South China, and consent form were obtained from study participants or their representatives.

2.2. Cell culture and treatments

THP-1 monocytes were treated with 160 nmol/L phorbol 12-myristate 13-acetate (PMA) to differentiate into macrophages. The THP-1-dervied macrophage, peritoneal macrophages extracted from $apoE^{-/-}$ mice and RAW264.7 cells obtained from ATCC were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2% penicillin-streptomycin. The macrophages were incubated with gypenosides (GP) that were directly added into the culture solution (final concentration of 50 μg/mL) for 48 h to detect relative protein expression.

2.3. LDL isolation and oxidization

Native lipoproteins (LDL: d = 1.025–1.063 g/mL) were isolated from human plasma by discontinuous density gradient ultracentrifugation as described previously [18]. Briefly, the density of plasma was adjusted to 1.006 g/mL with sodium chloride medium, and the plasma was centrifuged at 150 000 *g* for 24 h. The Very low-density lipoprotein (VLDL) and chylomicron-enriched layer was discarded. The density of the remaining fraction was adjusted to 1.063 g/mL with potassium bromide and then centrifuged at 150 000 *g* for 24 h to separate LDL from HDL fraction. The purified LDL was dialyzed for 96 h against PBS containing 0.3 mmol/L EDTA at 4 °C and degassed with N_2. LDL (200 μg protein/mL) was oxidized in the presence of 10 μmol/L $CuSO_4$ for 18–20 h at 37 °C. The purity of isolated LDL and ox-LDL was confirmed by the lipoprotein electrophoresis on an agarose gel. Oxidation of LDL was confirmed by measuring thiobarbituric acid-reactive substances (TBARS) with malonaldehyde bis (dimethyl acetal) (MDA) as the standard. TBARS content of ox-LDL and native LDL was 18.30 ± 0.62 and 0.94 ± 0.37 nmol/mg protein, respectively

($p < 0.01$). Ox-LDL was extensively dialyzed against tris-saline, kept in 50 μM Tris–HCl, 0.15 M NaCl and 2 μM EDTA at pH 7.4, and was used within 10 days. The level of endotoxin was measured by the E-Toxate kit (Sigma), which was consistently less than 0.005 EU/ml (lowest detection limit) [29].

2.4. RNA isolation and real time-quantitative PCR (RT-qPCR)

Total RNA was prepared from THP-1-derived macrophages, RAW264.7 cells or peritoneal macrophages using TRIzol reagent according to the manufacturer's instructions. Complementary DNA was

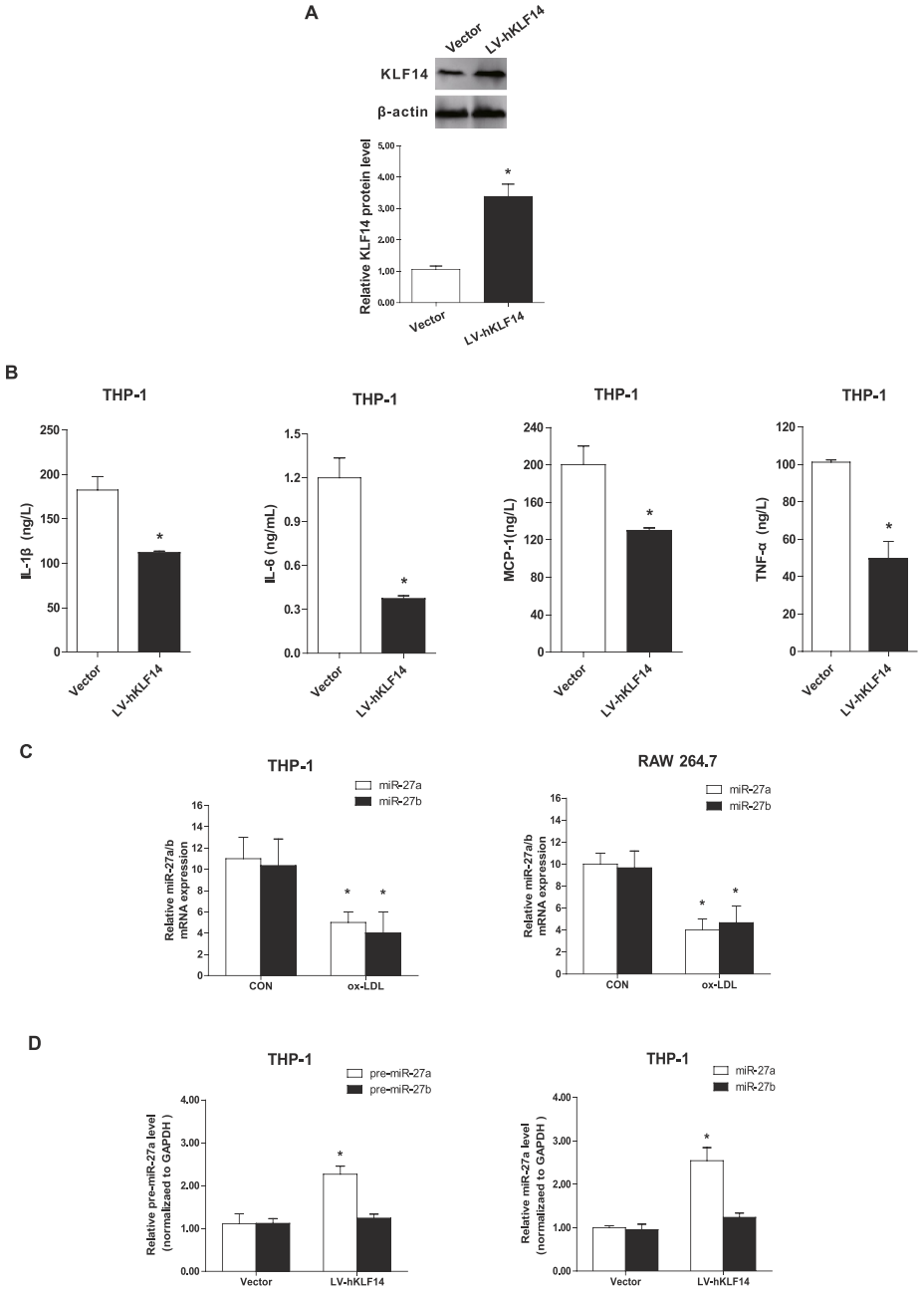


Fig. 1. KLF14 decreases proinflammatory cytokine secretion and lipid accumulation via binding to the miR-27a promoter in macrophages.
(A) Infection with recombinant lentiviral vector encoding human KLF14 (LV-hKLF14) results in overexpression of *KLF14* in THP-1 macrophages. (B) Overexpression of *KLF14* reduces the levels of proinflammatory cytokines (IL-1β, IL-6, MCP-1 and TNF-α) in ox-LDL treated THP-1 macrophages. $*p < 0.05$ *vs* vector group. (C) The expression of miR-27a/b down-regulation responded to ox-LDL treatment in THP-1 and RAW264.7 macrophage. $*p < 0.05$ *vs* CON group. (D) Overexpression of KLF14 with LV-KLF14 increases endogenous expression of precursor miR-27a (pre-miR-27a) and miR-27a, but not miR-27b, in THP-1 macrophages. $*p < 0.05$ *vs* vector group. (E) Expression of *KLF14* is downregulated in siRNA treated THP-1 macrophage. (F) Downregulation of *KLF14* with siRNA decreases the expression of pre-miR-27a and miR-27a, but not miR-27b, in the THP-1 macrophages. $*p < 0.05$ *vs* si-RNA-neg group. (G) After THP-1 macrophages were incubated with actinomycin D (Act D) and infected with AdKLF14 for 24 h, KLF14 mRNA and miR-27a levels were determined by real-time qPCR, respectively. $*p < 0.05$ *vs* vector group, $**p < 0.01$ *vs* vector group, $^{\#}p < 0.05$ *vs* DMSO group. (H) Two putative sites located at nt −1435∼ −1422 and −1521∼ −1508 in the promoter region of miR-27a with the binding sites of KLF14. Relative positions of the binding sites are predicted. (I) ChIP-PCR and ChIP-qPCR analysis of the KLF14 hit at the −1521∼ −1508 site at the miR-27a promoter in THP-1 macrophages. ChIP-q-PCR results are shown as fold enrichment compared with input. $**p < 0.01$ *vs* IgG group. (J) Mutations of the potential KLF14 binding sites verified by luciferase reporter gene assay show that miR-27a expression is dependent on KLF14-targeted binding site. $*p < 0.05$ *vs* WT group. (K) Luciferase reporter gene assay demonstrated that KLF14 can increase miR-27a promoter activity, but not KLF2, KLF4 and KLF11, in THP-1 macrophages infected with recombinant lentiviral vector. $**p < 0.01$ *vs* vector group. All data are expressed as mean ± S.D. from three independent experiments.

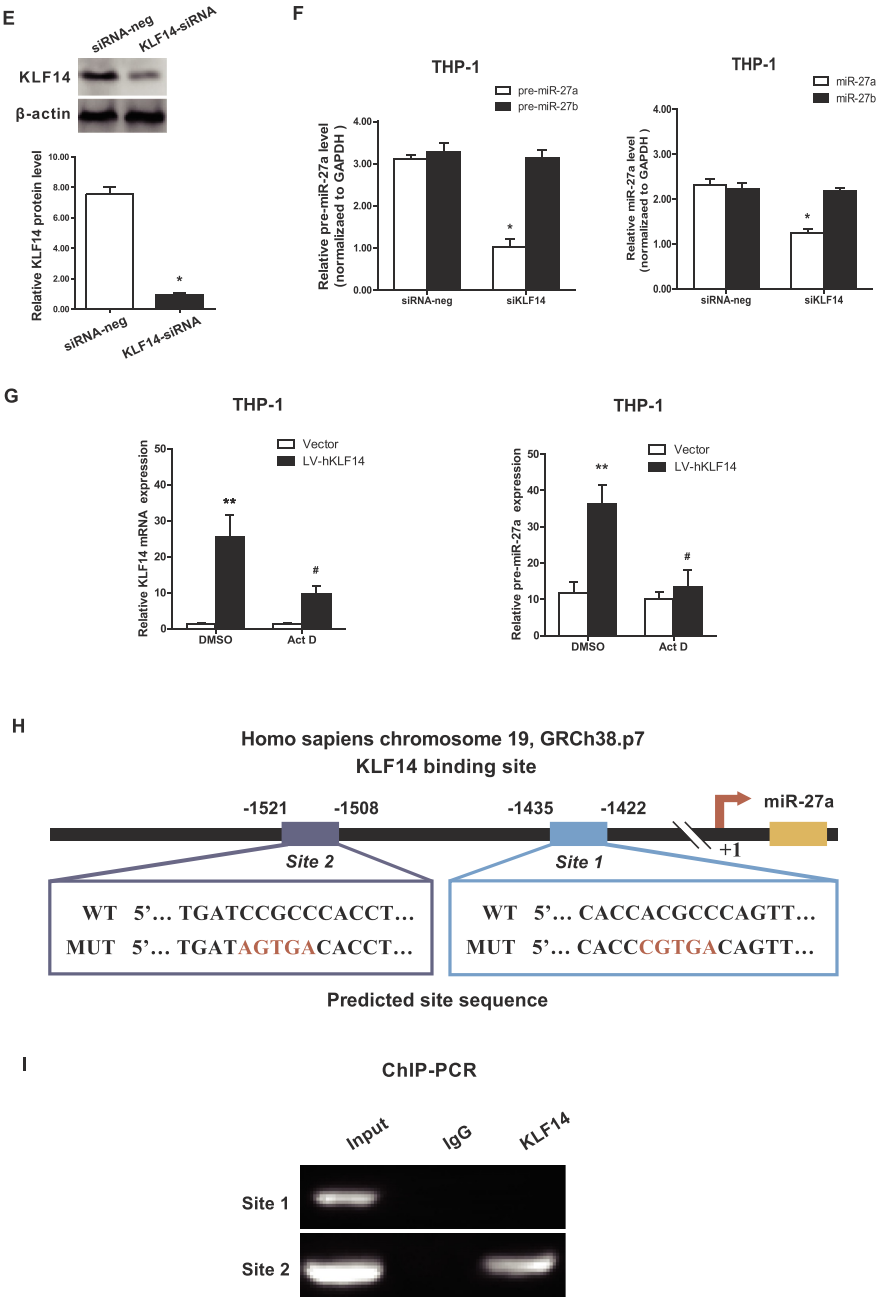


Fig. 1. (*continued*)

synthesized using a high-capacity cDNA reverse transcription kit (Takara, China), followed by 40-cycle 2-step PCR with sequence-specific primer pairs in the iCycler IQ Real-Time Detection System (Bio-Rad, U.S.A). All oligonucleotide sequences of the primers are shown below: KLF14, forward: 5′-CCTCAAGTCACACCAGCGTA-3′, and reverse: 5′-CGACCTCGGTA CTCGATCAT-3'; LPL, forward: 5′- GGGAGTT TGGCTCCA GAGTTT-3′, and reverse: 5′-TGTGTCTTCAGGGGTCCTTAG-3'; GAPHD, forward: 5′-AACTTTGG CATTGTGGAAGG-3′, and reverse: 5′-ACACATTGGGG GTAGGAACA-3'. All quantifications were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPHD), according to the formula ΔCt = [Ct] – [Ct (GAPHD)] and ΔΔCt = [ΔCT (sample)] – [ΔCT (control)].

2.5. Total protein extraction and Western blot analysis

Cells or tissues were washed twice with PBS, lysed with lysis buffer containing PMSF (94:6, V/V) and placed on ice for 25 min. After, the cell lysate was isolated by centrifugation at 12 000 rpm for 10 min at 4 °C. Protein concentrations in the supernatants were measured using the BCA assay. The proteins were then mixed with 5 × SDS loading buffer containing 10% β-mercaptoethanol and heated for 10 min. Equal amount of proteins was then subjected to 6% or 10% SDS-polyacrylamide gels for separation with subsequently electrotransferring to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk and then incubated with primary antibodies to LPL (ab21356, rabbit monoclonal antibody, 1:1000, ABCAM, U.K.), KLF14 (TA341450, rabbit polyclonal antibody, Origene, U.S.A.), β-actin (60008-1-Ig, mouse monoclonal antibody, Proteintech, U.S.A.) at 4 °C for 6–8 h, respectively. After incubation, the membranes were washed 3 times in TBST and incubated with secondary horseradish peroxidase-linked anti-rabbit or anti-mouse IgG (Beyotime, 1:1000, China) for 2 h. After washing, immune-active proteins were visualized with ECL Plus. β-actin was the internal control.

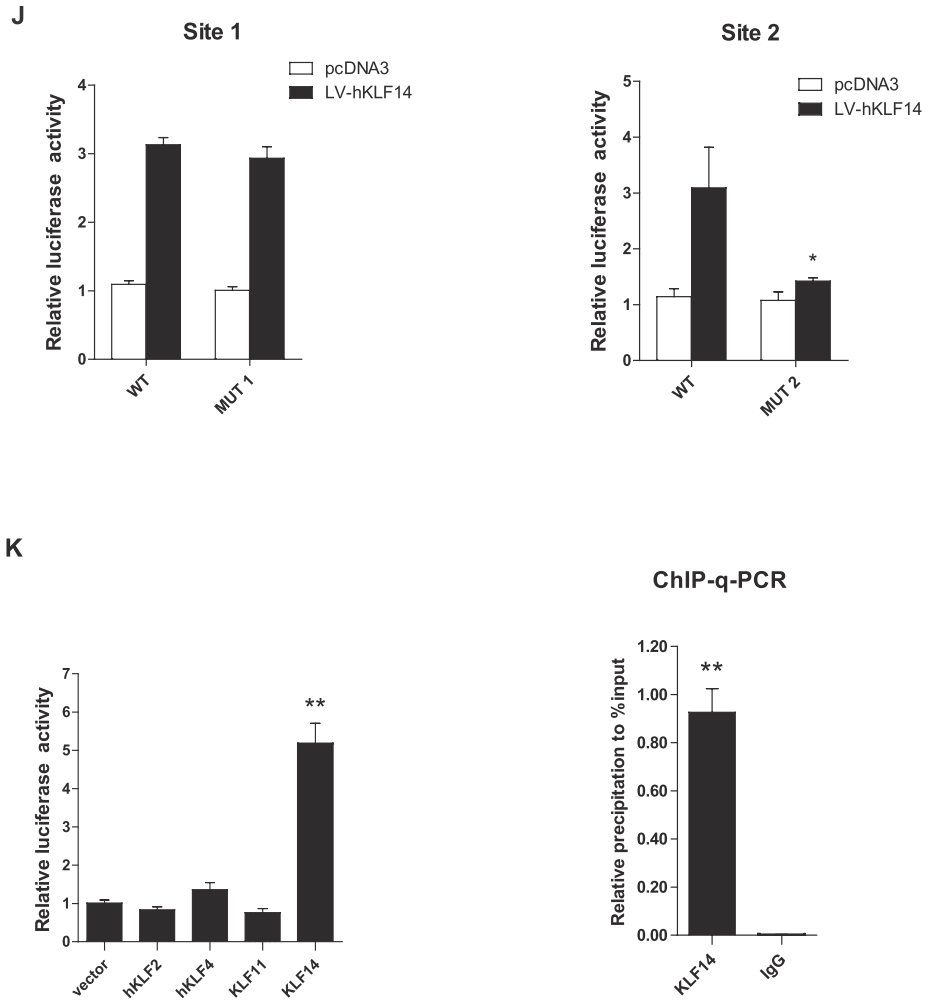


Fig. 1. (*continued*)

2.6. Luciferase reporter gene assay

The PCR was used to amplify the cDNA fragment (Primers: Sense: 5′-ATGGGAAAGAGTGGCTGGTATGTGT-3'; anti-sense: 5′-TAAAGGTTGG CTG GGACTCCCTG GC-3′) corresponding to the promotor region of 2000 bp of miR-27a from THP-1-derived macrophages. The luciferase vectors with the promoter of human wild-type miR-27a (−1435∼-1422: CACC ACGCCCAGTT, −1521∼-1508: TGATCCGCCCACCT) or mutated miR-27a (−1435∼-1422: CACCCGTGACAGTT, −1521∼-1508: TGATAGTG ACACCT) were constructed to normalize transfections. THP-1-derived macrophages were co-transfected with wild-type miR-27a or mutation miR-27a promotor reporter constructs and LV-KLF14.12 h later, the cells were washed with PBS and incubated with 10% FBS/RPMI for another 12 h. Analysis of luciferase activities was performed using the Dual-Glo Luciferase Assay System (Promega), which were normalized to the corresponding firefly luciferase activity and plotted as a percentage of the control. Data are the means of three independent experiments and represented as fold change in comparison with control group.

2.7. Enzyme-linked immunosorbent assay (ELISA)

THP-1-derived macrophages at a density of 3.0×10^5 cells/cm^2 were treated with KLF14 or DMSO (vehicle control) in the presence of ox-LDL (50 μg/ml). Culture media were collected, centrifuged at 4 °C for 10 min, and then examined for the presence of IL-1β, IL-6, MCP-1 and TNF-α using ELISA kits (ThermoFisher Scientific, U.S.A.) according to the manufacturers' instruction.

2.8. Transfection experiments

The human KLF14 cDNA was amplified by PCR and cloned into the pLV-EF1a-GFP vector. The construct (referred as LV-hKLF14 hereafter) and the empty vector were used to transfect cultured THP-1-derived macrophages at a multiplicity of infection of 100 transfecting units per cell in the presence of 8 mg/ml of polybrene. 24 h later, the cells were washed with fresh complete media. The human LPL expression vector were constructed with pLV-EF1a-RFP vector (LV-hLPL). The cells were cultured with bovine lpl (blpl) or transfected with LV-hLPL when treated with LV-hKLF14. The KLF14 small interfering RNA (siKLF14) was purchased from GenePharma. The THP-1-derived macrophages were transfected with siKLF14 using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. The serum-containing media (2% serum) were then added to cells before the experiments and/or functional assays were conducted. The effects of gene silencing or overexpression were confirmed using Western blot up to 48 h after transfection.

2.9. Lipid content assay by high-performance liquid chromatography (HPLC)

HPLC analysis of lipid content was conducted as described previously [28]. THP-1-derived macrophages were washed three times with PBS. Total proteins were extracted and measured using BCA Protein Assay kit (Beyotime). Cholesterol was extracted with isopropanol and stored at −20 °C. The stock solution was diluted with a cholesterol

standard calibration solution. 0.1 ml of cholesterol standard calibration solution or cell solution was supplemented with 10 ml reaction mixture. 0.4 U cholesterol oxidase in 10 ml of 0.5% NaCl was added for free cholesterol determination, or 0.4 U cholesterol oxidase plus 0.4 U of cholesterol esterase for total cholesterol measurement. The reaction solution was then incubated at 37 °C for 30 min, and 100 ml of methanol: ethanol (1:1) was added to stop the reaction. Each solution was placed on ice for 30 min and centrifuged at 1500 rpm for 10 min at 4 °C. After, 10 ml of supernatant was applied to a 2790 Chromatographer (Waters, U.S.A). The column was eluted for 12 min with PBS solution and monitored by absorbance at 226 nm. Data were analyzed with Total Chrom software from PerkinElmer.

2.10. Chromatin immunoprecipitation assay (CHIP)

The cells were cross-linked in 1% formaldehyde for 15 min at 37 °C, and then glycine solution was added to stop the reaction. SDS Lysis Buffer (Beyotime, China) and phenylmethylsulfonyl fluoride (PMSF) (Beyotime, China) were then added to the cells before sonication with the ultrasonic processor (Sonics, U.S.A) for 14 bursts of 4.5 s with 9s intervals under 60 W on the ice. Cell lysate was centrifuged at 12000 rpm for 10 min at 4 °C. The supernatant containing sheared chromatin was kept on the ice. After, the samples were subjected to immunoprecipitation using a ChIP assay kit (ABCAM, U.K.) and antibody against Elk-1 (ABCAM, U.K.) or antibodies against IgG (ABCAM, U.K.). The DNA was eluted, and then collected to analysis using agarose gel electrophoresis.

2.11. Mice

Male *apoE*$^{-/-}$ mice (8 weeks of age) were purchased from Changzhou Cavens Laboratory Animal Co. LTD (Jiang Su, China). The mice were injected via the tail vein with miR-27a antagomir (ANa), 2×10^8 TU/ml lentivirus particles of human LPL or KLF14 siRNA and then fed the high-fat diet (HFD) containing with or without 200 mg/kg/d GP for 12 weeks after a week of adaptive feeding with a chow diet.

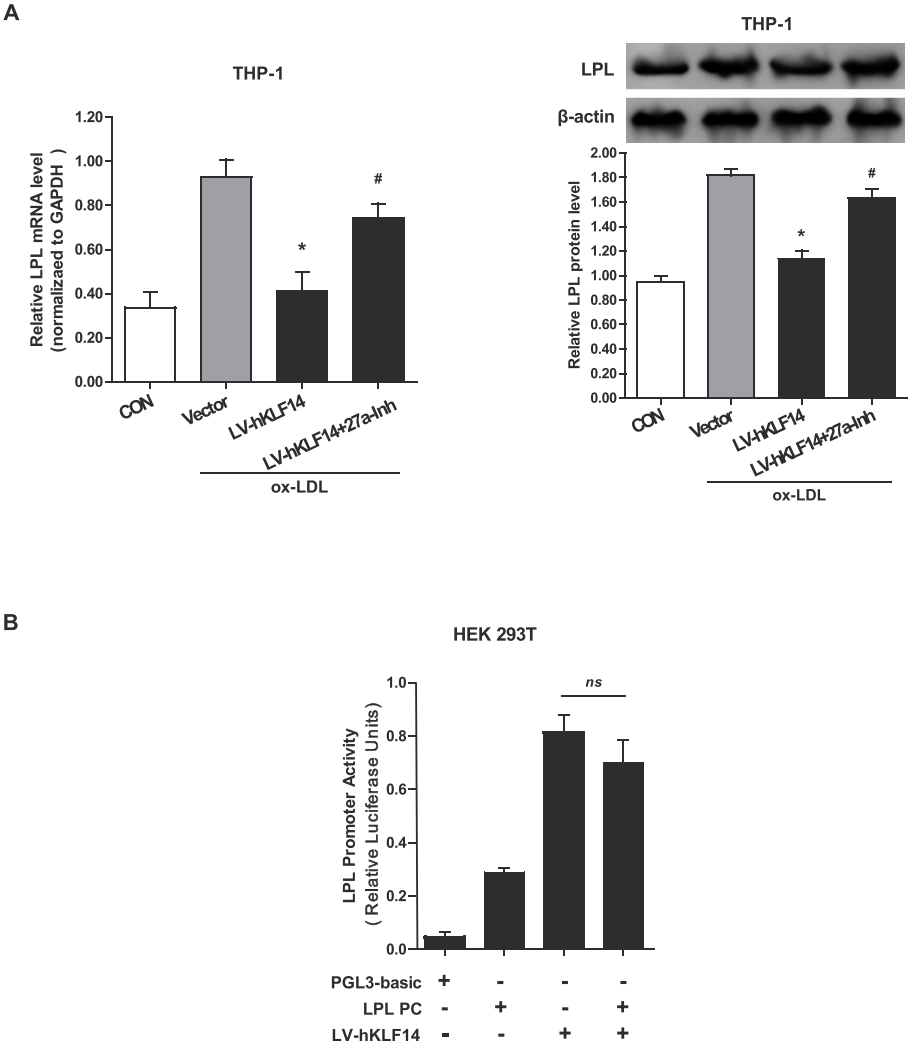


Fig. 2. LPL is involved in the effects of KLF14 on the secretion of proinflammatory cytokines and lipid accumulation in THP-1 macrophages.
(A) Overexpression of *KLF14* in ox-LDL-treated THP-1 macrophages through infection with LV-hKLF14 downregulates the mRNA expression of *LPL*. $^*p < 0.05$ *vs* vector group, $^{\#}p < 0.05$ *vs* LV-hKLF14 group. (B) Luciferase reporter gene assay demonstrated that KLF14 does not directly enhance *LPL* promoter activity. (C) LPL mediates the effects of KLF14 on the secretion of proinflammatory cytokines, such as IL-1β, IL-6, MCP-1 and TNF-α. $^*p < 0.05$, $^{**}p < 0.01$. (D) Oil red O assay demonstrated that LPL mediates KLF14-induced decrease of lipid accumulation in THP-1 macrophages. All data are expressed as mean ± S.D. from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

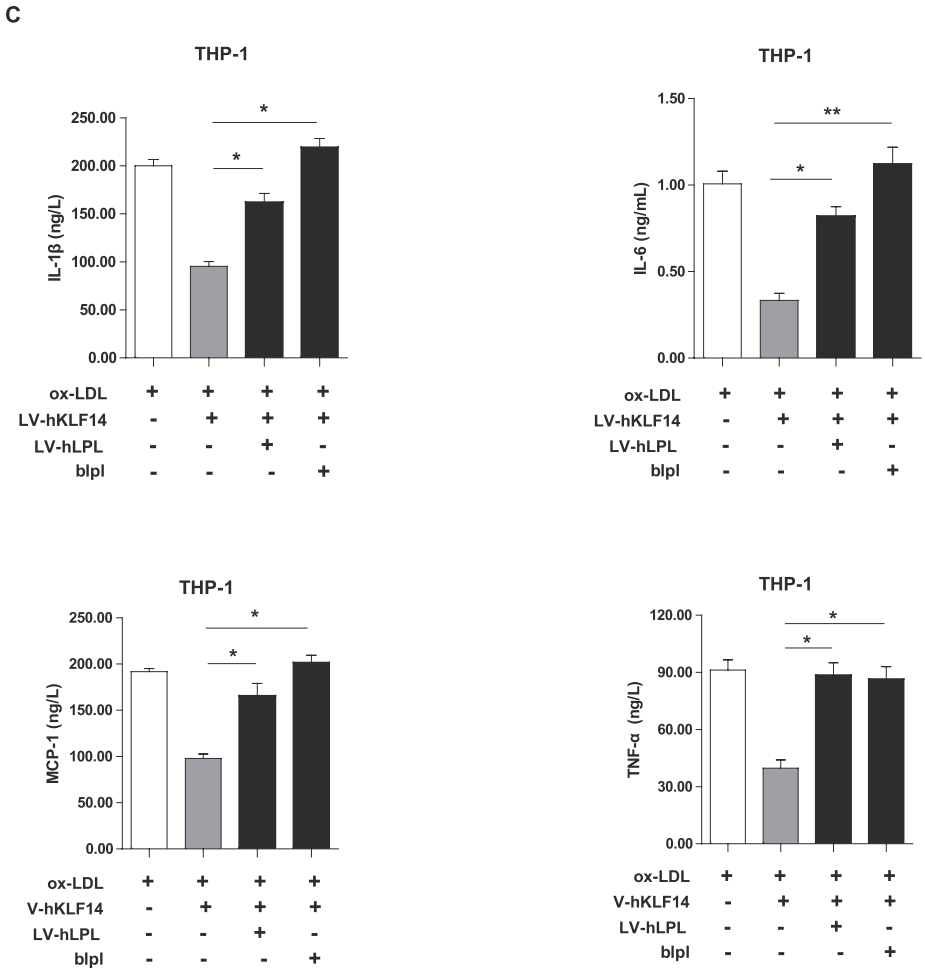


Fig. 2. (*continued*)

The animals were then euthanized for the collection of blood and tissues. Peritoneal macrophages were harvested 3 days after thioglycolate (2ml/mouse, Sigma, U.S.A.) intraperitoneal injection. The red blood cells and other contaminating cells were removed by red blood cell lysis buffer and MACS microbeads coating CD14 antibody. All procedures were performed in accordance with the Institutional Animal Ethics Committee and the University of South China Animal Care Guidelines for the Use of Experimental Animals.

2.12. Assessment of aortic atherosclerotic lesions

Mice were sacrificed by exsanguination under anesthesia. The heart and aorta were incubated with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde. Tissues were embedded, serially sectioned (6 μm) throughout the three aortic valves, and then stained with Oil Red O, hematoxylin-eosin (HE) stain and Masson stain. Images were captured and lesion areas were quantified with Image Pro Plus software.

2.13. Statistical analysis

All data were presented as means ± SD, and evaluated using one-way ANOVA and Student's t-test. $p < 0.05$ is considered statistically significant. All statistical analyses were performed with SPSS 13.0 software.

3. Results

3.1. KLF14 expression is downregulated in atherosclerotic lesions of aorta and ox-LDL-treated macrophages

Previous studies revealed a beneficial effect of hepatic klf14 on atherosclerosis through its enhancement of reverse cholesterol transport (RCT) capacity mediated by serum apoA-I and HDL [5]. To investigate the specific relationship between KLF14 and atherosclerosis, we first examined the expression of KLF14 in human atherosclerotic plaque lesions (APL) and in normal vascular tissue (NVT). The results showed that KLF14 mRNA and protein expression was significantly decreased by approximately 74.8% and 57.0%, respectively, in the atherosclerotic lesion compared with the normal tissue of human aorta (Supplementary Fig. 1A). We next detected the mRNA and protein expression of klf14 in C57BL/6 mice fed a regular chow or Western type diet (WD). Tissue homogenate of isolated aorta of mice fed WD displayed a noticeable down-regulation of klf14 expression (Supplementary Fig. 1B). We then assessed the expression of klf14 in the aorta of the *apoE*$^{-/-}$ mice fed high-fat diet (HFD) for 8 weeks, a well-established and accepted model for the study of dyslipidemia and atherosclerosis. Consistently, we found that klf14 mRNA and protein expression levels were decreased by 59.9% and 48.9%, respectively (Supplementary Fig. 1C). These results suggest that low expression of klf14 was present in atherosclerotic mice.

To further examine the expression of KLF14 *in vitro*, THP-1-derived macrophages were incubated with 50 μg/ml ox-LDL for different time

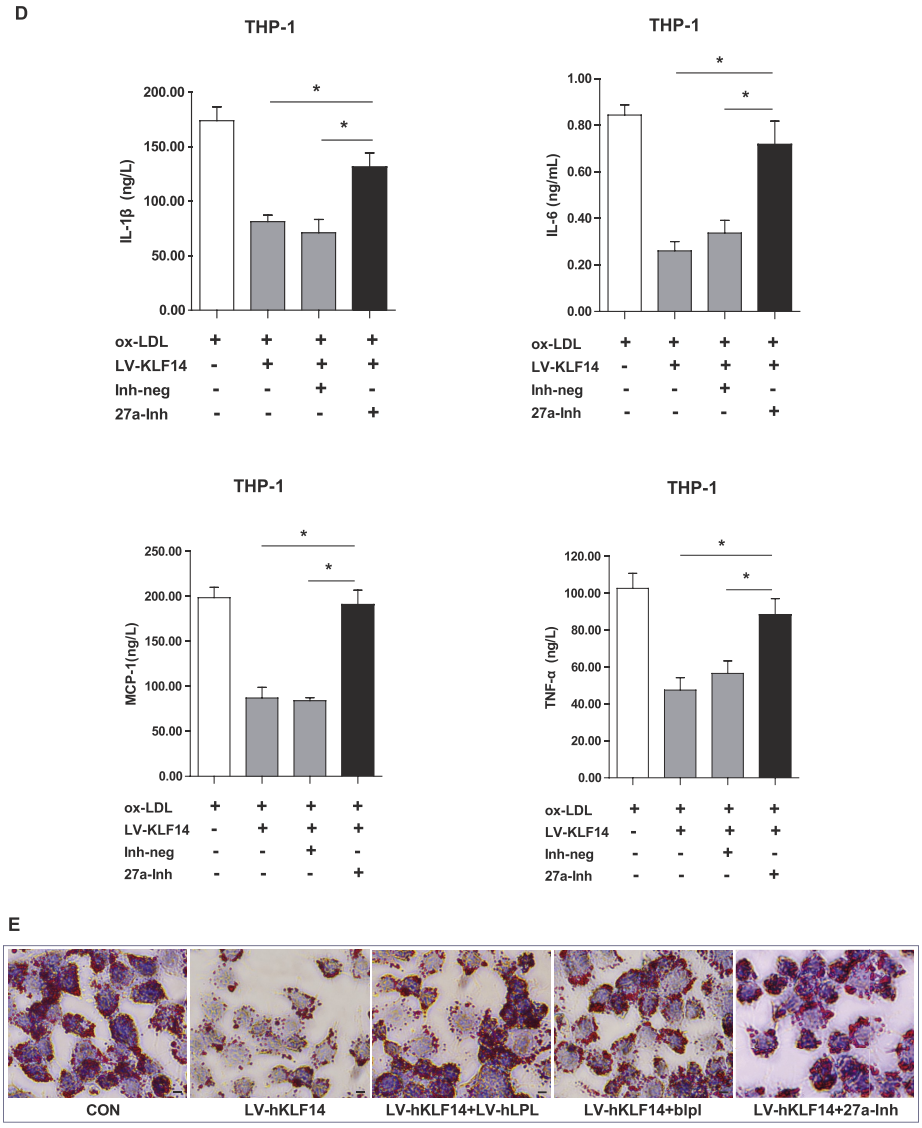


Fig. 2. (*continued*)

points (0, 12, 24, 48, 72 h). As shown in Supplementary Fig. 1D, KLF14 expression levels were markedly down-regulated in response to the incubation with ox-LDL for 48 h. Furthermore, similar results were obtained from peritoneal macrophages harvested from *apoE*$^{-/-}$ mice in response to the incubation with ox-LDL *in vitro* (Supplementary Fig. 1E). These findings suggest that KLF14 may be involved in the regulation of macrophage functions that are closely associated with the development of AS.

3.2. *KLF14 inhibits secretion of proinflammatory cytokines and lipid accumulation in macrophages through binding to the promoter of miR-27a*

Hepatic klf14 plays a critical role in coronary heart disease (CAD) [5]. Endothelial klf14, as a novel beneficial regulator of FGF/SK1 lipid signaling, regulates lipid metabolism [30]. However, whether these effects of KLF14 are mediated by its regulatory roles in macrophage functions that are known to have a key role in the development of atherosclerosis is unclear [3,4]. To examine whether KLF14 affected the secretion of proinflammatory cytokines and lipid accumulation, we infected ox-LDL-treated THP-1-derived macrophages with a recombinant lentiviral vector encoding human *KLF14* (LV-hKLF14) to overexpress KLF14 (Fig. 1A). LV-hKLF14 efficiently decreased the levels of proinflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) (Fig. 1B). LV-hKLF14 also significantly reduced the cellular levels of free cholesterol (FC), cholesterol ester (CE), and total cholesterol (TC) when compared with the control group (Supplementary Table 1).

The effects of KLF14 on the secretion of proinflammatory cytokines and lipid accumulation were consistent with what we observed in our previous study of miR-27a/b [28] and that miR-27a/b expression was down-regulated in response to ox-LDL treatment (Fig. 1C). Moreover, the promoter region of miR-27a/b contains binding sites of KLF14 predicted by bioinformatic sequence tools (JASPAR). Thus, it is possible that KLF14 regulates the expression of miR-27a/b. Indeed, we found that LV-hKLF14 dramatically up-regulated the expression of endogenous precursor (pre-miR-27a) and mature form (mat-miR-27a) of miR-27a without any detectable effect on the expression of miR-27b (Fig. 1D), whereas knockdown of *KLF14* by its siRNA efficiently repressed the expression of miR-27a (Fig. 1E and F). Furthermore, actinomycin D, a transcriptional inhibitor, blocked KLF14-induced up-regulation of pre-miR-27a in THP-1-derived macrophages (Fig. 1G), suggesting that KLF14 regulates the expression of miR-27a at the transcriptional level. To explore the potential underlying mechanisms,

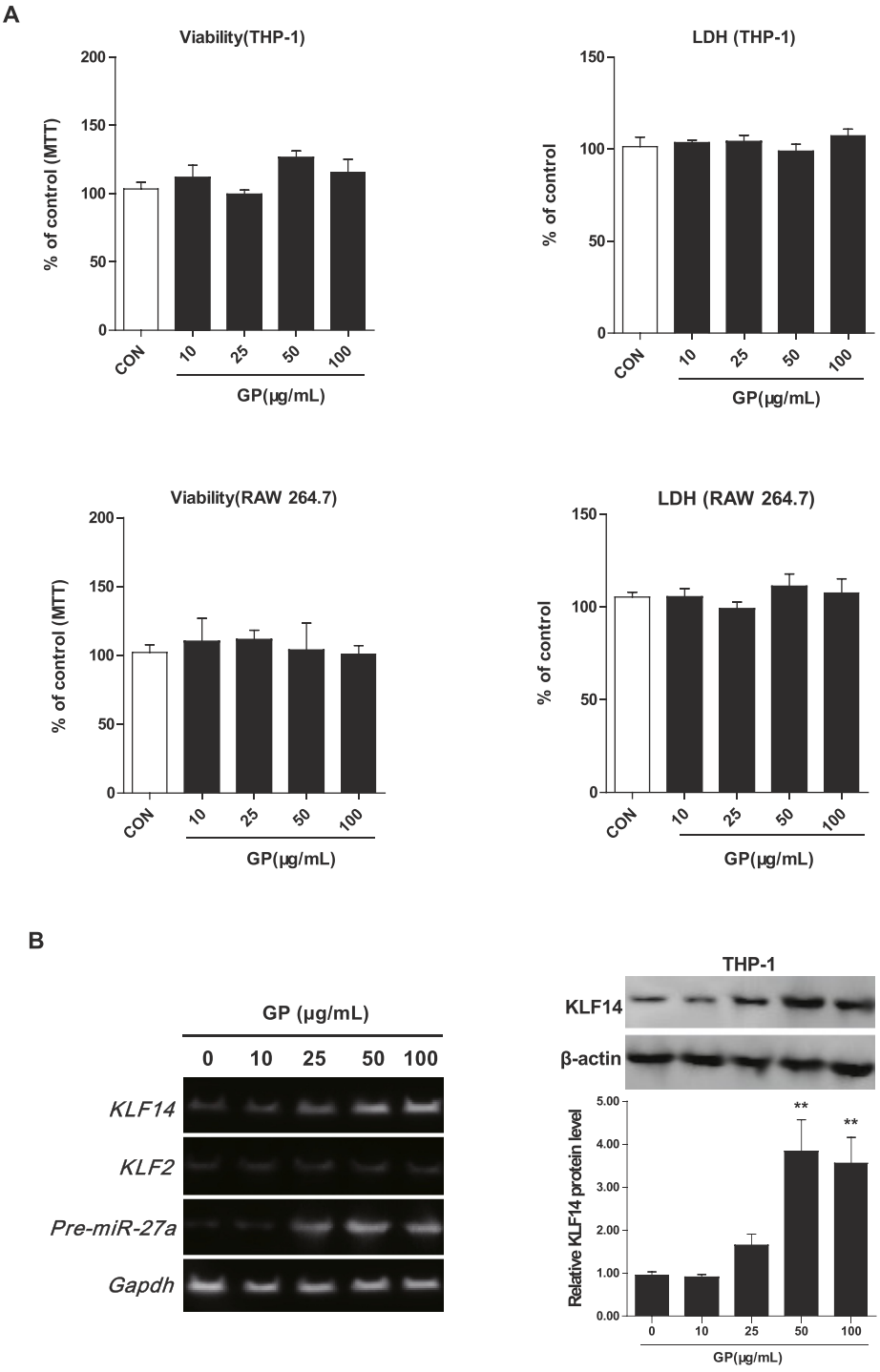


Fig. 3. Gypenosides (GP), as an activator of KLF14, depending on the KLF14/miR-27a/LPL pathway decreases lipid accumulation and proinflammatory cytokine secretion in THP-1 macrophages treated with ox-LDL.

(A) Treatment of THP-1 and RAW 264.7 macrophages with GP for 24 h did not affect cell viability and LDH release from macrophages via 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) analyses. (B and C) THP-1 macrophages treated with ox-LDL were incubated with different GP concentrations (0, 10, 25, 50, 100 μg/ml) for 24 h, followed by detection of the effects of GP on the expression of KLF14, pre-miR-27a and miR-27a by qRT-PCR and Western blot analyses. Administration of GP significantly up-regulated their expression in macrophage. $^{**}p < 0.01$ *vs* 0 h group, $^{*}p < 0.05$ *vs* CON group, $^{\#\#}p < 0.01$ *vs* ox-LDL group. (D) THP-1 macrophages transfected with siRNA to silence *KLF14* expression weakened the decrease of pre-miR-27a and miR-27a production induced by GP treatment. $^{*}p < 0.05$ *vs* siRNA-neg group. (E) ChIP-PCR and ChIP-q-PCR assays validated the occupancy of KLF14 and pol II at miR-27a promoter binding site in GP-treated THP-1 macrophages. $^{*}p < 0.05$ *vs* 0 h group. (F) Ox-LDL-treated THP-1 macrophages incubated with different concentrations of GP (0, 10, 25, 50, 100 μg/mL) or for time periods (0、6、12、24、48 h) were assessed for GP effects on LPL expression by Western blot and qRT-PCR analyses. Administration of GP down-regulated the expression of LPL in both concentration- and time-dependent manners. $^{*}p < 0.05$ *vs* 0 group. (G) Silencing *KLF14* expression (siKLF14) or inhibiting miR-27a (27a-Inh) reversed the trend of decreased LPL expression by GP. $^{*}p < 0.05$. (H) Silencing *KLF14* expression (siKLF14) significantly reversed the decrease trend of the effects of GP on proinflammatory cytokines (IL-1β, IL-6, MCP-1 and TNF-α) from ox-LDL-treated THP-1 macrophages in the presence of GP. $^{*}p < 0.05$ *vs* GP group. (I) Low expression of miR-27a resulted by inhibition (27a-Inh) or over-expression of *LPL* (LV-hLPL) or incubation with bovine lpl (blpl) demonstrated that miR-27a and LPL mediate the inhibitory effect of GP on the production of proinflammatory cytokines in THP-1 macrophages. $^{*}p < 0.05$ *vs* GP group, $^{**}p < 0.01$ *vs* GP group. (J) Oil red O staining assay demonstrated that GP-induced decrease of lipid accumulation was dependent on the KLF14/miR-27a/LPL pathway. All data are expressed as mean ± S.D. from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

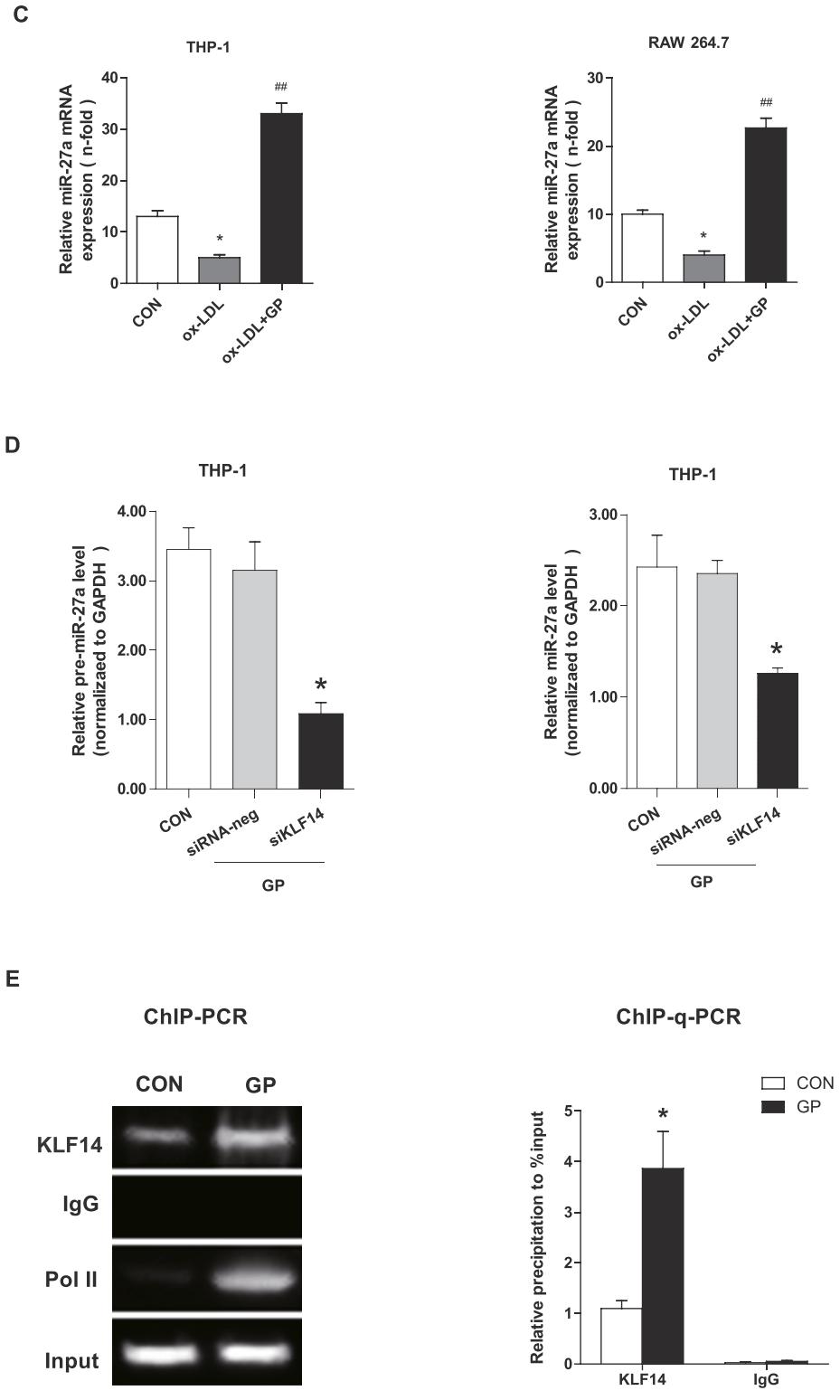


Fig. 3. (*continued*)

we examined whether KLF14, as a transcription factor, directly regulated miR-27a expression through binding to its promoter. We searched the binding potential of KLF14 to miR-27a and found two putative KLF14 bindings sites in human miR-27a promoter including CATGGT GAAAC and TGATCCGCCCACCT ocated at nt −1435∼-1422 and −1521∼-1508 (Fig. 1H). Interestingly, a direct target binding region of KLF14 that only harbors the distant nt −1521∼-1508 site (site 2) without nt −1435∼-1422 site (site 1) of miR-27a promoter was identified by ChIP assay (Fig. 1I). Similar results were confirmed by a luciferase reporter gene assay (Fig. 1J), indicating that miR-27a occupancy site 2 is critical for the transcriptional repression by KLF14. Given the similar DNA-binding preferences of KLF family members [31], we also examined whether other KLF transcription factors could regulate miR-27a expression. The results showed that KLF14, but not

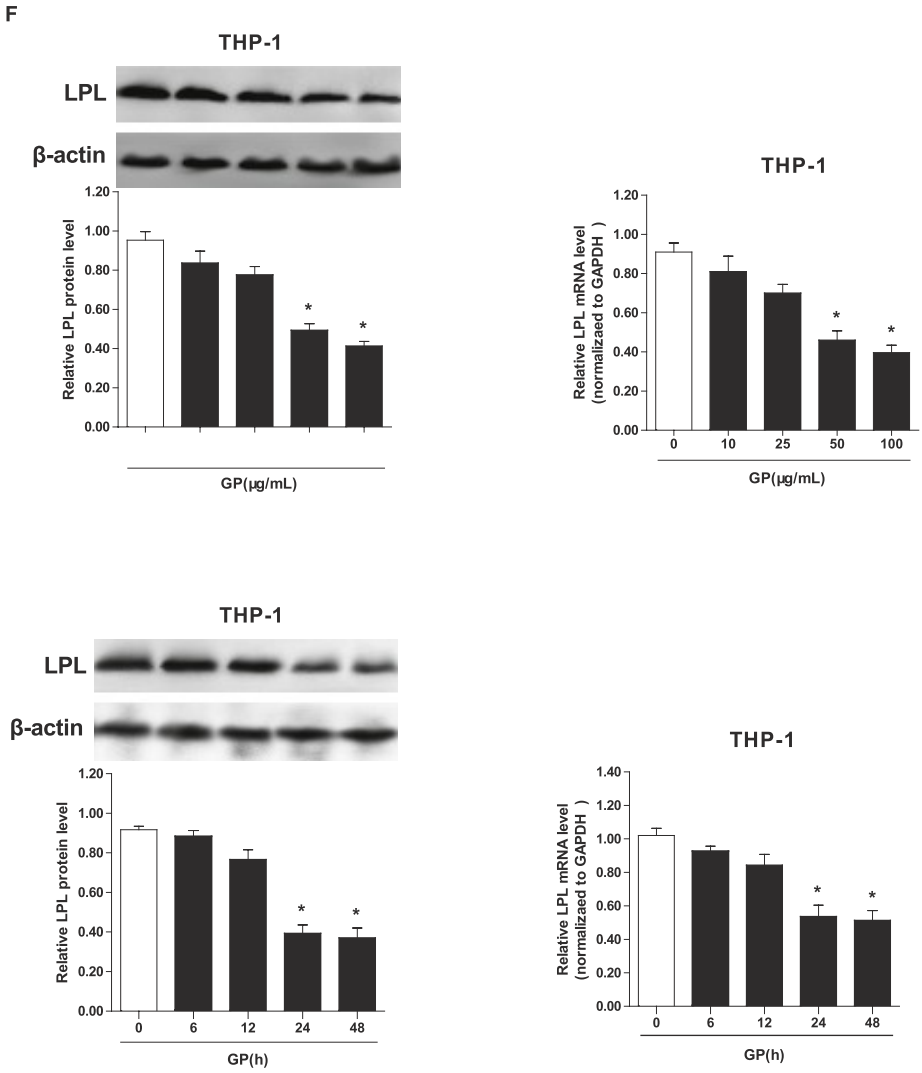


Fig. 3. (*continued*)

KLF2, KLF4, or KLF11 up-regulated miR-27a promoter activity in THP-1 macrophages (Fig. 1K), indicating the specificity of KLF14.

3.3. miR-27a-mediated LPL downregulation participates in the inhibitory effect of KLF14 on the secretion of proinflammatory cytokines and lipid accumulation in macrophages

We have previously demonstrated that downregulation of LPL, a miR-27 targeting gene, attenuates the secretion of proinflammatory cytokines and lipid accumulation in THP-1 macrophages [28]. Therefore, we explored whether LPL participated in the specific effects of KLF14 on macrophages and found that KLF14 overexpression markedly decreased LPL expression. This effect was independent of direct regulation of its promoter activity, whereas miR-27a inhibitor reversed the effect of KLF14 on LPL expression (Fig. 2A–B), suggesting that miR-27a mediates KLF14's action on LPL expression in macrophages.

To clarify the roles of LPL in KLF14 effects on proinflammatory cytokine secretion and lipid accumulation, the cells were treated with bovine lpl (blpl) or lentiviral vector encoding human LPL (LV-hLPL). ELISA assay showed that overexpression of LV-hLPL (Supplementary Fig. 2) and incubation with blpl dramatically blocked the down-regulation of KLF14 on proinflammatory cytokine secretion including IL-1β, IL-6, MCP-1 and TNF-α (Fig. 2C). In addition, LV-hLPL and blpl significantly enhanced the intracellular lipid accumulation (Fig. 2E), increased content of TC, FC and CE, and weakened the effects of KLF14 on lipid profiles (Supplementary Table 1). More interestingly, miR-27a inhibitor reversed the effects of the KLF14 overexpression on inflammation and lipid accumulation (Fig. 2D and E). These findings suggest that LPL plays an essential role in the regulatory effects of KLF14/miR-27a on inflammatory response and lipid metabolism.

3.4. Gypenosides acts as an activator for induction of KLF14 expression

Our results suggest that KLF14 serves as a novel modulator of miR-27a/LPL pathway, contributing to the lipid homeostasis and inflammation. Thus, we sought a strategy to activate endogenous KLF14 expression. It has been reported that gypenosides (GP) derived from a Chinese medicine Gynostemma Pentaphylla have exhibited cardiovascular protective effects [32,33]. We treated cells with 50 μg/ml GP for 24 h that exhibited a low cytotoxicity (Fig. 3A) and found that KLF14 expression was significantly increased in the GP-treated group cells incubated with oxLDL (Fig. 3B). Consistent with KLF14, GP also significantly increased the levels of pre-miR-27a and miR-27a as shown in Fig. 3B and C. To explore whether KLF14 played a role in the effects of

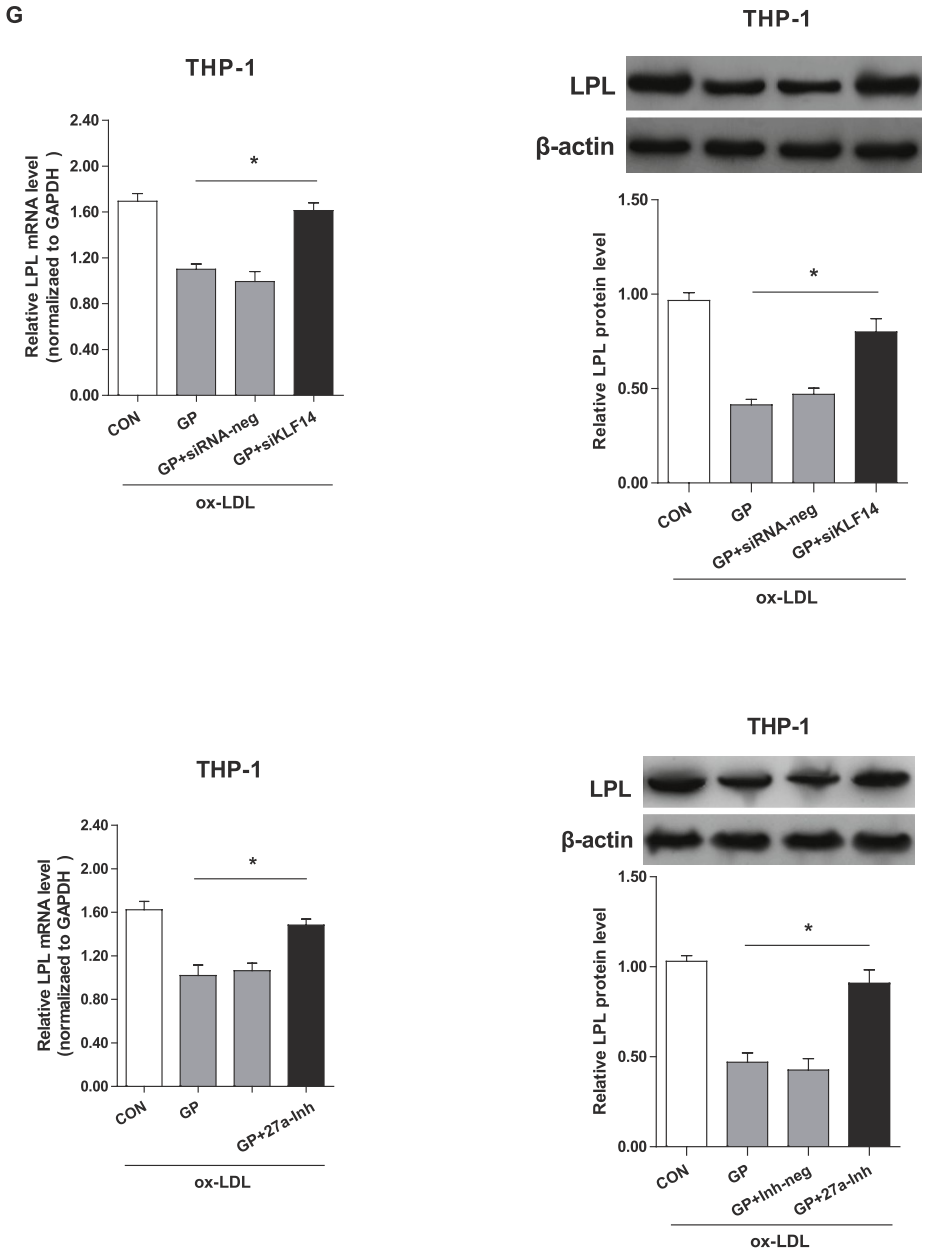


Fig. 3. (*continued*)

GP on the expression of miR-27, we knocked down *KLF14* expression via its specific siRNA. As expected, the silence of *KLF14* reduced GP-induced up-regulation of pre-miR-27a and mat-miR-27a production (Fig. 3D). A ChIP assay revealed that GP promoted binding of KLF14 to miR-27a promoter (Fig. 3E). These results suggest that GP promotes KLF14 expression, which then binds to the miR-27a promoter and consequently increases the level of miR-27a.

We also observed that GP obviously down-regulated LPL expression in both concentration- and time-dependent manners in ox-LDL-treated THP-1 macrophages (Fig. 3F). To confirm the contribution of KLF14/miR-27a to the effect of GP on LPL, the cells were treated with *KLF14* siRNA (siKLF14) or miR-27a inhibitor. Results showed that GP decreased LPL mRNA and protein expression, which was blocked by *KLF14* silence or miR-27a inhibitor in THP-1 macrophages treated with ox-LDL (Fig. 3G). Taking together, our results indicate that GP down-regulates LPL by the mechanisms of at least in part the activation of KLF14/miR-27a.

Next, we confirmed whether pharmacological effects of GP depended on the KLF14/miR-27a/LPL pathway in ox-LDL-treated THP-1 macrophages. GP noticeably reduced the levels of proinflammatory cytokines including IL-1β, IL-6, MCP-1 and TNF-α, which were inhibited by knockdown of KLF14 expression via siRNA (Fig. 3H). Similar results were observed when the cells were treated with miR-27a inhibitor or LPL overexpression (Fig. 3I). Moreover, GP reduced the formation of lipid droplets and the cellular levels of TC, FC, and CE, whereas knockdown of KLF14, miR-27a inhibitor, or LPL treatment significantly increased lipid accumulation in the presence of GP (Fig. 3J and Supplementary Table 2). These results further validate the key role

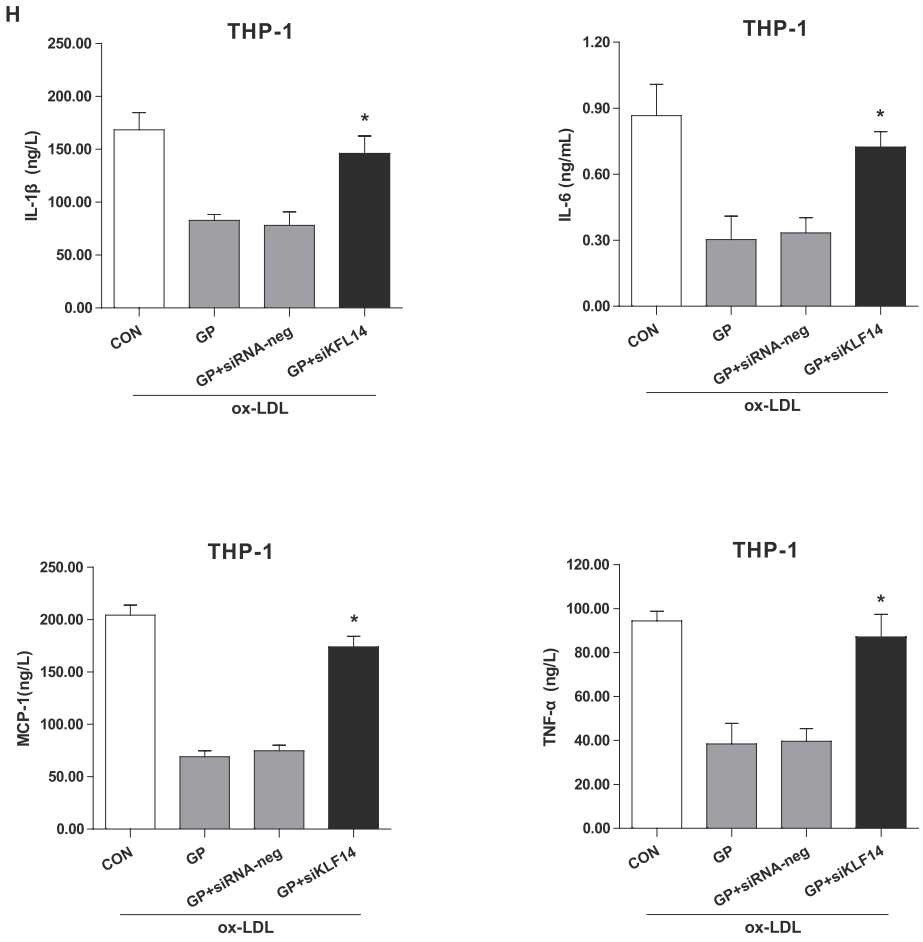


Fig. 3. (*continued*)

of the KLF14-dependent miR-27a/LPL signal pathway in the inhibitory effects of GP on proinflammatory cytokine secretion and lipid accumulation in macrophages.

3.5. Gypenosides delays the development of AS in $apoE^{-/-}$ mice

We further asked whether KLF14-dependent miR-27a/LPL pathway contributes to the effects of GP on AS *in vivo*. GP reduced the number and size of lesions in the aorta region (Fig. 4A and B), the plaque lesion areas (Figure 6C), and fiber content in the aortic sinus collagen (Fig. 4D) in $apoE^{-/-}$ mice, but these effects were blocked by ANa, siKLF14 or LV-hLPL that down- or up-regulate the expression of KLF14 or LPL, respectively (Supplementary Fig. 3), in both mouse peritoneal macrophage (MPM) and aorta of $apoE^{-/-}$ mice (Fig. 4A–D). Furthermore, less lipid deposition was observed in the aortic root when the mice were treated with GP, but siKLF14, ANa and LV-hLPL increased the lipid deposition (Fig. 4E). Together, these results demonstrate that GP inhibits the development of atherosclerotic lesions at least partially through the KLF14-dependent miR-27a/LPL pathway in $apoE^{-/-}$ mice.

GP also significantly decreased plasma levels of inflammatory factors including IL-1β, IL-6, MCP-1, and TNF-α (Fig. 4F), as well as plasma TC and LDL-C levels (Supplementary Table 3), but increased plasma HDL-C levels (Supplementary Table 3). On the other hand, inhibition of miR-27a by ANa or LV-hLPL treatment reduced HDL-C levels but increased TC and LDL-C levels (Supplementary Table 3). Thus, GP down-regulates plasma levels of inflammatory factors and cholesterol in $apoE^{-/-}$ mice.

4. Discussion

Krüppel-like factor (KLF) family, an important and evolutionarily conserved transcription factor, is composed of 17 members characterized by the presence of conserved DNA-binding domain, Cys_2/His_2 zinc fingers. The KLFs regulate the expression of target genes encoding proteins required for the important biological processes including proliferation, apoptosis, differentiation, and development [31]. Recently, GWAS shows that some SNPs near or in the *KLF14* gene, a member of KLFs, influence the risk of cardiovascular disease (CVD) [16]. KLF14 has also been reported to affect obesity and type 2 diabetes (T2D), which are closely associated with the onset and progress of atherosclerosis [9,14,34,35]. Variants *on KLF14* gene are closely associated metabolic syndrome as a result of type 2 diabetes [36–39]. Anunciado-Koza's study revealed that the adipose mesoderm-specific transcript (Mest) mRNA expression that is susceptive to the development of obesity has a very high correlation with *klf14* expression in both subcutaneous and visceral white adipose tissues (WAT) of C57BL/6J mice fed HFD [34]. Overexpression of klf14 in Hepa1-6 cells reversed the T2D-induced inhibition of glucose uptake and insulin sensitivity through the activation of the PI3K/Akt signaling pathway [40]. The increased age-related DNA methylation of *KLF14*, a blood-based

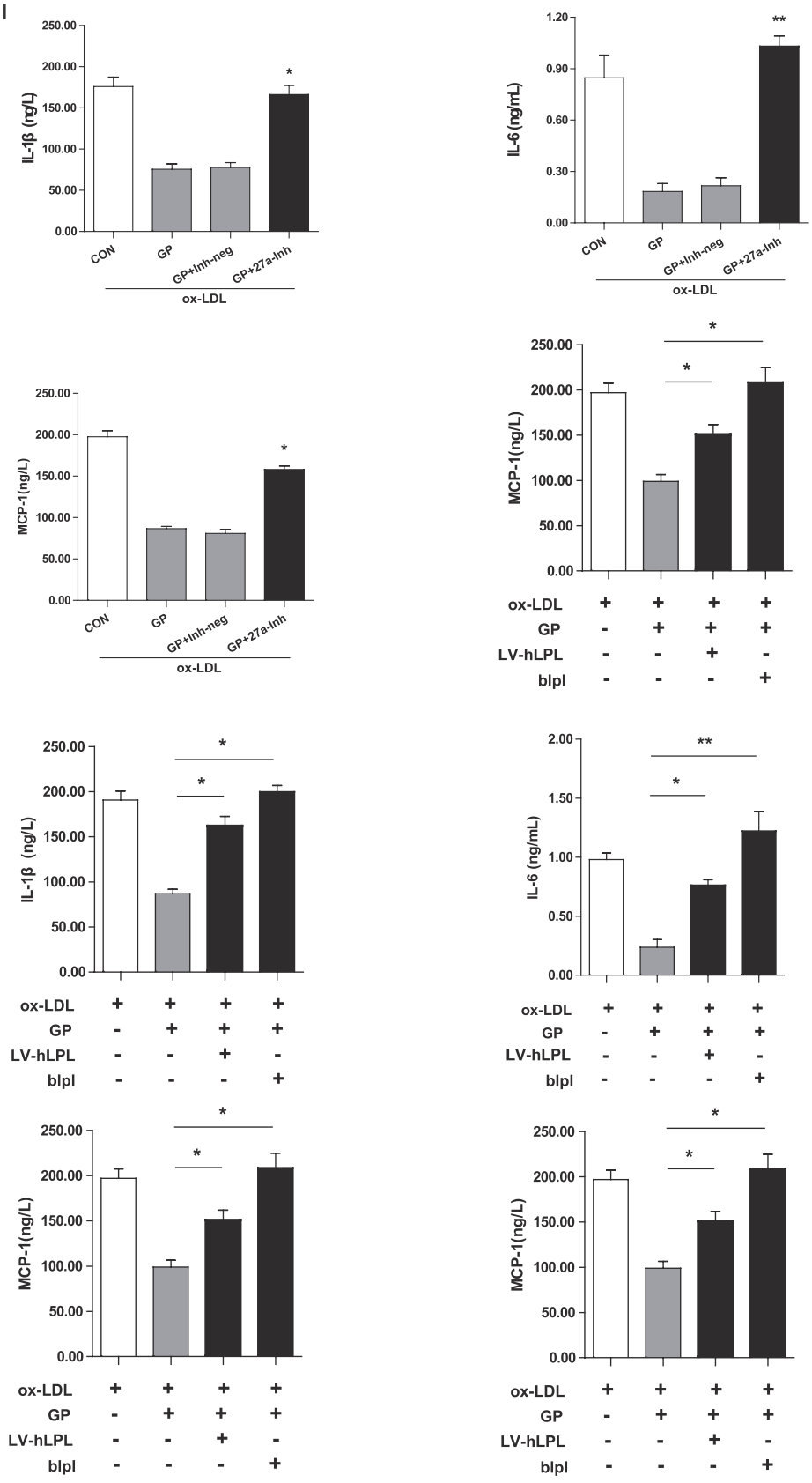


Fig. 3. (*continued*)

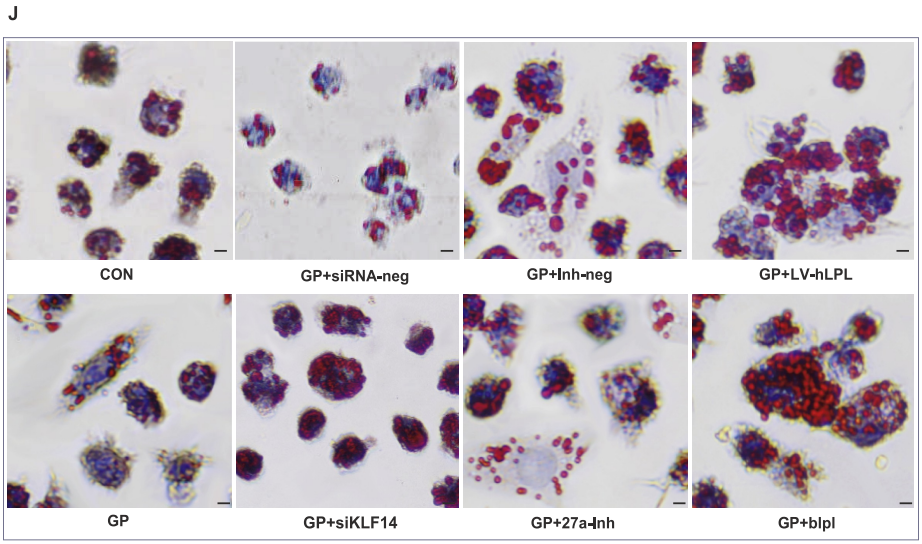


Fig. 3. (*continued*)

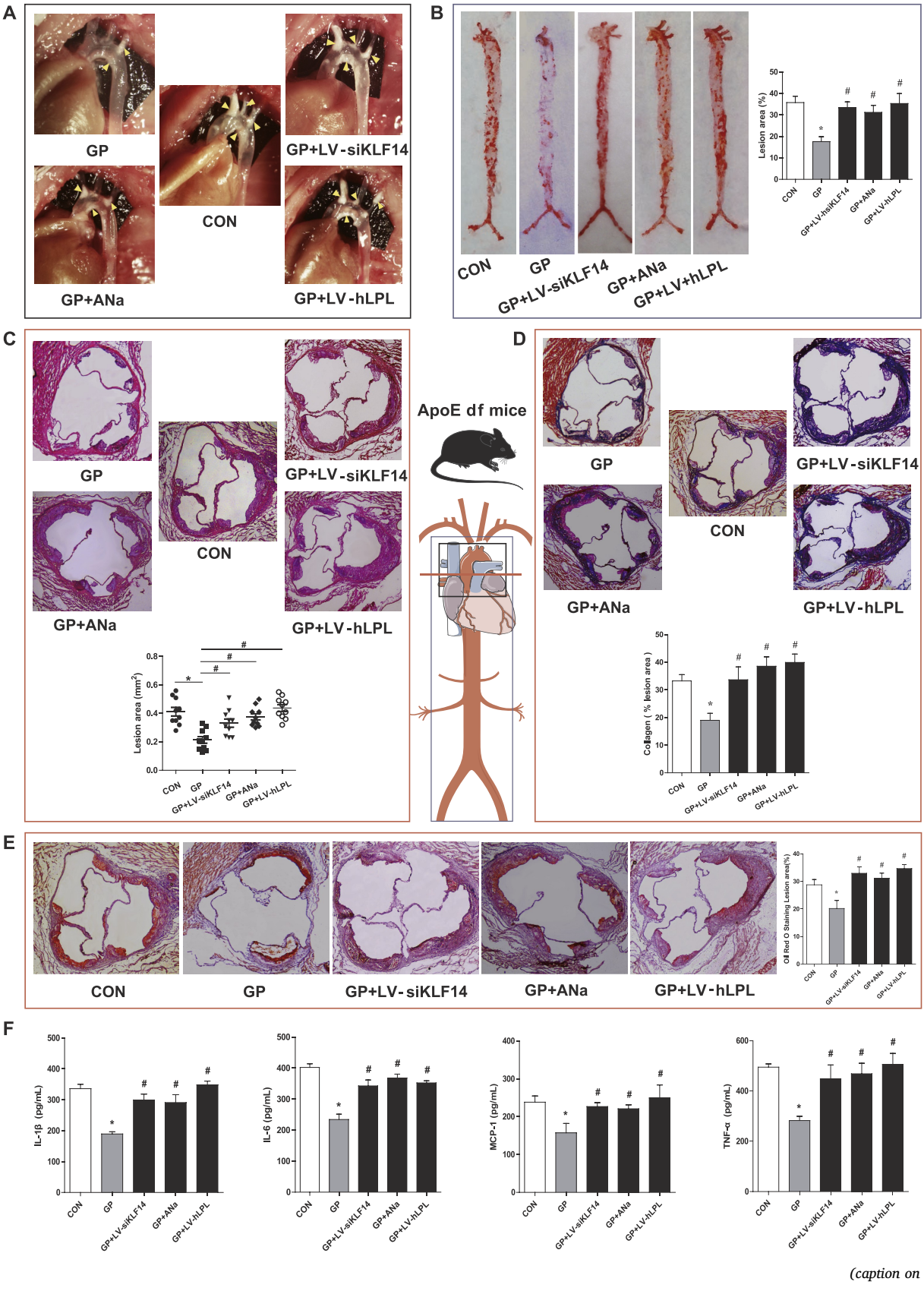
epigenetic biomarker, has been identified to associate with the severity of T2D [35]. Those findings suggest that KLF14 plays an essential role in the progress of glycol-metabolism homeostasis [9]. KLF14 is also a critical regulator of lipid metabolism. Liver-specific *klf14* expression markedly reduces atherosclerotic lesions through binding to *apoA-I* promoter sites and subsequent increasing apoA-I expression and reverse cholesterol transport (RCT) [5]. However, it still remains poorly understood how KLF14 expression is regulated in atherosclerotic plaque lesions of vertebrates. Our current study has revealed a reduction in KLF14 expression in the atherosclerotic plaques area when compared with that in the normal aorta tissue of human. A negative correlation between klf14 and the development of atherosclerosis is also observed in mice.

Although there has been a large amount of evidence on KLF14 as a potential regulator of AS [41], if and how KLF14 regulates lipid metabolism and inflammation have not been well defined. Here, we reported that KLF14 expression levels were obviously decreased in THP-1-derived macrophages and peritoneal macrophages in response to the incubation with ox-LDL. Furthermore, in *apoE*$^{-/-}$ mice, klf14 expression was significantly down-regulated in aortic sinus plaque area. These results suggest that macrophages in atherosclerotic plaques may low expression of KLF14. It has been reported that variants in *KLF14* influence atherosclerosis-related lipid profiles [15,42,43], which may be closely related with the expression of KLF14 target genes related lipid metabolism, such as *ARSD, TPMT, SLC7A10, APH1B, NINJ2, KLF13, GNB1, and MYL5* [10]. To explore the effects of KLF14 on cholesterol metabolism, intracellular levels of TC, FC, and CE were examined. Results obtained from HPLC showed that overexpression of KLF14 significantly reduce their levels. KLF14 also significantly down-regulated the levels of IL-1β, IL-6, MCP-1, and TNF-α. Thus, KLF14 plays an antiatherogenic role, promoting RCT to accelerate excessive cholesterol transportation from macrophages to the liver and decreasing secretion of inflammatory cytokines from macrophages [5]. However, the exact molecular mechanisms for KLF14's action on macrophages remain poorly understood and need to be further investigated.

Our previous studies have shown that miR-27a/b represses cholesterol accumulation and inflammatory cytokine secretion in the macrophages through inhibiting LPL expression via directly targeting its 3′UTR [28]. Given that miR-27a/b acts as an important modulator to the development of atherosclerosis, it posttranscriptionally represses the expression of target genes and is regulated by a variety of factors as well, such as ELAVL1, hepatitis C virus (HCV), and Pitx2c [44–46]. KLF14, a member of the SP/XKLF transcription family factors, transcriptionally regulates the expression of genes required for important biological and pathological processes. Our current study revealed that miR-27a/b expression is down-regulated by KLF14 in macrophages at the transcriptional level. One of the two putative KLF14 binding sites in the promoter region of miR-27a is a direct target of KLF14 but not other KLF members tested, suggesting that KLF14 regulates miR-27a expression through binding its promoter site.

In the current study, we confirmed that miR-27a mediates the negative effect of KLF14 on the expression of LPL without direct influence on its promoter activity. Klf14 can significantly ameliorate macrophage infiltration and reduce lpl expression in the aortic lesion regions of *apoE*$^{-/-}$ mice. KLF14 also inhibits LPL-mediated inflammatory cytokine secretion and lipid accumulation via miR-27a in the macrophages. However, it is unknown how KLF14 specifically regulates gene expression in an atherosclerosis-related manner and if KLF14 transcriptionally regulates other genes related to lipid metabolism, such as lecithin cholesterol amyl transferase (LCAT), acyl coenzyme a-cholesterol acyltransferase 1 (ACAT1), CD36, ATP-binding cassette transporter (ABCA1 and ABCG1) and sterol regulatory element binding protein-1 (SREBP1). Considering the potential antiatherogenic capability of KLF14, it will be important to address these issues.

Gypenosides (GP) has been widely used for its beneficial effects on immunomodulation, lipid metabolism, and the maintenance of cardiovascular health [47–51]. GP was found to inhibit high-fat diet-induced AS in rats [52]. It was previously reported that those effects might be associated with its specific anti-oxidation [33,53,54]. However, it remains to be elucidated whether GP affects inflammation and lipid metabolism in macrophages as one of the underlying mechanisms for its therapeutic effects. Here, we reported that GP increases the expression of miR-27a through klf14. Therefore, miR-27a attenuates lipid accumulation and the pro-inflammatory cytokine secretion and consequently leads to a reduction in atherosclerotic lesions in *apoE*$^{-/-}$ mice, which is at least partially contributed by repressing the expression of lpl as described in our previous study [28]. As expected, we confirmed the contribution of KLF14-dependent miR-27a/LPL pathway to the inhibitory effects of GP on proinflammatory cytokine secretion and lipid accumulation in macrophages. We also found that GP significantly



(caption on next page)

Fig. 4. Administration of gypenosides delays the development of aortic AS in *apoE*$^{-/-}$ mice.
Eight-week-old male *apoE*$^{-/-}$ mice (n = 10 mice per group), fed a high-fat diet (HFD) containing 200 mg/kg/d GP, were given saline or siKLF14 or miR-27a antagomir (ANa) or human LPL lentiviral vector (LV-hLPL), respectively, via tail vein injection every 2 days for 8 weeks. (A) Gypenosides alleviated atherosclerotic plaque in *apoE*$^{-/-}$ mice. Plaques (arrows) in aortic arches and thoracic aortas of representative *apoE*$^{-/-}$ mice are shown. (B) Gypenosides decreased atherosclerotic lesion areas in *apoE*$^{-/-}$ mice. Representative images and quantification of atherosclerotic lesion areas in *en face* analysis of the aorta with Oil red O staining. (C) Effects of gypenosides on aortic atherosclerotic lesions in aortic root in *apoE*$^{-/-}$ mice. Representative micrographs were obtained from hematoxylin-eosin (HE) staining of cross-sections of aortic sinus in *apoE*$^{-/-}$ mice. (D) Gypenosides decreased the aortic sinus lesion areas in *apoE*$^{-/-}$ mice. Representative micrographs were obtained from Masson staining of cross-sections of aortic sinus in *apoE*$^{-/-}$ mice. (E) Gypenosides reduced lipid accumulation in the aortic sinus of *apoE*$^{-/-}$ mice. Characterization of aortic sinus atherosclerotic lesion areas was performed by Oil red O staining. Total Oil red O staining positive area was determined using IMAGEPRO PLUS Software. Images of representative sections from each group are accompanied by bar charts. (F) Gypenosides reduced the plasma levels of inflammatory factors (IL-1β, IL-6, MCP-1 and TNF-α). Each data point represents one individual animal. The horizontal lines denote the mean of each group. All results are expressed as mean ± S.D. $^{*}p < 0.05$ *vs* CON group, $^{\#}p < 0.05$ *vs* GP group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

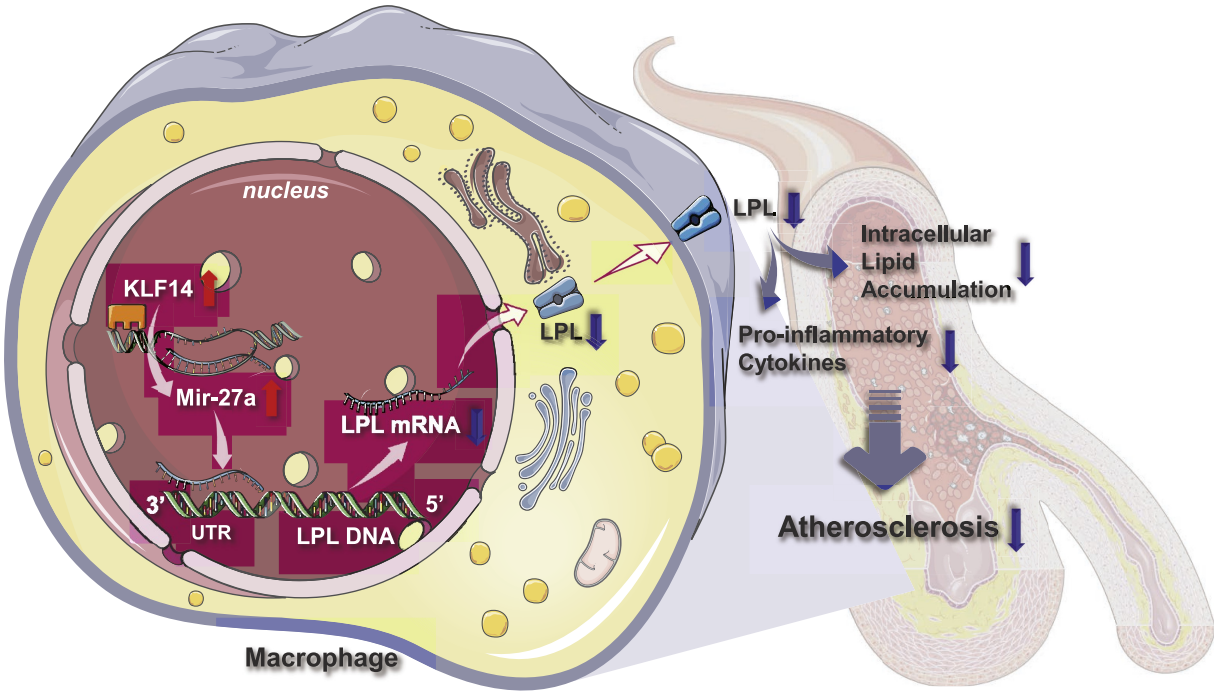


Fig. 5. Proposed model for the mechanism by which gypenosides (GP) induces KLF14 overexpression and delays atherosclerosis.
Administration of GP induces upregulation of KLF14 expression. KLF14, as a transcription factor, binds to mir-27a promoter to accelerate transcriptional level and expression of mir-27a, which can specifically inhibit the expression of LPL via its RNA 3′UTR, and then attenuate lipid accumulation and secretion of proinflammatory cytokines *in vivo*, a key link in the progression of atherogenesis. This mechanism may also account for further atherosclerosis.

delayed the development of atherosclerotic lesions in *apoE*$^{-/-}$ mice fed HFD, at least partially as a result of klf14-induced inhibition of lpl by miR-27a (Fig. 5). We are currently investigating the molecular mechanism by which GP influences the expression of KLF14 since further understanding of the underlying mechanisms of KLF14 functions will help us to treat AS by targeting different signaling molecules in different cells, tissues, and species.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Financial support

The authors gratefully acknowledge the financial supports from the National Natural Science Foundation of China of China (81800386), the Hunan Provincial Natural Science Foundation of China (2017JJ2234, 2018JJ3453) and the start-up funds for Ph.Ds in University of South China (2017XQD12, 2017XQD05).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.08.012.

References

[1] K.J. Moore, F.J. Sheedy, E.A. Fisher, Macrophages in atherosclerosis: a dynamic balance, Nat. Rev. Immunol. 13 (2013) 709–721.
[2] C.K. Glass, J.L. Witztum, Atherosclerosis. the road ahead, Cell 104 (2001) 503–516.
[3] M. Pennings, I. Meurs, D. Ye, R. Out, M. Hoekstra, T.J. Van Berkel, M. Van Eck, Regulation of cholesterol homeostasis in macrophages and consequences for atherosclerotic lesion development, FEBS Lett. 580 (2006) 5588–5596.
[4] I. Tabas, Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol, Cell Death Differ. 11 (Suppl 1) (2004) S12–S16.
[5] Y. Guo, Y. Fan, J. Zhang, G.A. Lomberk, Z. Zhou, L. Sun, A.J. Mathison, M.T. Garcia-Barrio, J. Zhang, L. Zeng, L. Li, S. Pennathur, C.J. Willer, D.J. Rader, R. Urrutia, Y.E. Chen, Perhexiline activates KLF14 and reduces atherosclerosis by modulating ApoA-I production, J. Clin. Investig. 125 (2015) 3819–3830.
[6] D.I. Chasman, G. Pare, S. Mora, J.C. Hopewell, G. Peloso, R. Clarke, L.A. Cupples, A. Hamsten, S. Kathiresan, A. Malarstig, J.M. Ordovas, S. Ripatti, A.N. Parker, J.P. Miletich, P.M. Ridker, Forty-three loci associated with plasma lipoprotein size,

concentration, and cholesterol content in genome-wide analysis, PLoS Genet. 5 (2009) e1000730.
[7] B.B. McConnell, V.W. Yang, Mammalian Kruppel-like factors in health and diseases, Physiol. Rev. 90 (2010) 1337–1381.
[8] K.S. Small, M. Todorcevic, M. Civelek, J.S. El-Sayed Moustafa, X. Wang, M.M. Simon, J. Fernandez-Tajes, A. Mahajan, M. Horikoshi, A. Hugill, C.A. Glastonbury, L. Quaye, M.J. Neville, S. Sethi, M. Yon, C. Pan, N. Che, A. Vinuela, P.C. Tsai, A. Nag, A. Buil, G. Thorleifsson, A. Raghavan, Q. Ding, A.P. Morris, J.T. Bell, U. Thorsteinsdottir, K. Stefansson, M. Laakso, I. Dahlman, P. Arner, A.L. Gloyn, K. Musunuru, A.J. Lusis, R.D. Cox, F. Karpe, M.I. McCarthy, Regulatory variants at KLF14 influence type 2 diabetes risk via a female-specific effect on adipocyte size and body composition, Nat. Genet. 50 (2018) 572–580.
[9] L. Wang, X. Tong, F. Gu, L. Zhang, W. Chen, X. Cheng, L. Xie, Y. Chang, H. Zhang, The KLF14 transcription factor regulates hepatic gluconeogenesis in mice, J. Biol. Chem. 292 (2017) 21631–21642.
[10] M. Civelek, A.J. Lusis, Conducting the metabolic syndrome orchestra, Nat. Genet. 43 (2011) 506–508.
[11] W. Hu, H. Lu, J. Zhang, Y. Fan, Z. Chang, W. Liang, H. Wang, T. Zhu, M.T. Garcia-Barrio, D. Peng, Y.E. Chen, Y. Guo, Kruppel-like factor 14, a coronary artery disease associated transcription factor, inhibits endothelial inflammation via NF-kappaB signaling pathway, Atherosclerosis 278 (2018) 39–48.
[12] C. Iwaya, H. Kitajima, K. Yamamoto, Y. Maeda, N. Sonoda, H. Shibata, T. Inoguchi, DNA methylation of the Klf14 gene region in whole blood cells provides prediction for the chronic inflammation in the adipose tissue, Biochem. Biophys. Res. Commun. 497 (2018) 908–915.
[13] K.S. Small, M. Todorcevic, M. Civelek, J.S. El-Sayed Moustafa, X. Wang, M.M. Simon, J. Fernandez-Tajes, A. Mahajan, M. Horikoshi, A. Hugill, C.A. Glastonbury, L. Quaye, M.J. Neville, S. Sethi, M. Yon, C. Pan, N. Che, A. Vinuela, P.C. Tsai, A. Nag, A. Buil, G. Thorleifsson, A. Raghavan, Q. Ding, A.P. Morris, J.T. Bell, U. Thorsteinsdottir, K. Stefansson, M. Laakso, I. Dahlman, P. Arner, A.L. Gloyn, K. Musunuru, A.J. Lusis, R.D. Cox, F. Karpe, M.I. McCarthy, Author Correction: regulatory variants at KLF14 influence type 2 diabetes risk via a female-specific effect on adipocyte size and body composition, Nat. Genet. 50 (2018) 1342.
[14] K.S. Small, A.K. Hedman, E. Grundberg, A.C. Nica, G. Thorleifsson, A. Kong, U. Thorsteindottir, S.Y. Shin, H.B. Richards, G. Consortium, M. Investigators, D. Consortium, N. Soranzo, K.R. Ahmadi, C.M. Lindgren, K. Stefansson, E.T. Dermitzakis, P. Deloukas, T.D. Spector, M.I. McCarthy, T.C. Mu, Identification of an imprinted master trans regulator at the KLF14 locus related to multiple metabolic phenotypes, Nat. Genet. 43 (2011) 561–564.
[15] P. Huang, R.X. Yin, K.K. Huang, X.N. Zeng, T. Guo, Q.Z. Lin, J. Wu, D.F. Wu, H. Li, S.L. Pan, Association of the KLF14 rs4731702 SNP and serum lipid levels in the Guangxi Mulao and Han populations, BioMed Res. Int. 2013 (2013) 231515.
[16] X. Chen, S. Li, Y. Yang, X. Yang, Y. Liu, Y. Liu, W. Hu, L. Jin, X. Wang, Genome-wide association study validation identifies novel loci for atherosclerotic cardiovascular disease, J. Thromb. Haemost. 10 (2012) 1508–1514.
[17] J.B. Lingrel, R. Pilcher-Roberts, J.E. Basford, P. Manoharan, J. Neumann, E.S. Konaniah, R. Srinivasan, V.Y. Bogdanov, D.Y. Hui, Myeloid-specific Kruppel-like factor 2 inactivation increases macrophage and neutrophil adhesion and promotes atherosclerosis, Circ. Res. 110 (2012) 1294–1302.
[18] X. Liao, N. Sharma, F. Kapadia, G. Zhou, Y. Lu, H. Hong, K. Paruchuri, G.H. Mahabeleshwar, E. Dalmas, N. Venteclef, C.A. Flask, J. Kim, B.W. Doreian, K.Q. Lu, K.H. Kaestner, A. Hamik, K. Clement, M.K. Jain, Kruppel-like factor 4 regulates macrophage polarization, J. Clin. Investig. 121 (2011) 2736–2749.
[19] J. Kobayashi, H. Mabuchi, Lipoprotein lipase and atherosclerosis, Ann. Clin. Biochem. 52 (2015) 632–637.
[20] I.J. Goldberg, Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis, J. Lipid Res. 37 (1996) 693–707.
[21] M. Takahashi, H. Yagyu, F. Tazoe, S. Nagashima, T. Ohshiro, K. Okada, J. Osuga, I.J. Goldberg, S. Ishibashi, Macrophage lipoprotein lipase modulates the development of atherosclerosis but not adiposity, J. Lipid Res. 54 (2013) 1124–1134.
[22] K. Wilson, G.L. Fry, D.A. Chappell, C.D. Sigmund, J.D. Medh, Macrophage-specific expression of human lipoprotein lipase accelerates atherosclerosis in transgenic apolipoprotein e knockout mice but not in C57BL/6 mice, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 1809–1815.
[23] T. Koike, J. Liang, X. Wang, T. Ichikawa, M. Shiomi, H. Sun, T. Watanabe, G. Liu, J. Fan, Enhanced aortic atherosclerosis in transgenic Watanabe heritable hyperlipidemic rabbits expressing lipoprotein lipase, Cardiovasc. Res. 65 (2005) 524–534.
[24] P.P. He, X.P. Ouyang, Y.Y. Tang, L. Liao, Z.B. Wang, Y.C. Lv, G.P. Tian, G.J. Zhao, L. Huang, F. Yao, W. Xie, Y.L. Tang, W.J. Chen, M. Zhang, Y. Li, J.F. Wu, J. Peng, X.Y. Liu, X.L. Zheng, W.D. Yin, C.K. Tang, MicroRNA-590 attenuates lipid accumulation and pro-inflammatory cytokine secretion by targeting lipoprotein lipase gene in human THP-1 macrophages, Biochimie 106 (2014) 81–90.
[25] Y.C. Lv, Y.Y. Tang, J. Peng, G.J. Zhao, J. Yang, F. Yao, X.P. Ouyang, P.P. He, W. Xie, Y.L. Tan, M. Zhang, D. Liu, D.P. Tang, F.S. Cayabyab, X.L. Zheng, D.W. Zhang, G.P. Tian, C.K. Tang, MicroRNA-19b promotes macrophage cholesterol accumulation and aortic atherosclerosis by targeting ATP-binding cassette transporter A1, Atherosclerosis 236 (2014) 215–226.
[26] G.P. Tian, W.J. Chen, P.P. He, S.L. Tang, G.J. Zhao, Y.C. Lv, X.P. Ouyang, K. Yin, P.P. Wang, H. Cheng, Y. Chen, S.L. Huang, Y. Fu, D.W. Zhang, W.D. Yin, C.K. Tang, MicroRNA-467b targets LPL gene in RAW 264.7 macrophages and attenuates lipid accumulation and proinflammatory cytokine secretion, Biochimie 94 (2012) 2749–2755.
[27] M. Zhang, J.F. Wu, W.J. Chen, S.L. Tang, Z.C. Mo, Y.Y. Tang, Y. Li, J.L. Wang, X.Y. Liu, J. Peng, K. Chen, P.P. He, Y.C. Lv, X.P. Ouyang, F. Yao, D.P. Tang, F.S. Cayabyab, D.W. Zhang, X.L. Zheng, G.P. Tian, C.K. Tang, MicroRNA-27a/b regulates cellular cholesterol efflux, influx and esterification/hydrolysis in THP-1 macrophages, Atherosclerosis 234 (2014) 54–64.
[28] W. Xie, L. Li, M. Zhang, H.P. Cheng, D. Gong, Y.C. Lv, F. Yao, P.P. He, X.P. Ouyang, G. Lan, D. Liu, Z.W. Zhao, Y.L. Tan, X.L. Zheng, W.D. Yin, C.K. Tang, MicroRNA-27 prevents atherosclerosis by suppressing lipoprotein lipase-induced lipid accumulation and inflammatory response in apolipoprotein E knockout mice, PLoS One 11 (2016) e0157085.
[29] G.J. Zhao, Z.C. Mo, S.L. Tang, X.P. Ouyang, P.P. He, Y.C. Lv, F. Yao, Y.L. Tan, W. Xie, J.F. Shi, Y. Wang, M. Zhang, D. Liu, D.P. Tang, X.L. Zheng, G.P. Tian, C.K. Tang, Chlamydia pneumoniae negatively regulates ABCA1 expression via TLR2-Nuclear factor-kappa B and miR-33 pathways in THP-1 macrophage-derived foam cells, Atherosclerosis 235 (2014) 519–525.
[30] T.M. de Assuncao, G. Lomberk, S. Cao, U. Yaqoob, A. Mathison, D.A. Simonetto, R.C. Huebert, R.A. Urrutia, V.H. Shah, New role for Kruppel-like factor 14 as a transcriptional activator involved in the generation of signaling lipids, J. Biol. Chem. 289 (2014) 15798–15809.
[31] G. Lomberk, R. Urrutia, The family feud: turning off Sp1 by Sp1-like KLF proteins, Biochem. J. 392 (2005) 1–11.
[32] C. Circosta, R. De Pasquale, F. Occhiuto, Cardiovascular effects of the aqueous extract of Gynostemma pentaphyllum Makino, Phytomedicine 12 (2005) 638–643.
[33] H. Yu, Q. Guan, L. Guo, H. Zhang, X. Pang, Y. Cheng, X. Zhang, Y. Sun, Gypenosides alleviate myocardial ischemia-reperfusion injury via attenuation of oxidative stress and preservation of mitochondrial function in rat heart, Cell Stress Chaperones 21 (2016) 429–437.
[34] R.P. Anunciado-Koza, J. Manuel, R.A. Koza, Molecular correlates of fat mass expansion in C57BL/6J mice after short-term exposure to dietary fat, Ann. N. Y. Acad. Sci. 1363 (2016) 50–58.
[35] K. Bacos, L. Gillberg, P. Volkov, A.H. Olsson, T. Hansen, O. Pedersen, A.P. Gjesing, H. Eiberg, T. Tuomi, P. Almgren, L. Groop, L. Eliasson, A. Vaag, T. Dayeh, C. Ling, Blood-based biomarkers of age-associated epigenetic changes in human islets associate with insulin secretion and diabetes, Nat. Commun. 7 (2016) 11089.
[36] A.S. Dimas, V. Lagou, A. Barker, J.W. Knowles, R. Magi, M.F. Hivert, A. Benazzo, D. Rybin, A.U. Jackson, H.M. Stringham, C. Song, A. Fischer-Rosinsky, T.W. Boesgaard, N. Grarup, F.A. Abbasi, T.L. Assimes, K. Hao, X. Yang, C. Lecoeur, I. Barroso, L.L. Bonnycastle, Y. Bottcher, S. Bumpstead, P.S. Chines, M.R. Erdos, J. Graessler, P. Kovacs, M.A. Morken, N. Narisu, F. Payne, A. Stancakova, A.J. Swift, A. Tonjes, S.R. Bornstein, S. Cauchi, P. Froguel, D. Meyre, P.E. Schwarz, H.U. Haring, U. Smith, M. Boehnke, R.N. Bergman, F.S. Collins, K.L. Mohlke, J. Tuomilehto, T. Quertemous, L. Lind, T. Hansen, O. Pedersen, M. Walker, A.F. Pfeiffer, J. Spranger, M. Stumvoll, J.B. Meigs, N.J. Wareham, J. Kuusisto, M. Laakso, C. Langenberg, J. Dupuis, R.M. Watanabe, J.C. Florez, E. Ingelsson, M.I. McCarthy, I. Prokopenko, M. Investigators, Impact of type 2 diabetes susceptibility variants on quantitative glycemic traits reveals mechanistic heterogeneity, Diabetes 63 (2014) 2158–2171.
[37] M.C. Ng, R. Saxena, J. Li, N.D. Palmer, L. Dimitrov, J. Xu, L.J. Rasmussen-Torvik, J.M. Zmuda, D.S. Siscovick, S.R. Patel, E.D. Crook, M. Sims, Y.D. Chen, A.G. Bertoni, M. Li, S.F. Grant, J. Dupuis, J.B. Meigs, B.M. Psaty, J.S. Pankow, C.D. Langefeld, B.I. Freedman, J.I. Rotter, J.G. Wilson, D.W. Bowden, Transferability and fine mapping of type 2 diabetes loci in african Americans: the candidate gene association resource plus study, Diabetes 62 (2013) 965–976.
[38] R.L. Hanson, T. Guo, Y.L. Muller, J. Fleming, W.C. Knowler, S. Kobes, C. Bogardus, L.J. Baier, Strong parent-of-origin effects in the association of KCNQ1 variants with type 2 diabetes in American Indians, Diabetes 62 (2013) 2984–2991.
[39] S. Elouej, I. Rejeb, R. Attaoua, M. Nagara, O.K. Sallem, I. Kamoun, M. Chargui, H. Jamoussi, Z. Turki, A. Abid, C.B. Slama, S. Bahri, H.B. Romdhane, S. Abdelhak, R. Kefi, F. Grigorescu, Gender-specific associations of genetic variants with metabolic syndrome components in the Tunisian population, Endocr. Res. (2016) 1–10.
[40] M. Yang, Y. Ren, Z. Lin, C. Tang, Y. Jia, Y. Lai, T. Zhou, S. Wu, H. Liu, G. Yang, L. Li, Kruppel-like factor 14 increases insulin sensitivity through activation of PI3K/Akt signal pathway, Cell. Signal. 27 (2015) 2201–2208.
[41] W. Xie, L. Li, X.L. Zheng, W.D. Yin, C.K. Tang, The role of Kruppel-like factor 14 in the pathogenesis of atherosclerosis, Atherosclerosis 263 (2017) 352–360.
[42] A.K. Nair, P. Piaggi, N.A. McLean, M. Kaur, S. Kobes, W.C. Knowler, C. Bogardus, R.L. Hanson, L.J. Baier, Assessment of established HDL-C loci for association with HDL-C levels and type 2 diabetes in Pima Indians, Diabetologia 59 (2016) 481–491.
[43] S. Elouej, I. Rejeb, R. Attaoua, M. Nagara, O.K. Sallem, I. Kamoun, M. Chargui, H. Jamoussi, Z. Turki, A. Abid, C. Ben Slama, S. Bahri, H. Ben Romdhane, S. Abdelhak, R. Kefi, F. Grigorescu, Gender-specific associations of genetic variants with metabolic syndrome components in the Tunisian population, Endocr. Res. 41 (2016) 300–309.
[44] R. Singaravelu, R. Chen, R.K. Lyn, D.M. Jones, S. O'Hara, Y. Rouleau, J. Cheng, P. Srinivasan, N. Nasheri, R.S. Russell, D.L. Tyrrell, J.P. Pezacki, Hepatitis C virus induced up-regulation of microRNA-27: a novel mechanism for hepatic steatosis, Hepatology 59 (2014) 98–108.
[45] Y.C. Lu, S.H. Chang, M. Hafner, X. Li, T. Tuschl, O. Elemento, T. Hla, ELAVL1 modulates transcriptome-wide miRNA binding in murine macrophages, Cell Rep. 9 (2014) 2330–2343.
[46] E. Lozano-Velasco, A. Contreras, C. Crist, F. Hernandez-Torres, D. Franco, A.E. Aranega, Pitx2c modulates Pax3+/Pax7+ cell populations and regulates Pax3 expression by repressing miR27 expression during myogenesis, Dev. Biol. 357 (2011) 165–178.
[47] H.K. Zhang, Y. Ye, Z.N. Zhao, K.J. Li, Y. Du, Q.M. Hu, J.F. He, Neuroprotective effects of gypenosides in experimental autoimmune optic neuritis, Int. J. Ophthalmol. 10 (2017) 541–549.
[48] H. Li, H. Ying, A. Hu, Y. Hu, D. Li, Therapeutic effect of gypenosides on nonalcoholic steatohepatitis via regulating hepatic lipogenesis and fatty acid oxidation,

Biol. Pharm. Bull. 40 (2017) 650–657.
[49] S.H. Gou, H.F. Huang, X.Y. Chen, J. Liu, M. He, Y.Y. Ma, X.N. Zhao, Y. Zhang, J.M. Ni, Lipid-lowering, hepatoprotective, and atheroprotective effects of the mixture Hong-Qu and gypenosides in hyperlipidemia with NAFLD rats, J. Chin. Med. Assoc. 79 (2016) 111–121.
[50] K. Li, Y. Du, Q. Fan, C.Y. Tang, J.F. He, Gypenosides might have neuroprotective and immunomodulatory effects on optic neuritis, Med. Hypotheses 82 (2014) 636–638.
[51] Y.H. Yang, J. Yang, Q.H. Jiang, Hypolipidemic effect of gypenosides in experimentally induced hypercholesterolemic rats, Lipids Health Dis. 12 (2013) 154.
[52] S.H. Gou, B.J. Liu, X.F. Han, L. Wang, C. Zhong, S. Liang, H. Liu, Y. Qiang, Y. Zhang, J.M. Ni, Anti-atherosclerotic effect of Fermentum Rubrum and Gynostemma pentaphyllum mixture in high-fat emulsion- and vitamin D3-induced atherosclerotic rats, J. Chin. Med. Assoc. 81 (2017) 398–408.
[53] L. Li, L. Jiao, B.H. Lau, Protective effect of gypenosides against oxidative stress in phagocytes, vascular endothelial cells and liver microsomes, Cancer Biother. 8 (1993) 263–272.
[54] Y. Quan, Y. Yang, H. Wang, B. Shu, Q.H. Gong, M. Qian, Gypenosides attenuate cholesterol-induced DNA damage by inhibiting the production of reactive oxygen species in human umbilical vein endothelial cells, Mol. Med. Rep. 11 (2015) 2845–2851.