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Basic Science

Functions of Monocytes and Macrophages and the Associated Effective Molecules and Mechanisms at the Early Stage of Atherosclerosis

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Objective: This study aimed to explore the functions and possible underlying regulatory molecules and mechanisms of monocytes and macrophages under early atherosclerotic conditions.

Methods: THP-1-derived monocytes or macrophages were induced by 50 μg/ml oxidized low density lipoprotein (ox-LDL) for 24 hours, and the degree of lipid metabolism and inflammation were determined. In addition, we identified differentially expressed genes, noncoding ribonucleic acids (RNAs), pathways and mechanisms by RNA sequencing, and performed further correlation analysis and molecular expression verification.

Results: Monocytes could not form foam cells with oil red O staining directly and had low levels of lipids as determined by total cholesterol and triglycerides assays, cholesterol uptake molecules CD36, the class A macrophage scavenger receptor and lectin-like oxidized low-density lipoprotein receptor-1 and cholesterol efflux molecules ATP binding cassette transporter A1, ATP binding cassette transporter G1 and liver X receptor α, and inflammatory factors, which were markedly different from those in macrophages. Additionally, sequencing data showed obviously differentially expressed genes, microRNAs and long noncoding RNAs in the atherosclerotic group. We identified 15 upregulated and downregulated genes, and 10 biological processes and pathways involved in atherosclerosis. Specifically, fatty acid desaturase 2 and apolipoprotein A1 in the peroxisome proliferator-activated receptor signaling pathway were differentially expressed in stimulated macrophages, whereas no changes were observed in the monocyte groups. Furthermore, correlation analysis showed differential expressed lncRNAs targeting miRNAs and mRNAs, and 24 competing endogenous RNA (ceRNA) networks of long noncoding RNA-microRNA-messenger RNA in early oxidative macrophages.

Conclusions: Monocytes did not directly participate in lipid metabolism before differentiation into macrophages at the early stage in vitro. Furthermore, noncoding RNAs and ceRNA networks might play important roles in regulating the lipid metabolism of macrophages at the early stage of atherosclerosis.

Key Words: Atherosclerosis • Competing endogenous RNA • Lipid metabolism • Macrophage • Monocyte • Noncoding RNA

INTRODUCTION

Atherosclerosis is a chronic disease that causes severe cardiovascular events worldwide.[1] Monocytes and macrophages have been shown to play important roles in the development and progression of atherosclerosis according to many studies on the pathophysiology of atherosclerosis.[2] Previous studies have indicated that macrophages that engulf modified lipoproteins are dif-

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ferentiated from monocytes, which are recruited at the injury site of blood vessels and then adhere to and pass through the endothelium. These macrophages can phagocytize lipids to accelerate the progression of atherosclerotic plaque formation.[3] However, no studies have clearly shown whether monocytes can directly regulate lipid metabolism, form foam cells, produce an inflammatory response, and affect plaque progression at the initial stage of atherosclerosis. Therefore, this study aimed to construct a microenvironment for atherosclerosis to evaluate the differences and functions of monocytes and macrophages at the early stage of atherosclerosis.

In addition, recent studies have indicated that monocyte- and macrophage-related immunometabolism or their origin and status in the microenvironment can influence the fate of atherosclerotic plaques and vice versa.[4,5] Importantly, the specific genetic, transcriptional and metabolic changes of these cells in the microenvironment are important for disease outcomes, but little is known of the mechanisms involved. Therefore, we performed transcriptome sequencing of early injured macrophages to further explore the underlying key molecules, changes and related mechanisms that cause the occurrence and progression of atherosclerosis. We hypothesized that there may be negative regulatory correlations between long noncoding RNA (lncRNA) and miRNA (microRNA), and miRNA and messenger RNA (mRNA), thus providing a theoretical basis for targeted interventions of atherosclerosis at an early stage.

MATERIALS AND METHODS

Culture and treatment of THP-1 cells

The THP-1 cell line (National Infrastructure of Cell Line Resource) was cultured in RPMI1640 (HyClone) containing 10% fetal bovine serum at 37 °C with 5% CO_2, and the cells were subcultured at 80% confluence. THP-1 cells were seeded into 6-well culture plates and treated with phorbol-12-myristate acetate (PMA; 100 nM; Solarbio) for 72 hours. Then, oxidized low density lipoprotein (ox-LDL) (50 μg/mL; Yiyuan Biotechnologies) was added to the model groups for another 24 hours, and an equivalent amount of sterile phosphate buffered saline (PBS) was added to the control groups. Grouping was as follows: 1) monocyte control: THP-1, 2) monocyte model group: THP-1 + 50 μg/mL ox-LDL, 3) macrophage control: THP-1 + 100 nM PMA, and 4) macrophage model group: THP-1 + 100 nM PMA + 50 μg/mL ox-LDL.

Hematoxylin and eosin (H&E) and oil red O staining

Cells were seeded onto coverslips in 6-well plates and subjected to different treatments. Then, the cells were washed, fixed with 4% paraformaldehyde, and stained with H&E or oil red O staining to observe the cell morphology and foam cell formation, respectively. Stained cells were observed and imaged under a microscope.

Determination of total cholesterol (TC) and triglycerides (TG)

Total cholesterol and TG Quantification Assay Kits (Solarbio, Beijing) were used to determine the concentrations of TC and TG in cells, respectively. The assay kit reagents were equilibrated to room temperature before use. The specific experimental procedures, including working solution and standard preparation, cell disruption and measurement, were performed according to the manufacturer's instructions.

Quantitative real-time reverse transcriptase polymerase chain reaction

Total RNA from cells was isolated using TRIzol reagent (Invitrogen) and reverse transcribed to complementary DNA (cDNA) using a reverse transcription kit (Solarbio, Beijing). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR Master Mix (Solarbio, Beijing) with a Molarray PCR system (MA-6000, Molarray Ltd., Suzhou). The specific primer sequences are provided in Table 1. Experiments were performed in triplicate, and the data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, respectively. Relative expressions were calculated with the $2^{-\Delta\Delta CT}$ method.

Enzyme-linked immunosorbent assay (ELISA) detection

Cells were incubated in 6-well plates and subjected to different treatments. At the endpoint of the experiment, cell supernatants in all groups were collected and centrifuged at 3000 rpm for 5 minutes. Partial supernatants were used to detect the concentrations of tumour necrosis factor α (TNFα), interleukin-6 (IL-6) and inter-

Table 1. Primer sequences for PCR

Target gene		Primers sequences
GAPDH	F	5′-TCAAGAAGGTGGTGAAGCAGG-3′
	R	5′-GCGTCAAAGGTGGAGGAGTG-3′
CD36	F	5′-GTAGGACTTTCCTGCAGAATACCA-3′
	R	5′-GCTCTGGTTCTTATTCACAAATCA-3′
SR-A	F	5′-ACAGCTTTGCTTCCTCCGAA-3′
	R	5′-CTTCAGGAGTTGAGCTGCCA-3′
LOX-1	F	5′-TTGTCCGCAAGACTGGATCTG-3′
	R	5′-CGAGACAGCCCCATCCAGAA-3′
ABCA1	F	5′-GGCTGAGGGAACATGGCTT-3′
	R	5′-GCAGCAGCTGACATGTTTGT-3′
ABCG1	F	5′-TCGACCAGCTTTACGTCCTG-3′
	R	5′-GTGGTAGGTTGGGCAGTTCA-3′
LXRα	F	5′-CAGTCTCGGTGGGATTGCG-3′
	R	5′-CTCCACCGCAGAGTCTTCTTA-3′
TNF-α	F	5′-CATCCAACCTTCCCAAACGC-3′
	R	5′-CGAAGTGGTGGTCTTGTTGC-3′
IL-6	F	5′-CAATGAGGAGACTTGCCTGGT-3′
	R	5′-AGCTGCGCAGAATGAGATGA-3′
IL-10	F	5′-ACGGCGCTGTCATCGATTTCT-3′
	R	5′-CAGAGCCCCAGATCCGATTTT-3′
APOA1	F	5′-GCCTGTGGGATGATGTTGAA-3′
	R	5′-AGCCCTGGTCTGTCCTTTGC-3′
FADS2	F	5′-TCCCCACTCCCCAGACTCCA-3′
	R	5′-CGGGTCATCAGCCACTACGC-3′

ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; APOA1, apolipoprotein A1; CD36, scavenger receptor CD36; FADS2, fatty acid desaturase 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin-6; IL-10, interleukin-10; LOX-1, lectin-like oxidized low-density lipoprotein receptor-1; LXRα, liver X receptor α; SR-A, the class A macrophage scavenger receptor; TNFα, tumour necrosis factor α.

leukin-10 (IL-10) with commercial ELISA kits (R&D) in accordance with the manufacturer's directions.

RNA sequencing

Based on the above grouping and detection, we selected the macrophage control and ox-LDL-induced groups for further sequencing and analysis. Each group was prepared with at least 3×10^6 cells and three biological replicates. The cells were washed with sterile RNase-free PBS and lysed with RNase-free TRIzol. RNA degradation was detected on 1.5% agarose gel, and the concentration and purity of RNA were measured. We used transcriptome sequencing to construct an RNA library. A total of 1.5 μg RNA per sample was used as input material for rRNA removal using a Ribo-Zero rRNA Removal Kit (Epicentre, Madison, WI, USA). Sequencing libraries were generated using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes were added to assign sequences to each sample. After adenylation of the 3′ ends of DNA fragments, the NEBNext adaptor with hairpin loop structure was ligated to prepare for hybridization. To preferentially select insert fragments of 150-200 bp in length, the library fragments were purified with AMPure XP Beads (Beckman Coulter, Beverly, USA). Then, 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 minutes before PCR. PCR was then performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index(X) Primer. Finally, PCR products were purified (AMPure XP system), and the library quality was assessed by qPCR on an Agilent Bioanalyzer 2100.

Sequencing data analysis

Adaptor sequences and low-quality sequence reads were removed from the data sets. Raw sequences were transformed into clean reads after data processing. These clean reads were then mapped to the reference genome sequence. Only reads with a perfect match were further analyzed and annotated based on the reference genome. HISAT2 software was used to map reads to the reference genome. Quantification of gene expression levels was estimated by fragments per kilobase of transcript per million fragments mapped, i.e., FPKM. MiRNA expression levels were estimated for each sample, mapped back to the precursor sequence, and the read count for each miRNA was obtained from the mapping results. Quantification of lncRNA expression levels was performed using StringTie to calculate FPKM values of lncRNAs.

Differential expression analysis of two conditions was performed using the DESeq R package. DESeq provides statistical methods for determining differentially expressed genes using a model based on negative binomial distribution. The resulting p-values were adjusted using Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with a p-value < 0.05 and absolute value of log 2 (fold change) > 1 determined by DESeq were assigned as being differentially expressed.

Gene function was annotated based on the GO and KEGG databases. Pathway annotation analysis of differ-

entially expressed genes can help further interpret gene functions. KEGG pathway enrichment analysis was performed on genes using clusterProfiler R. The enrichment analysis used a hypergeometric test to find KEGG pathways that were significantly enriched compared to the entire genomic background.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Differences between the experimental groups were compared using the Student's t-test or one-way analysis of variance (ANOVA). All statistical analyses were performed using SPSS 17.0 and GraphPad Prism 6. $p < 0.05$ was considered to be statistically significant.

RESULTS

Foam cell formation and quantification of TC and TG in cells from different groups

THP-1 cells grew in suspension, while macrophages that differentiated from THP-1 cells upon stimulation attached to plates. The THP-1 cells did not adhere to the culture plate even after ox-LDL induction. Therefore, THP-1 cells in the control group and ox-LDL model group could not be stained with H&E or oil red O dyes and presented in semi-suspension. Conversely, THP-1 macrophages presented as round or stretched shapes and spindle or polymorphic shapes in the control group and ox-LDL-induced group, respectively. Furthermore, after ox-LDL stimulation, macrophages phagocytized abundant lipid droplets as demonstrated by oil red O staining compared with the control group (Figure 1A).

After ox-LDL stimulation, we detected the concentrations of TC and TG in the cells of different groups to determine the degree of lipid metabolism. The results showed that the concentrations of TC and TG in the THP-1 monocyte and macrophage model groups were higher than those in the respective control groups. However, the levels in the macrophage model group were significantly higher than those in its control group, while there were no marked differences between the monocyte model and control groups. Overall, macrophages had higher levels of TC and TG than THP-1 monocytes (Figure 1B).

Expression of cholesterol uptake and efflux molecules in THP-1 monocytes and macrophages

Furthermore, we detected the levels of lipid metabolism-related molecules involved in cholesterol uptake and efflux in ox-LDL-induced THP-1 monocytes and macrophages. The results showed that the relative mRNA levels of CD36, SR-A and LOX-1 in macrophages were higher than those in monocytes, and that ox-LDL-induced macrophages had obviously higher levels than non-stimulated macrophages. However, there were no differences in gene expressions between the monocyte control and ox-LDL-induced groups (Figure 2A).

In addition, macrophages had obviously higher levels of the cholesterol efflux molecules ABCA1, ABCG1 and LXRα than monocytes, but the expressions decreased after macrophages were stimulated with ox-LDL. However, there were no differences in the expressions of these molecules between the monocyte control and ox-LDL-inducing groups (Figure 2B). Taken together, these findings indicated that ox-LDL-induced macrophages

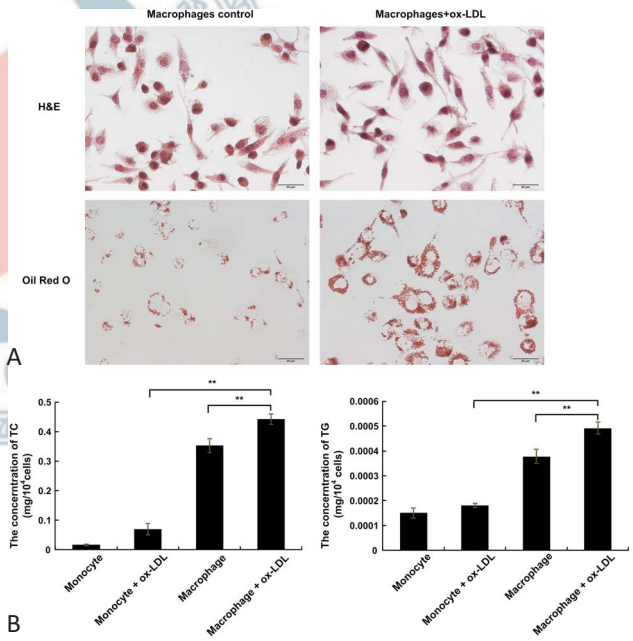

Figure 1. *Foam cell formation and quantification of TC and TG in cells from different groups. THP-1 cells were treated with or without PMA for 72 hours to induce monocytes or macrophages and then stimulated with ox-LDL for 24 hours. H&E and oil red O staining were performed to observe the morphology and foam cell formation of cells (A). The concentrations of TC and TG in cells were determined in the different groups to assess the degree of lipid metabolism (B). n = 3, scale bar = 20 μm, ** p < 0.01. H&E, hematoxylin and eosin; ox-LDL, oxidized low density lipoprotein; PMA, phorbol-12-myristate acetate; TC, total cholesterol; TG, triglycerides.*

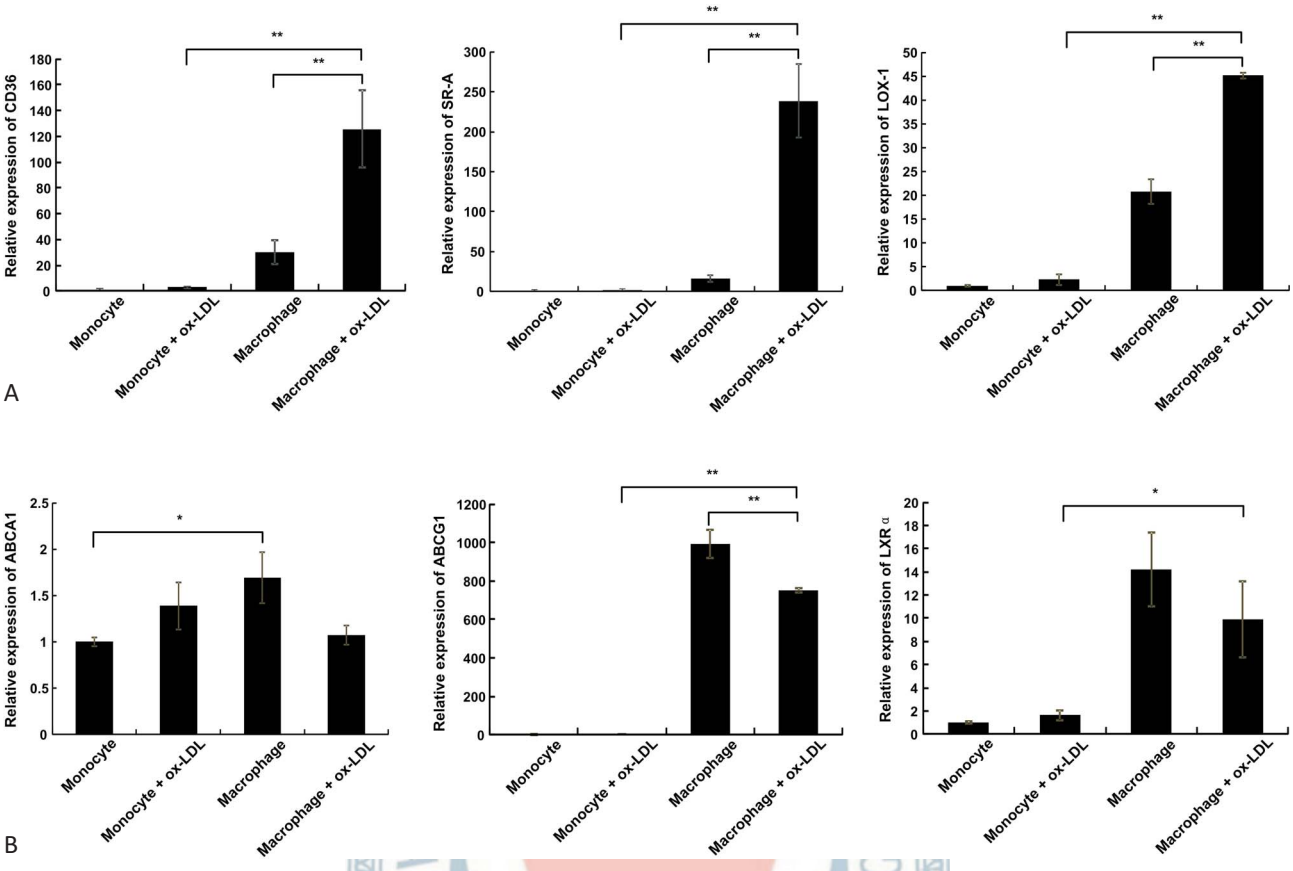

Figure 2. *The expression of cholesterol uptake and efflux molecules in THP-1 monocytes and macrophages. The relative mRNA levels of the cholesterol uptake molecules CD36, SR-A and LOX-1 (A) and the cholesterol efflux molecules ABCA1, ABCG1 and LXRα (B) were measured by PCR to reflect the abilities of monocytes and macrophages to regulate lipid metabolism after exposure moderate concentrations of ox-LDL stimulation at the initial stage. N = 3, * p < 0.05, ** p < 0.01. ox-LDL, oxidized low density lipoprotein; PCR, polymerase chain reaction.*

had a stronger ability to take up cholesterol and a decreased ability to efflux cholesterol, while these effects were not observed in THP-1 monocytes.

Expression and secretion of inflammatory molecules by THP-1 monocytes and macrophages

Atherosclerosis is not only associated with cholesterol metabolism, but is also related to inflammatory reactions. Our results showed that the expressions of TNF-α and IL-6 increased in the macrophage control group compared to the THP-1 monocyte groups, while the levels of IL-10 were nearly unchanged. Furthermore, ox-LDL-induced macrophages had markedly higher levels of TNF-α, IL-6 and IL-10 than unstimulated macrophages, but there were no obvious changes in monocytes (Figure 3A). In addition, we found that ox-LDL-induced macrophages had higher levels of IL-10 than untreated macrophages (Figure 3A), which may have been due to the moderate concentration of ox-LDL used in our research. Similarly, ELISA results showed that the concentrations of TNF-α, IL-6 and IL-10 were similar to those obtained by PCR (Figure 3B). These results indicated that a moderate concentration of ox-LDL could induce macrophages to secrete inflammatory factors, while monocytes were unable to produce inflammation-related molecules at the early stage of oxidative injury.

Differentially expressed molecules between the control and atherosclerotic groups

The above results showed that monocytes had limited ability to form foam cells and regulate lipid metabolism compared to macrophages after ox-LDL induction. Therefore, we determined the differentially expressed genes, miRNAs and lncRNAs by transcriptome sequencing between the macrophage control and model groups to reflect the significant differences under-

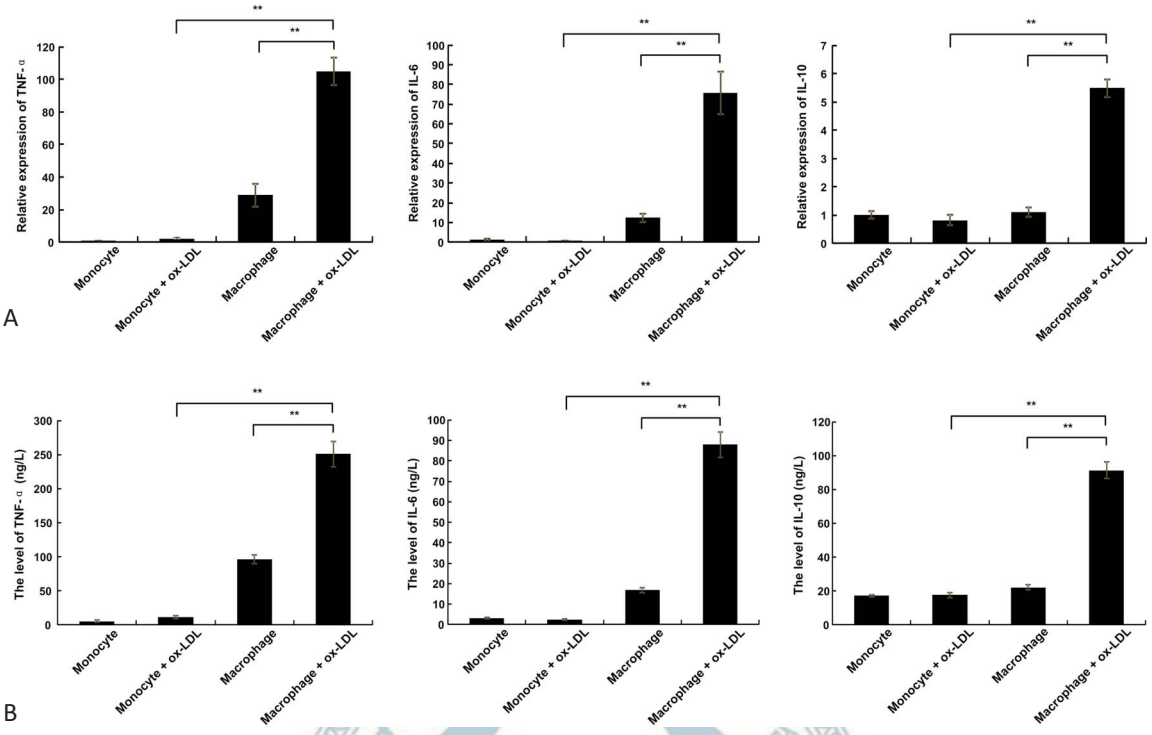

Figure 3. *The expression and secretion of inflammatory molecules in THP-1 monocytes and macrophages. The gene expression and secretion levels of TNF-α, IL-6 and IL-10 were detected by PCR (A) and ELISA (B) in different groups at the early stage of atherosclerotic oxidative injury to indicate the degree of inflammatory response and the secretion of inflammatory factors. n = 3, ** p < 0.01. ELISA, enzyme-linked immunosorbent assay; IL, interleukin; PCR, polymerase chain reaction; TNF-α, tumour necrosis factor α.*

lying the progression of atherosclerosis. The results showed that there were 437 markedly differentially expressed genes, of which 175 were upregulated and 262 were downregulated in the ox-LDL-induced group compared to the control group (Figure 4A). Recent studies have suggested that noncoding RNAs might be important molecules that regulate the progression of diseases.[3,6,7] Data analysis showed that ox-LDL-stimulated macrophages had 233 significantly differentially expressed miRNAs and that 212 were downregulated compared to the control group (Figure 4A). Similarly, the volcano plot identified 323 lncRNAs with changes in expression after ox-LDL stimulation, of which 112 were upregulated (Figure 4A). The top three differentially upregulated and downregulated genes, miRNAs and lncRNAs are shown in Figure 4B.

Differentially expressed lipid metabolism-related genes and pathways in different groups

According to the above results, we further analyzed the associations between the differentially expressed molecules and atherosclerosis. The cluster heat map in Figure 5A shows the specific differentially expressed genes between the two groups, of which 9 were significantly upregulated and 6 were markedly downregulated. Moreover, the cluster heat map indicated good consistency within both groups. Specifically, the 9 upregulated genes were LY96, FCGR3A, FADS2, SPHK2, IL7R, LPAR4, P2RY1, PARD6A and PAFAH1B3, and the 6 downregulated genes were Human_newGene_47464, APOA1, ABCA13, ITGA2, ABCA1 and JAG1 based on the log 2 (fold change) value between the two groups (Figure 5B-C). Importantly, these genes were highly related to atherosclerosis based on functional annotation. Furthermore, we identified differentially expressed pathways and biological processes between these two groups, including ATP binding component (ABC) transporters, TNF signaling pathway and calcium signaling pathways and many metabolic processes (Figure 5D-E). The results showed that these pathways were highly correlated to lipid metabolism in atherosclerosis.

Among these pathways, we found a strong associa-

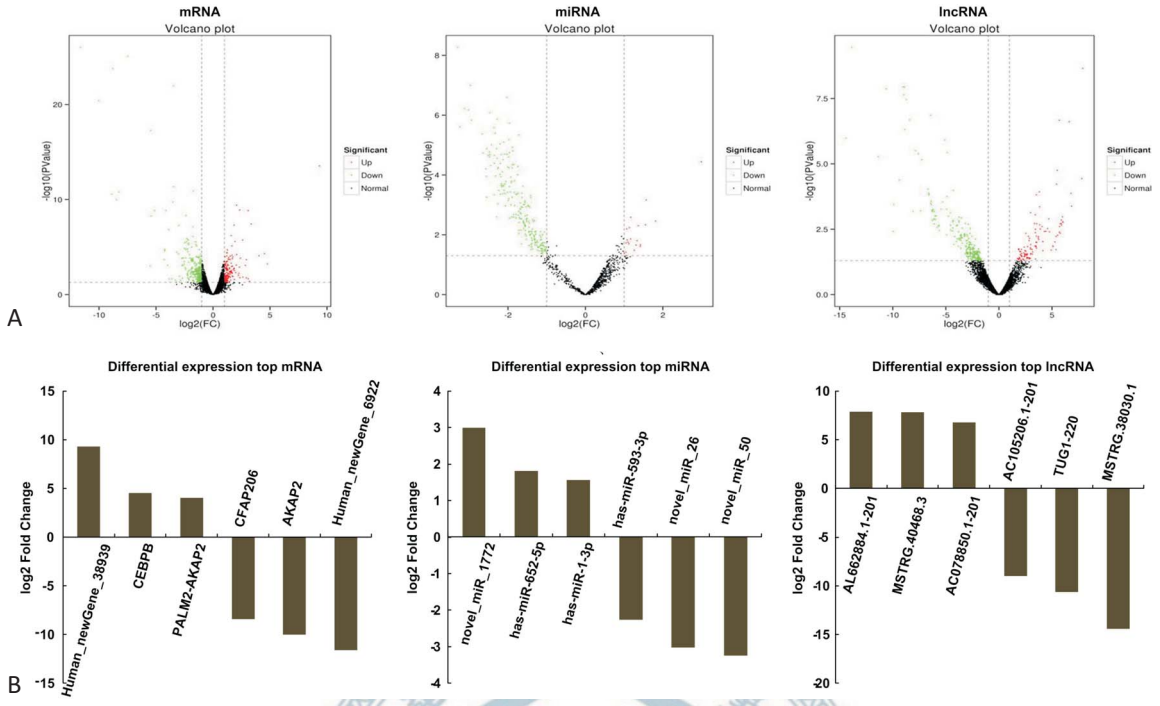

Figure 4. *Differentially expressed molecules between the control and atherosclerotic groups. Due to the ability of macrophages to form foam cells and regulate lipid metabolism and inflammation after ox-LDL stimulation, the macrophage control and ox-LDL-induced groups were chosen for transcriptome sequencing. The overall differential expression of genes (mRNA), miRNAs and lncRNAs are presented as volcano plots, and all significantly upregulated (red spots) and downregulated RNAs (green spots) are also shown (A). Among them, three upregulated and three downregulated of the top 10 significantly differentially expressed mRNAs, miRNAs and lncRNAs were analyzed and are listed (B). n = 3, log 2|fold change| > 1. lncRNA, long noncoding RNA; mRNA, messenger RNA; miRNA, microRNA; ox-LDL, oxidized low density lipoprotein.*

tion between the peroxisome proliferator-activated receptor (PPAR) signaling pathway and lipid metabolism. Moreover, the FADS2 and APOA1 genes, which are part of this pathway, were differentially expressed and had opposite trends between the two groups. We further detected the expressions of these two genes in monocytes and the ox-LDL-induced group. As expected, compared to the macrophage group, the expression of APOA1 was decreased in ox-LDL-stimulated macrophages, while FADS2 was markedly increased. However, the expressions of both genes were low in the monocyte group and did not obviously change after ox-LDL stimulation (Figure 5F-G). These data further demonstrated that, unlike macrophages, monocytes stimulated at the early stage of atherosclerosis had little ability to form foam cells and regulate lipid metabolism directly.

Noncoding RNAs might regulate lipid metabolism through a competing endogenous RNA network mechanism

Based on the above sequencing results, further combined analyses were performed among differentially expressed RNAs, focusing on the lncRNAs which mRNAs and miRNAs targeted and the competing endogenous RNA (ceRNA) network between lncRNAs and miRNAs, and miRNAs and mRNAs via miRNA response element (MRE). The results showed that 58 and 323 differential lncRNAs targeted differential mRNAs and 54 for miRNAs, which may have affected the expressions and functions of mRNA directly or indirectly (Figure 6A). In addition, we identified several ceRNA networks consisting of 24 specific differential upregulated and downregulated pathways (Figure 6B), including 6 downregulated and 5 upregulated lncRNAs, 1 downregulated and 4 upregulated miRNAs, and 8 downregulated and 8 upregulated mRNAs (Figure 6C). There were negative regulatory relations among them, which might play important roles in atherosclerosis.

DISCUSSION

The pathophysiological mechanisms of atherosclerosis

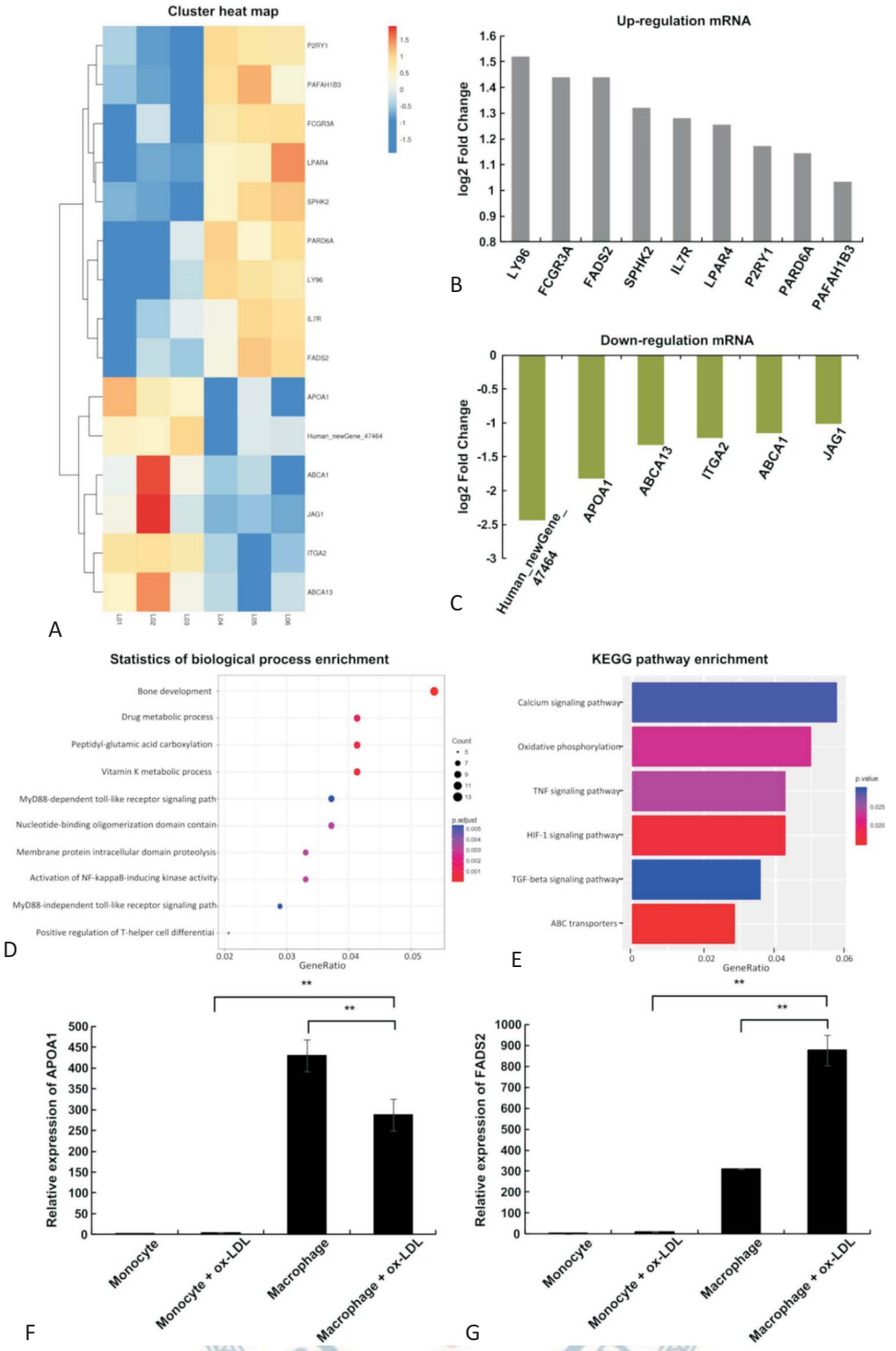

Figure 5. *Differentially expressed lipid metabolism-related genes and pathways in different groups. Further analysis of the 437 differentially expressed genes showed that 15 significantly differentially expressed genes were present in the cluster heat map and had high consistencies within the groups (A). Among them, 9 markedly upregulated (B) and 6 downregulated (C) genes were related to atherosclerosis based on genetic function. Differentially expressed biological processes (D) and KEGG pathway enrichment (E) partially associated with lipid metabolism were summarized. In addition, the levels of the downregulated gene APOA1 (F) and upregulated gene FADS2 (G) in the PPAR signaling pathway were verified in the monocyte and macrophage control and ox-LDL-induced groups. n = 3, ** p < 0.01. APOA1, apolipoprotein A1; FADS2, fatty acid desaturase 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; ox-LDL, oxidized low density lipoprotein; PPAR, peroxisome proliferator-activated receptor.*

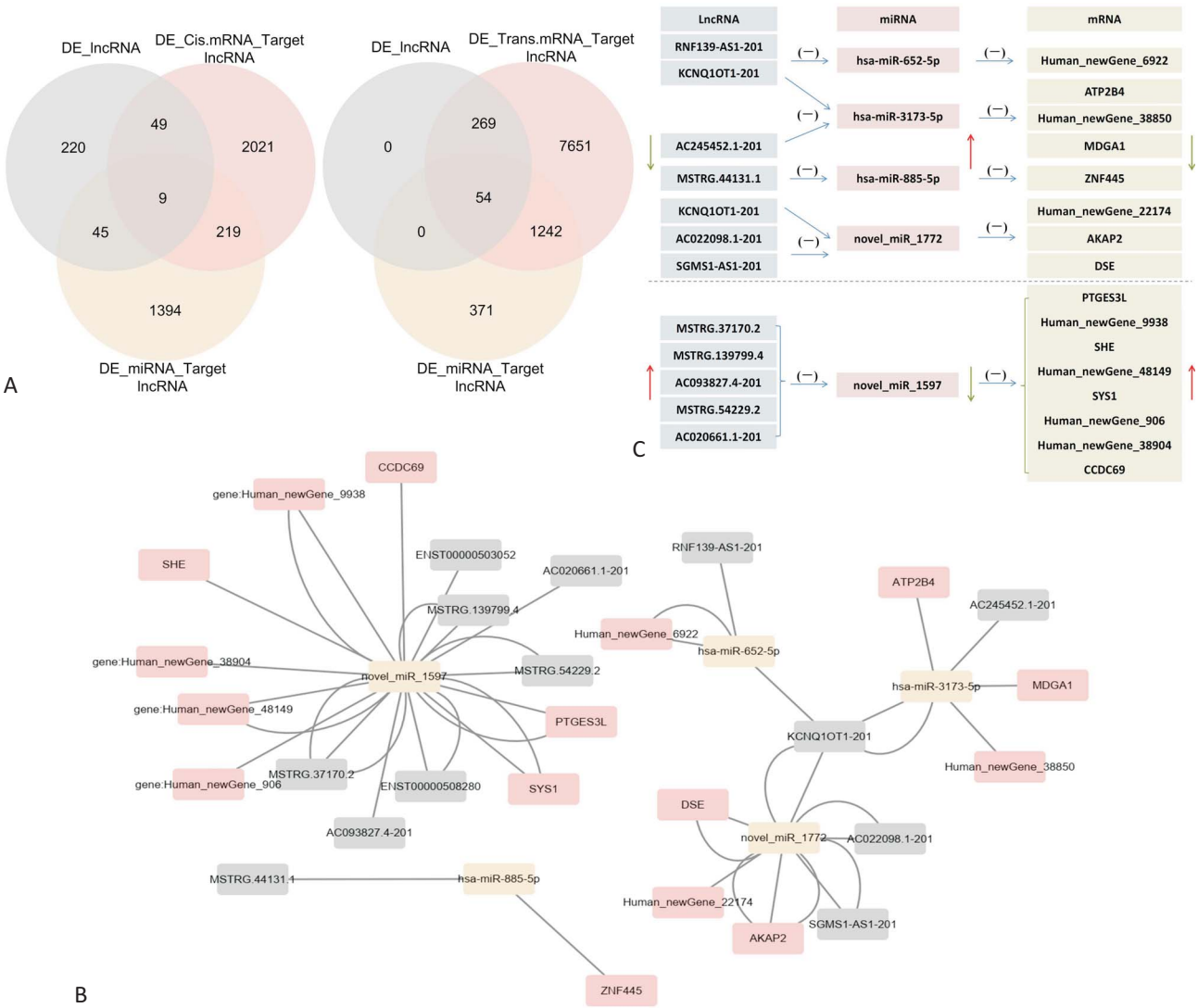

Figure 6. *The mechanisms of noncoding RNAs involved in oxidative macrophages. Correlation analysis of sequencing results between control and moderate ox-LDL induced macrophages. Differentially expressed lncRNAs directly targeted differential miRNAs and mRNAs (A). In addition, specific ceRNA networks of lncRNA-miRNA-mRNA including specific mutually regulated molecules and upregulated and downregulated pathways regulated the expressions and functions of mRNAs and phenotypes in oxidative macrophages (B). n = 3, gray, yellow and pink mean lncRNA, miRNAs and mRNAs, respectively. In detail, the specific 7, 1 and 8 downregulated, and 5, 4 and 8 upregulated lncRNAs, miRNAs and mRNAs were listed, respectively (C). And the regulatory direction and relationship among them were also showed. Blue, red and green mean lncRNA, miRNAs and mRNAs, respectively. Abbreviations are in Figure 4. ceRNA, competing endogenous RNA.*

rosis involve endothelial cell damage, recruitment and adhesion of monocytes at the initial stage, and macrophage differentiation and phagocytosis at both early and advanced stages.[8,9] The effects of macrophages in atherosclerosis have been studied extensively, but few studies have examined the regulatory effects of monocytes at the initial phase. Except for recruitment to the injury site and differentiation into macrophages, it is unknown whether monocytes are directly involved in cholesterol uptake and efflux, the formation of foam cells, and the secretion of inflammatory factors. In this study, we aimed to investigate the function of monocytes at the initial phase, and provide a theoretical basis for the identification of new targets for the treatment and prevention of atherosclerosis.

The most important role of macrophages differentiated from monocytes is to phagocytize accumulated modified lipoprotein to form foam cells.[10,11] However, it

has not been clearly shown whether monocytes can directly phagocytize lipoproteins to clear oxidized lipids before differentiating into macrophages. On this basis, we used a moderate concentration of ox-LDL to stimulate THP-1 monocytes and THP-1-derived macrophages for a short 24-hour period. We found that monocytes presented as cell suspensions even after ox-LDL stimulation and could not directly phagocytize modified lipoprotein to form foam cells that could be stained with oil red O and as demonstrated by the TC and TG levels, while the results from ox-LDL-treated macrophages were consistent with previous studies. Thus, monocytes had a much weaker ability to form foam cells compared to monocyte-derived macrophages.

Essentially, the phenomenon of excessive ox-LDL accumulation in macrophages reflects the instability of lipid metabolism. Therefore, we detected the expressions of lipid metabolism-related molecules involved in cholesterol uptake (CD36, SR-A, and LOX-1) and efflux (ABCA1, ABCG1, and LXRα) in ox-LDL-induced THP-1 monocytes and macrophages.[8,10] The results showed that compared to the monocyte control group, there were no significant differences in the expressions of cholesterol uptake- and efflux-related molecules in the ox-LDL-induced group. Furthermore, the expression levels in the monocyte groups were considerably lower than those in the macrophage groups, indicating that monocytes did not directly participate in cholesterol metabolism abnormalities at the early stage of atherosclerosis before their differentiation into macrophages in vitro.

In addition, atherosclerosis is also a persistent chronic inflammatory state.[12] Previous studies have shown that monocytes could participate in the inflammatory response only after they differentiated into macrophages upon stimulation. However, few studies have shown their secretory ability at the undifferentiated stage of early injury. Therefore, we detected the levels of inflammatory factors in the monocyte and macrophage groups. The results showed that monocytes secreted very low levels of TNF-α and IL-6 even after ox-LDL stimulation, whereas macrophages secreted increased levels of inflammatory factors after oxidative damage. In addition, the level of IL-10 was also increased in ox-LDL-stimulated macrophages because of the moderate concentration of ox-LDL used in this study. We used a moderate concentration of ox-LDL stimulation to mimic the early to middle stages of atherosclerosis rather than the late-stage atherosclerotic plaque microenvironment with severe inflammation. When macrophages were stimulated with a moderate concentration of ox-LDL (50 μg/ml), they engulfed modified lipoprotein to remove excess oxidized lipids in the environment. These macrophages with phagocytic and anti-inflammatory functions were mainly polarized to alternatively activated phenotypes, which can explain the increased secretion of IL-10 in this situation.

Our results showed that monocytes did not have the ability to form foam cells, regulate lipid metabolism and secrete inflammatory factors directly before they differentiated into macrophages at the early stage of atherosclerosis at the cellular level. However, recent studies have suggested that the immunometabolism of macrophages and monocytes, as well as cellular changes and intermolecular interactions in the local microenvironment, might affect the progression of atherosclerosis.[4,13] Specifically, noncoding RNAs, including lncRNAs and miRNAs, are important regulatory molecules that can directly (via lncRNA-bound or miRNA-bound) or indirectly (via lncRNA-bound and retroregulated miRNA) regulate gene expression, affect epigenetic modifications and then affect the regulatory mechanisms of many diseases, including cancers, immunological diseases and cardiovascular diseases, but their effects on atherosclerosis need to be researched further.[14-17] At present, treatments of atherosclerosis mainly involve the reduction of plasma LDL-C concentration by inhibiting relevant targets of cholesterol uptake receptors and suppression of inflammation through drugs.[18-20] Research on noncoding RNA-mediated regulatory mechanisms and cellular immunometabolic changes in early atherosclerotic plaques is still in its infancy. Therefore, this study compared the differentially expressed genes, noncoding RNAs and regulatory mechanisms between macrophage control and induced groups to further explore the mechanism underlying atherosclerosis at the early stage, which can provide theoretical support for new targets for the early intervention of atherosclerosis.

We performed transcriptome sequencing to further explore the regulatory mechanisms of atherosclerosis. The results showed that ox-LDL-induced macrophages had hundreds of differentially expressed genes, miRNAs,

lncRNAs and pathways compared to the control group, which included upregulated and downregulated genes. Specifically, we identified 9 significantly upregulated genes and 6 downregulated genes. When analyzed in the GO functional and KEGG pathway databases, most of these genes were involved in regulatory mechanisms and pathways, including many metabolic processes, ABC transporters, and calcium and transforming growth factor beta signaling pathways. We identified 10 differentially expressed biological processes and 10 pathways that may be involved in the regulation of lipid metabolism directly or indirectly, indicating the close association of these molecules to the progression of atherosclerosis.[21] To further verify whether monocytes also had similar effects and were involved in regulatory mechanisms, we investigated the expressions of the FADS2 and APOA1 genes in the PPAR signaling pathway, which may be correlated with lipid metabolism. The expression trend in induced macrophages was the same as the sequencing results, while the expression of the two genes was not significantly changed in the ox-LDL-induced monocyte group.

Further, according to previous studies and our results, the mechanism of noncoding RNAs involved in regulating the expressions and functions of mRNAs may be a direct targeting effect or ceRNA regulatory networks, which could influence transcription, post-transcription and epigenetic modification.[1,3] The ceRNA network is a kind of regulatory mechanism between lncRNAs and miRNAs, miRNAs and mRNAs. LncRNA specifically binds to the complementary region of miRNA and inhibits the expression of miRNA. This then influences the effects of miRNA on binding to the 3′ UTR region of mRNA, suppressing the translation and promoting the degradation of mRNA, finally affecting the expressions and functions of miRNA and mRNA.[6,22,23] We found 24 such specific ceRNA pathways of noncoding RNAs to regulate the expressions of mRNAs. We further analyzed and predicted the results of sequencing in early oxidative macrophages, and found that lncRNAs may be involved in regulating foam cell formation, lipid metabolism or inflammatory response at the early stage by directly targeting mRNA or through a ceRNA network mechanism or by targeting miRNA. Further in vivo studies are needed to elucidate the specific mechanisms.

CONCLUSIONS

In conclusion, this study indicated that monocytes might not directly participate in cholesterol metabolism during the early stage of ox-LDL induction or uptake of modified lipoprotein to form foam cells, secrete inflammatory factors or affect metabolism-related mechanisms and gene expressions before they differentiate into macrophages. Macrophages stimulated with moderate concentrations of ox-LDL had significantly differentially expressed genes, noncoding RNAs and pathways. On this basis, the ceRNA network of lncRNA-miRNA-mRNA might play an important role in atherosclerosis. Further in vivo and in vitro studies are needed to investigate the specific regulatory mechanisms and molecular interactions underlying these differences.

ACKNOWLEDGMENTS

None.

CONFLICTS OF INTEREST

All the authors declare no conflict of interest.

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ETHICS APPROVAL

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Chinese PLA General Hospital.

CONSENT FOR PUBLICATION

All authors give the full consent to publish the present article.

DATA AVAILABILITY

Data and study materials are available.

AUTHORS' CONTRIBUTION STATEMENTS

Liyuan Jin designed the basic experiment, performed the experiment, collected and analyzed the data and wrote the initial manuscript; Zihui Deng, Yongyi Bai and Ping Ye designed the basic experiment and revised the article. All authors read and approved the final manuscript.

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