

Integrated analysis of long noncoding RNA and mRNA profiling ox-LDL-induced endothelial dysfunction after atorvastatin administration

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Abstract

Background: Long noncoding RNAs (lncRNAs) play a key role in the development of endothelial dysfunction. However, few lncRNAs associated with endothelial dysfunction after atorvastatin administration have been reported.

Methods: In the present study, differentially expressed (DE) genes in ox-LDL versus control and ox-LDL+atorvastatin versus control were detected. Bioinformatics analysis and integrated analysis of mRNAs and lncRNAs were conducted to study the mechanisms of endothelial dysfunction after atorvastatin administration and to explore the regulation functions of lncRNAs.

Results: Here, 532 DE mRNAs and 532 DE lncRNAs were identified (among them, 195 mRNAs and 298 lncRNAs were upregulated, 337 mRNAs and 234 lncRNAs were downregulated) after ox-LDL treatment for 24 hours (fold change ≥ 2.0 , $P < .05$). After ox-LDL treatment following atorvastatin administration, 750 DE mRNAs and 502 DE lncRNAs were identified (among them, 149 mRNAs and 218 lncRNAs were upregulated and 601 mRNAs and 284 lncRNAs were downregulated). After atorvastatin administration, 167 lncRNAs and 262 mRNAs were still DE. Q-PCR validated the results of microarrays.

Conclusion: Chronic inflammatory response, nitric oxide biosynthetic process, microtubule cytoskeleton, cell proliferation and cell migration are regulated by lncRNAs, which also participated in the mainly molecular function and biological processes underlying endothelial dysfunction. Atorvastatin partly improved endothelial dysfunction, but the aspects beyond recovery were mainly concentrated in cell cycle, mitosis, and metabolism. Further exploration is required to explicit the mechanism by which lncRNAs participate in endothelial dysfunction.

Abbreviations: BP = biological processes, CC = cellular component, DE = differentially expressed, ECGS = endothelial cell growth supplement, ECM = endothelial cell medium, FBS = fetal bovine serum, GO = Gene Ontology, HAECs = human aortic endothelial cells, lncRNAs = long noncoding RNAs, MF = molecular function, ox-LDL = oxidized low density lipoprotein, P/S = penicillin/streptomycin solution.

Keywords: atorvastatin, endothelial dysfunction, lncRNA, mRNA, ox-LDL

1. Introduction

Endothelial dysfunction is one of the first recognizable signs of the development of atherosclerosis and plays a key role in vascular remodeling, including endothelial cell apoptosis and changes in endothelial-derived factors.^[1–3] The main mechanism of endothelial dysfunction is the imbalance between vasodilating

and vasoconstricting compounds.^[4,5] As a representative indicator of endothelial dysfunction, oxidized low density lipoprotein (ox-LDL) reduces endothelial-dependent vasodilation by inhibiting NO synthesis and bioavailability.^[6,7] To explore the progressive development of endothelial injury and develop more effective preventions and treatments for atherosclerosis, we studied the molecular mechanism of endothelial dysfunction induced by ox-LDL.

Atorvastatin is considered one of the most effective drugs for treating hyperlipidemia and preventing cardiovascular disease. In addition to its lipid-lowering effect, atorvastatin improves the function of endothelial cells by reducing oxidative stress and endothelial inflammation.^[8,9] However, endothelial dysfunction is not completely cured after atorvastatin administration.^[10–12] Additionally, the adverse events of atorvastatin, such as insulin resistance and musculoskeletal problems, have received much attention. The endothelial status after atorvastatin administration thus deserves attention.

Long noncoding RNAs (lncRNAs) are considered the emerging stars in gene regulation, epigenetics, and human disease.^[13] Several studies have reported that lncRNAs are involved in atherosclerotic processes by causing vascular injury and remodeling and angiogenesis.^[14] However, few lncRNAs associated with endothelial dysfunction after atorvastatin administration have been reported.

To study the regulation of lncRNAs in endothelial function after atorvastatin administration, we performed an integrated

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analysis of transcriptome and lncRNA microarray data to profile the global long noncoding RNA of endothelial dysfunction caused by ox-LDL following atorvastatin administration. We hope to provide new insights to help protect the vascular endothelium in atherosclerosis.

2. Materials and methods

2.1. Cell culture

Human aortic endothelial cells (HAECs, ScienCell, Cat. #6100, San Diego, CA) were cultured with endothelial cell medium (ECM, ScienCell, Cat. #100) and supplemented with 5% fetal bovine serum (FBS, ScienCell, Cat. #0025), 1% endothelial cell growth supplement (ECGS, ScienCell, Cat. #1052), and 1% penicillin/streptomycin solution (P/S, ScienCell, Cat. #0503) at 37°C in a humidified 5% CO₂ incubator. HAECs were cultured in fibronectin-precoated 6-well plates at a density of 7000 cells/cm² for 24 hours in the presence of ox-LDL (80 µg/mL, Union-Biology Co., Ltd, Beijing, China) or ox-LDL (80 µg/mL) following atorvastatin (0.05 µmol/L, Jialin Pharma Ltd, Beijing, China). Untreated HAECs were used as a negative control. Cells were serum-starved overnight before treatment. All samples were assayed in triplicate.

2.2. RNA extraction and purification

Total RNA was extracted using TRIZOL Reagent (Life Technologies, Cat. #15596-018, Carlsbad, CA) following the manufacturer's instructions. RNA integrity was verified using a NanoDrop ND-20003.2 Spectrophotometer and Agilent Bio-analyzer 2100 (Agilent Technologies, Santa Clara, CA). Total RNA was further purified by the RNeasy mini kit (QIAGEN, Cat. #74106, GmbH, Dusseldorf, Germany) and the RNase-Free DNase Set (QIAGEN, Cat. #79254).

2.3. RNA amplification and labeling

Total RNA was amplified and labeled by the Low Input Quick Amp WT Labeling Kit (Agilent Technologies, Cat. #5190-2943), following the manufacturer's instructions. Labeled cRNAs were purified by the RNeasy mini kit (QIAGEN, Cat. #74106).

2.4. Microarray hybridization

Each slide was hybridized with 1.65 µg of Cy3-labeled cDNA using the Gene Expression Hybridization Kit (Agilent Technologies, Cat. #5188-5242) in a hybridization oven (Agilent Technologies, Cat. #G2545A), according to the manufacturer's instructions. After 17 hours of hybridization, slides were washed in staining dishes (Thermo Shandon, Cat. #121, Waltham, MA) with Gene Expression Wash Buffer Kit (Agilent Technologies, Cat. #5188-5327), following the manufacturer's instructions.

2.5. Microarray scan

Slides were scanned by an Agilent Microarray Scanner (Agilent Technologies, Cat. #G2565CA) with the default settings: dye channel: green, scan resolution = 3 µm, PMT 100%, and 20 bits. Data were extracted with Feature Extraction software 10.7 (Agilent Technologies). Raw data were normalized by the Quantile algorithm in the limma packages in R. LncRNA and mRNA microarray assays were performed by the Biotechnology Corporation (Shanghai, China).

2.6. Selection of differentially expressed genes

Differentially expressed (DE) genes were selected by fold-change (fold change (linear) ≤ 0.5 or fold change (linear) ≥ 2) and Student *t* test (*P*-value < .05 or < .01) after normalization of raw data. A heatmap was used to display the gene expression levels in different samples by clustering the samples and genes. Gene Ontology (GO) adapted Fisher test, the cluster profiler packages in R/Bioconductor, and KEGG pathway analysis were used to analyze the functions of the genes in the pathway.

2.7. LncRNA target prediction (cis, trans)

Cis: the genes located approximately 10 kb either upstream or downstream of the lncRNA were considered possible target genes; *Trans*: the complementary sequences were aligned in Blast, the complementary energy between two sequences was calculated with RNA-plex, and then the genes with *e* ≤ -30 were considered possible target genes.

2.8. Quantitative real-time PCR (q-PCR)

To validate the results of the microarrays, 16 DE genes related to endothelial dysfunction were chosen for q-PCR, including 8 lncRNAs (lnc-C17orf81-4:4, lnc-TK1-2:1, lnc-KIAA0101-1:3, lnc-RP1-32I10.10.1-2:1, lnc-KLHL10-2:1, lnc-PKLR-1:1, lnc-LCNL1-2:1, and NONHSAT061051) and their corresponding target mRNAs (ACADVL, BIRC5, KIAA0101, KIAA1644, ACLY, FDPS, PTGDS, and ICAM1), which were related to endothelial dysfunction. Total RNA was isolated and q-PCR for lncRNAs and mRNAs was performed according to standard protocols. Complementary DNA was synthesized using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Cat. #04379012001, Germany), and q-PCR was performed by using LightCycler 480 SYBR Green I Master (Roche, Cat. #04707516001), according to the manufacturer's instructions, using a LightCycler480 instrument (Roche). The primer sequences are listed in Table 1 (Sangon Biotech, Shanghai, China). The data were calculated and presented by the 2^{-ΔΔCt} method.

3. Results

3.1. DE lncRNAs

The DE lncRNAs are shown in a heatmap (Fig. 1A and B). In total, 532 DE lncRNAs were identified (298 were upregulated,

Table 1
Primer sequences.

LncRNA or mRNA	Forward primer (5'-3')	Reverse primer (5'-3')
lnc-C17orf81-4:4	TGGACCTTAGCCAGACCCAACC	GCTCTGATTATCCCTTCCCTTACCG
lnc-TK1-2:1	AAACAGTAGAGGAGCCAGGGA	TGAGCTGCAGGTTCCCTTATCT
lnc-KIAA0101-1:3	AACAGTGCCCTACAGCCTACA	AGAATACCAGGGTAACAAGG
lnc-RP1-32I10.10.1-2:1	CACCGGAACACTCTTCAGCTA	ACTTCATCTTTGGAGACTTTACTTT
lnc-KLHL10-2:1	AGTAGGGTTCTGGCTCTGTT	GGCTGTATCGTCATCCGTGGG
lnc-PKLR-1:1	CCTCAGTCAGCACCCCTAACGA	CAGAATGAAGGAGACCCAGAA
lnc-LCNL1-2:1	CCCCAGGGCTGAGTTAAAGGA	TGAATGAATGGAGTGGCTCACCTAT
NONHSAT061051	GGCAGAAATGTATGTGGGTGG	GATCAAATGGGGCTGGGACTT
ACADVL	CCTTAGCCAGACCCAACCGA	CCTTTGAACATTTCCACAGCA
IFI27	CGAGGGTACTGGGAAACAGAG	AGGGTGGACAGAGTGGGACAT
BIRC5	AGCCAGTGCCAGTTTATTT	ACTTCTCCGCGATTTCCCTCA
KIAA0101	AACAGTTGATACTGGATGGC	TACCAGGGTAACAAGGAGAC
KIAA1644	TACCCTGTTGAATGACCCTAA	AAACTGCGTATGAGAAACTGAC
ACLY	CCCTTCCCTTAGCCTCTTCTC	AGTAGGTTCTCTGGCTCTGTT
FDPS	TCATTGAACAGTACCGACAC	AGGTCACCTTCTCCGCTTGTA
PTGDS	GCAGTCCCTCATCCTAGCCTTTCG	GAGTGGCCAGGGTTCCTTTGG
ICAM1	CCATGAGGACATACAACCTGGGAAAT	GCTGGCATCCGTGAGGAAGTG

LncRNAs = long noncoding RNAs.

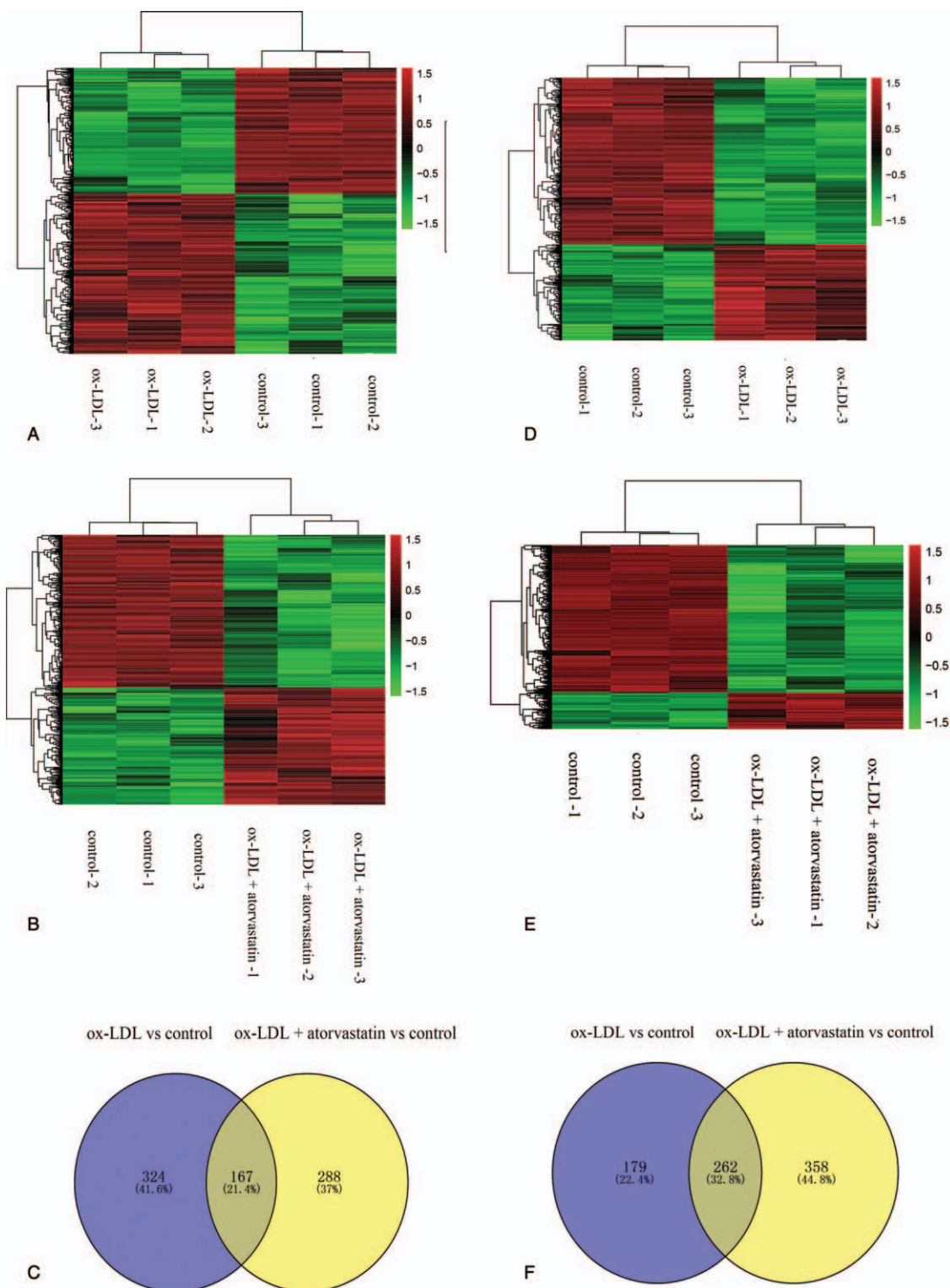


Figure 1. DE lncRNAs. (A) Heatmap of ox-LDL group vs control group. (B) Heatmap of ox-LDL + atorvastatin group vs control group. Black represents 0, and indicates no difference between groups. Red stands for high expression and green represents low expression. (C) Gene number of ox-LDL vs control and ox-LDL + atorvastatin vs control was shown in Venn diagram. DE mRNAs. (D) Heatmap of ox-LDL group vs control group. (E) Heatmap of ox-LDL + atorvastatin group vs control group. (F) Gene numbers of ox-LDL vs control and ox-LDL + atorvastatin vs control are shown in a Venn diagram.

and 234 were downregulated) after 24 hours of ox-LDL exposure (fold change ≥ 2.0 , $P < .05$). After ox-LDL treatment following atorvastatin administration, 502 DE lncRNAs were identified (218 were upregulated and 284 were downregulated). A Venn

diagram showed that 167 lncRNAs existed in both comparisons, which suggested that atorvastatin did not have any effect on these lncRNAs (Fig. 1C). Among them, lnc-TK1-2:1, lnc-KLHL10-2:1, and lnc-PKLR-1:1 were validated by q-PCR. The top 10 up- or

Table 2

The top 10 up/downregulated lncRNAs and mRNAs.

lncRNA/mRNA	P	Fold change	Regulation	lncRNA/mRNA	P	Fold change	Regulation
ENST00000384772	.007888	20.64936	Up (ox-LDL vs Control)	ENST00000516250	5.16E-05	0.222699	Down (ox-LDL vs Control)
NR_026797	.002899	12.781	Up (ox-LDL vs Control)	lnc-C15orf37-2:1	3.8E-05	0.221793	Down (ox-LDL vs Control)
NR_027256	.000375	9.650484	Up (ox-LDL vs Control)	lnc-CCNL1-2:1	6.96E-05	0.21591	Down (ox-LDL vs Control)
ENST00000443123	.034948	9.345414	Up (ox-LDL vs Control)	lnc-BLCAP-5:1	.023629	0.213761	Down (ox-LDL vs Control)
ENST00000421771	.004038	7.110715	Up (ox-LDL vs Control)	lnc-TTC9-3:1	.000131	0.206738	Down (ox-LDL vs Control)
NR_026797	.019822	6.064061	Up (ox-LDL vs Control)	ENST00000580734	3.41E-05	0.203913	Down (ox-LDL vs Control)
ENST00000418415	.010122	5.874603	Up (ox-LDL vs Control)	lnc-DROSHA-9:1	.042251	0.202453	Down (ox-LDL vs Control)
ENST00000445839	.007544	5.543008	Up (ox-LDL vs Control)	lnc-PKLR-1:1	.003418	0.1928	Down (ox-LDL vs Control)
NR_125760	.018167	5.298528	Up (ox-LDL vs Control)	NR_036685	.027342	0.170143	Down (ox-LDL vs Control)
NR_038845	.006423	5.268761	Up (ox-LDL vs Control)	lnc-SUM01-4:1	.005665	0.124476	Down (ox-LDL vs Control)
NR_106759	.000844	9.852135	Up (ox-LDL + atorvastatin vs Control)	lnc-STRN3-12:1	.001909	0.040448	Down (ox-LDL + atorvastatin vs Control)
NR_027256	.003943	5.893429	Up (ox-LDL + atorvastatin vs Control)	lnc-KIAA0101-1:3	.001227	0.065251	Down (ox-LDL + atorvastatin vs Control)
lnc-GOS2-3:2	.005714	5.879277	Up (ox-LDL + atorvastatin vs Control)	lnc-TXNDC3-1:1	.009571	0.13474	Down (ox-LDL + atorvastatin vs Control)
lnc-NRSN1-5:1	.004836	5.255841	Up (ox-LDL + atorvastatin vs Control)	lnc-DROSHA-9:1	.003774	0.140445	Down (ox-LDL + atorvastatin vs Control)
lnc-ITIH4-2:1	.012844	5.111992	Up (ox-LDL + atorvastatin vs Control)	lnc-KDM1A-3:1	.001483	0.148287	Down (ox-LDL + atorvastatin vs Control)
lnc-CADM1-7:1	.007189	4.996836	Up (ox-LDL + atorvastatin vs Control)	lnc-C15orf37-2:1	.007363	0.155802	Down (ox-LDL + atorvastatin vs Control)
lnc-MEGF9-1:9	.00202	4.786497	Up (ox-LDL + atorvastatin vs Control)	lnc-TK1-2:1	.000547	0.166857	Down (ox-LDL + atorvastatin vs Control)
NR_002147	.025902	4.636121	Up (ox-LDL + atorvastatin vs Control)	NONHSAT028897	.002105	0.170971	Down (ox-LDL + atorvastatin vs Control)
ENST00000457813	.033558	4.514217	Up (ox-LDL + atorvastatin vs Control)	NR_036685	.019141	0.177205	Down (ox-LDL + atorvastatin vs Control)
ENST00000455838	.002257	4.433267	Up (ox-LDL + atorvastatin vs Control)	ENST00000578704	.01753	0.180371	Down (ox-LDL + atorvastatin vs Control)
HSPA6	.007981	29.7559	Up (ox-LDL vs Control)	SYTL5	.030195	0.144949	Down (ox-LDL vs Control)
AKR1B10	.021803	18.85684	Up (ox-LDL vs Control)	ACAT2	4.93E-05	0.137569	Down (ox-LDL vs Control)
NROB1	.004348	15.79368	Up (ox-LDL vs Control)	OAS1	.010977	0.121841	Down (ox-LDL vs Control)
AKR1C1	.000365	10.09501	Up (ox-LDL vs Control)	TM7SF2	.001653	0.121095	Down (ox-LDL vs Control)
ABCC3	.011947	10.03225	Up (ox-LDL vs Control)	ANKRD1	.001269	0.104363	Down (ox-LDL vs Control)
AKR1C2	.003664	9.717916	Up (ox-LDL vs Control)	SLC13A5	.004358	0.103209	Down (ox-LDL vs Control)
CSF2	.011379	9.017859	Up (ox-LDL vs Control)	MX2	.008037	0.064098	Down (ox-LDL vs Control)
HTRA3	1.42E-05	8.969243	Up (ox-LDL vs Control)	NPPB	.002594	0.061316	Down (ox-LDL vs Control)
CXCL8	.028565	8.324693	Up (ox-LDL vs Control)	PCSK9	6.31E-06	0.04527	Down (ox-LDL vs Control)
CDK5RAP2	4.92E-05	8.247509	Up (ox-LDL vs Control)	FABP3	1.01E-06	0.03848	Down (ox-LDL vs Control)
ADH1A	.010697	27.78759	Up (ox-LDL + atorvastatin vs Control)	PCSK9	.003316	0.018511	Down (ox-LDL + atorvastatin vs Control)
NROB1	.000554	19.48146	Up (ox-LDL + atorvastatin vs Control)	KIF20A	.001456	0.020963	Down (ox-LDL + atorvastatin vs Control)
ABCC3	.005862	17.96786	Up (ox-LDL + atorvastatin vs Control)	FAM64A	1.47E-05	0.030398	Down (ox-LDL + atorvastatin vs Control)
ENPP2	.000358	13.77148	Up (ox-LDL + atorvastatin vs Control)	CA9	.000663	0.03468	Down (ox-LDL + atorvastatin vs Control)
ADH1C	.017138	13.3708	Up (ox-LDL + atorvastatin vs Control)	FABP3	.003527	0.043354	Down (ox-LDL + atorvastatin vs Control)
CCL11	.0017	13.3499	Up (ox-LDL + atorvastatin vs Control)	SPC25	.000588	0.047384	Down (ox-LDL + atorvastatin vs Control)
AKR1B10	.026925	12.03057	Up (ox-LDL + atorvastatin vs Control)	BIRC5	.001536	0.049487	Down (ox-LDL + atorvastatin vs Control)
HTRA3	.000339	9.790495	Up (ox-LDL + atorvastatin vs Control)	UBE2C	9.84E-05	0.052743	Down (ox-LDL + atorvastatin vs Control)
PLIN4	.004499	7.910962	Up (ox-LDL + atorvastatin vs Control)	HIST1H3G	.001797	0.053107	Down (ox-LDL + atorvastatin vs Control)
CDK5RAP2	.003416	6.797091	Up (ox-LDL + atorvastatin vs Control)	PLK1	4.98E-05	0.054217	Down (ox-LDL + atorvastatin vs Control)

lncRNAs=long noncoding RNAs, ox-LDL=oxidized low density lipoprotein.

downregulated lncRNAs are listed in Table 2. Among them, NR_027256, lnc-C15orf37-2:1, and NR_036685 remained unchanged in both comparisons.

3.2. DE mRNAs

The DE mRNAs are shown in a heatmap (Fig. 1D and E). In total, 532 DE mRNAs were identified (195 were upregulated, and 337 were downregulated) after 24 hours of ox-LDL exposure for (fold change ≥ 2.0 , $P < .05$). After ox-LDL treatment following atorvastatin administration, 750 differential mRNAs were identified (149 were upregulated, and 601 were downregulated). A Venn diagram showed that 262 mRNAs existed in both comparisons, which suggested that atorvastatin did not have any effect on these mRNAs (Fig. 1F); among them, BIRC5, KIAA1644, ACLY, and FDPS were validated by q-PCR. The top 10 up- or downregulated mRNAs are listed in Table 2. Among them, AKR1B10, NROB1, ABCC3, HTRA3, CDK5RAP2, PCSK9, and FABP3 remained unchanged in both comparisons.

3.3. DE lncRNAs chromosomal distribution and subtype analysis

According to chromosomal location, the DE lncRNAs were located in every chromosome, mostly in chromosomes 1, 17, and 2, after 24 hours of ox-LDL exposure (Fig. 2A). After ox-LDL treatment following atorvastatin administration, the top 3 chromosomes in which DE lncRNAs were located were 1, 2, and 17 (Fig. 2B). To identify the relationship between DE lncRNAs and their associated protein-coding genes, DE lncRNAs were classified into 6 groups using subtype analysis, and the results suggested that intergenic lncRNAs were mostly altered in both comparisons (Fig. 2C and D).

3.4. Function analysis

In GO enrichment analysis, GO terms and gene product annotations are widely used to classify different genes and annotate genes into 3 categories: biological process (BP),

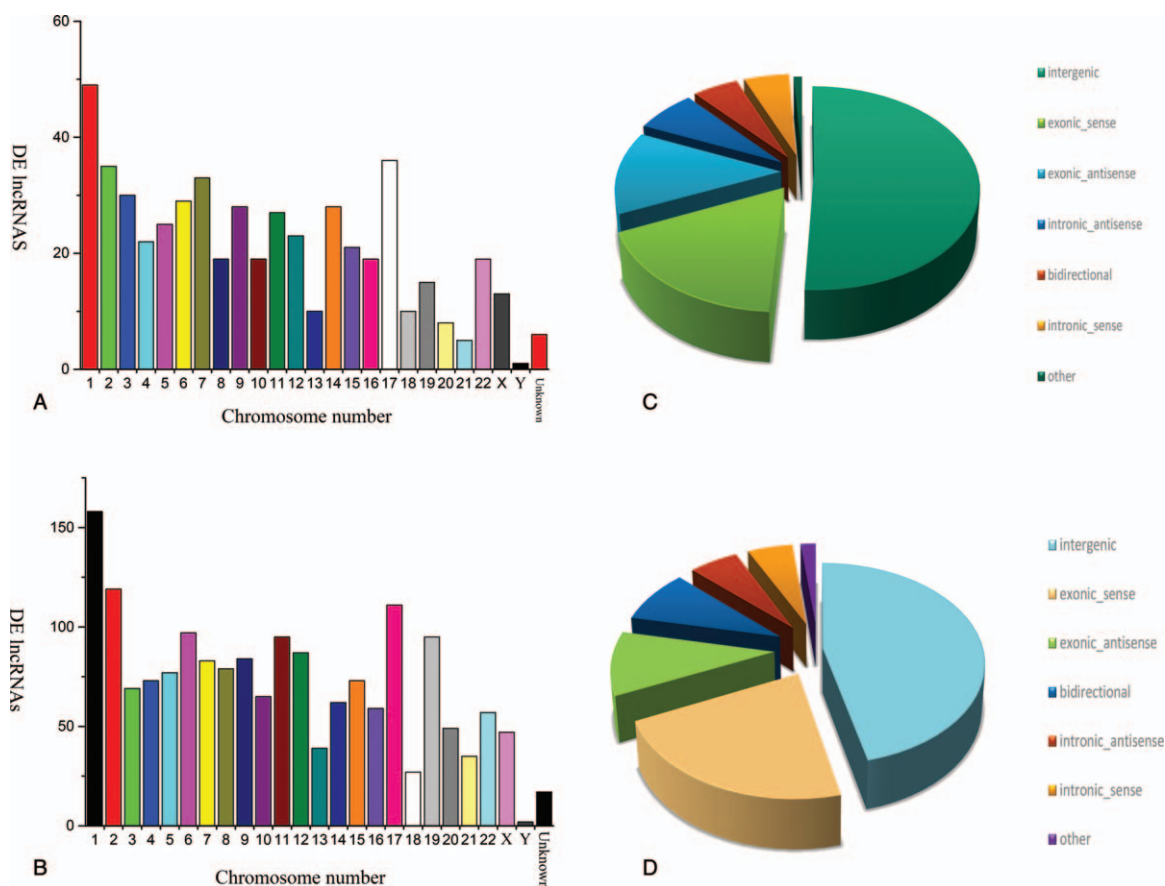


Figure 2. Location and classification of DE lncRNAs. (A) Location of DE lncRNAs in ox-LDL group vs control group in different chromosomes, (B) location of DE lncRNAs in ox-LDL+atorvastatin group vs control group in different chromosomes, (C) pie charts summarized classification of DE lncRNAs in ox-LDL group vs control group, (D) pie charts summarizing classification of DE lncRNAs in ox-LDL+atorvastatin group vs control group.

cellular component (CC), and molecular function (MF). All differential genes were sorted by GO with an associated *P*-value. The top 30 enrichments are listed in Fig. 3A and B. The most enriched GO terms associated with endothelial dysfunction in each class were steroid metabolic process (GO ID: GO:0008202; Type: BP; *P*-value: 2.362e-13), extracellular region (GO ID: GO:0005576; Type: CC; *P*-value: 3.821e-08), and oxidoreductase activity (GO ID: GO: 0016491; Type: MF; *P*-value: 2.474e-08) after ox-LDL treatment for 24 hours. After atorvastatin intervention, the most enriched GO associated with the overlap mRNAs were steroid metabolic process (GO ID: GO: 0008202; Type: BP; *P*-value: 2.832e-16), condensed chromosome, centromeric region (GO ID: GO: 0000779; Type: CC; *P*-value: 1.857e-08), and alditol: NADP⁺ 1-oxidoreductase activity (GO ID: GO:004032; Type: MF; *P*-value: 6.884e-07).

In KEGG enrichment analysis, the pathways were enriched to find significant differences. The top 10 significantly enriched pathways are listed in Fig. 3C and D. The most enriched pathways were steroid biosynthesis (8 genes), terpenoid backbone biosynthesis (7 genes), and PPAR signaling pathway (8 genes), after 24 hours of ox-LDL treatment. After the atorvastatin intervention, the most enriched pathways of the overlap mRNAs were terpenoid backbone biosynthesis (7 genes), steroid biosynthesis (7 genes), and steroid hormone biosynthesis (6 genes).

3.5. lncRNA target prediction

We predicted the lncRNA targets prediction to explore the potential role of lncRNAs. After treatment with ox-LDL for 24 hours, the DE lncRNAs included 1630 *cis* and *trans* target genes. KEGG analysis was used to determine the function of the protein-coding transcripts by *cis* or *trans* prediction. As shown in Fig. 3E, the *cis* and *trans* target genes were mostly enriched in the biosynthesis of unsaturated fatty acids, p53 signaling pathway, terpenoid backbone biosynthesis, glycerolipid metabolism, NOD-like receptor signaling pathway and PPAR signaling pathway. For the DE lncRNAs that overlapped the ox-LDL group versus control group and ox-LDL+atorvastatin group versus control group, Fig. 3F shows that, their *cis* and *trans* target genes were still mostly enriched in the biosynthesis of unsaturated fatty acids, citrate cycle (TCA cycle), glycerolipid metabolism, NOD-like receptor signaling pathway, p53 signaling pathway, and PPAR signaling pathway. These pathways were also consistent with the results of the KEGG analysis of DE mRNAs, which suggests that these biological processes and molecular functions might be regulated by corresponding DE lncRNAs.

3.6. lncRNA-mRNA coexpression network

To determine the lncRNA-mRNA functional interactions, the correlation of DE lncRNAs and DE mRNAs were analyzed to establish a coexpression network. There were 1120 lncRNA-

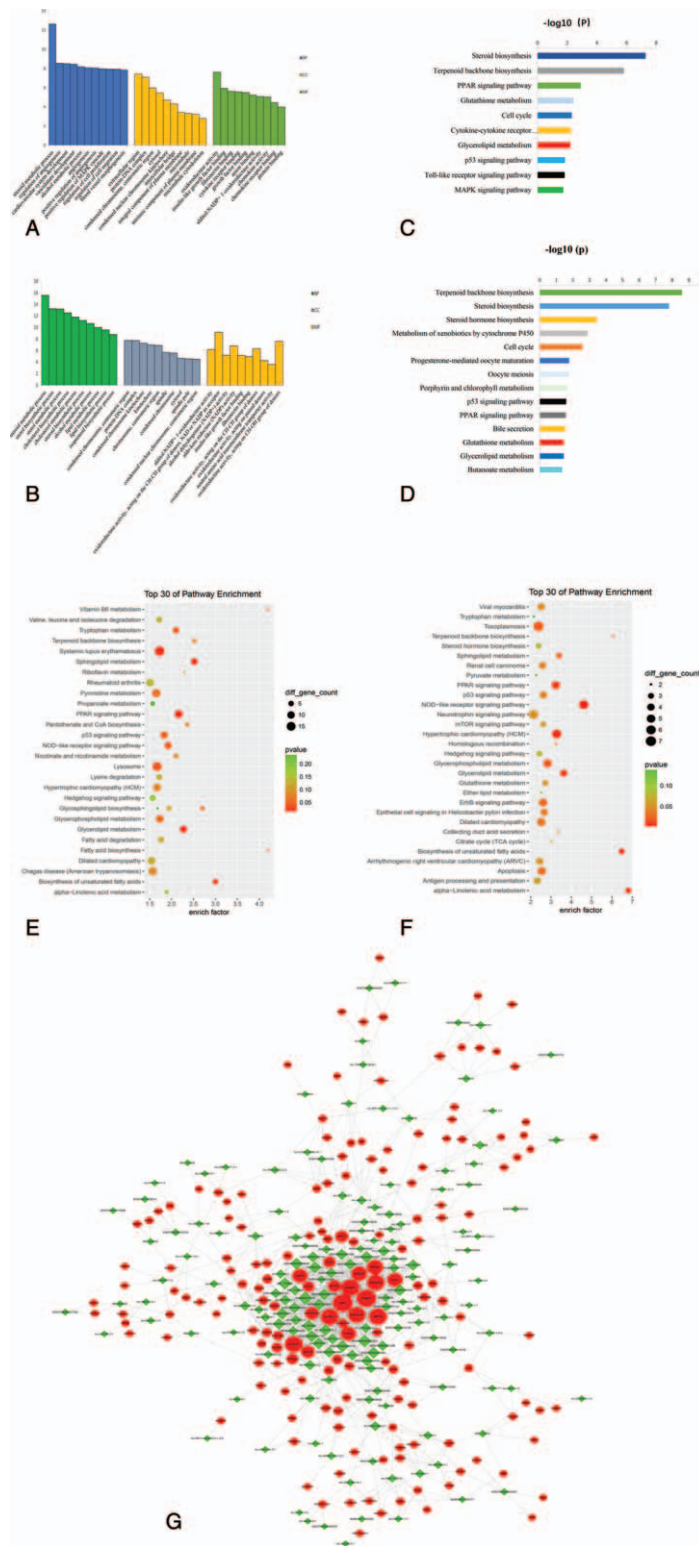


Figure 3. GO and KEGG enrichment analysis of DE mRNAs. (A) GO enrichment of ox-LDL group vs control group; (B) GO enrichment of overlap in ox-LDL group vs control group and ox-LDL + atorvastatin group vs control group. Top 30 in 3 categories: cellular component, biological process, and molecular function, are summarized; x-axis indicates subcategories, and y-axis indicates $-\log_{10}(P)$. (C) KEGG enrichment of ox-LDL group vs control group; (D) KEGG enrichment of overlap of ox-LDL group vs control group and ox-LDL + atorvastatin group vs control group. Top 10 pathways of DE mRNAs are summarized. y-axis indicates functional pathways, and x-axis indicates $-\log_{10}(P)$. KEGG analysis of DE lncRNA target genes. (E) DE lncRNAs of ox-LDL group vs control group. (F) DE lncRNAs of overlap of ox-LDL group vs control group and ox-LDL + atorvastatin group vs control group. (G) lncRNA-mRNA coexpression network of ox-LDL group vs control group.

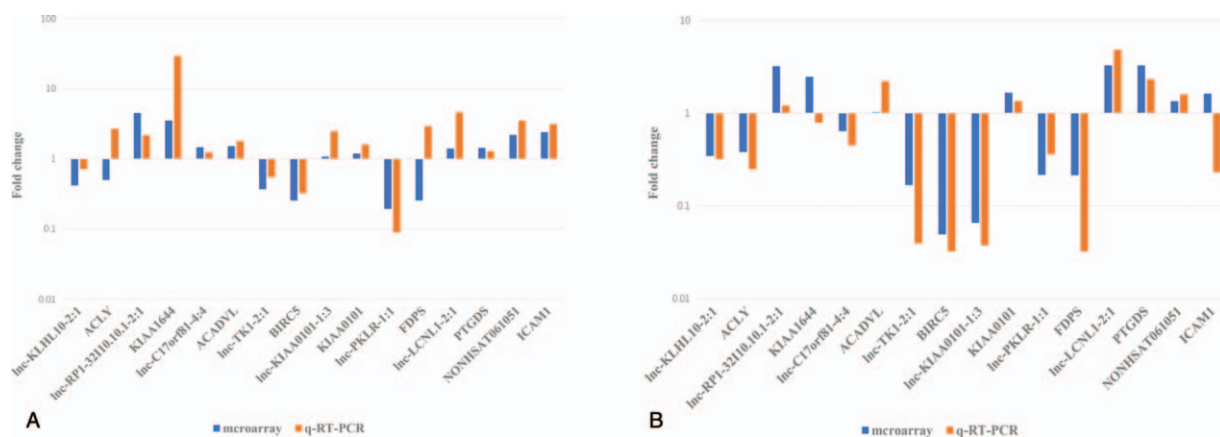


Figure 4. q-PCR validation of DE genes compared to microarrays. (A) ox-LDL group vs control group, (B) ox-LDL + atorvastatin group vs control group.

mRNA pairs in ox-LDL group versus control group, among them, 323 key genes (106 upregulated, 217 downregulated) were detected. As key genes in the network, lnc-TK1-2:1, lnc-PKLR-1:1, BIRC5, KIAA1644, ACLY, and FDPS were validated by q-PCR. The detailed network is shown in Fig. 3G.

3.7. Microarray verification by q-PCR

All the q-PCR results were consistent with the microarrays results except for ACLY and FDPS in the ox-LDL group versus control group and KIAA1644 and ICAM1 in the atorvastatin + ox-LDL group versus control group. Among the 8 DE lncRNAs in the ox-LDL group, 5 lncRNAs were upregulated, and 3 lncRNAs were downregulated; their corresponding mRNAs had a direct correlation in fold-change with the associated lncRNAs (Fig. 4A). In the ox-LDL + atorvastatin versus control group, the finding of 3 upregulated lncRNAs and 5 downregulated lncRNAs was consistent with the microarray results. ACLY, BIRC5, FDPS, and PTGDS demonstrated a positive correlation with the associated lncRNAs (lnc-KLHL10-2:1, lnc-TK1-2:1, lnc-PKLR-1:1, and lnc-LCNL1-2:1), while ACADVL and KIAA0101 demonstrated a negative correlation with the corresponding lncRNAs (lnc-C17orf81-4:4 and lnc-KIAA0101-1:3) (Fig. 4B).

4. Discussion

Endothelial dysfunction has been associated with atherosclerosis, acting as an early leading cause to cardiovascular events.^[15,16] Many studies have shown that ox-LDL precedes the development of endothelial dysfunction. lncRNAs play a regulatory role in important biological processes that contribute to endothelial dysfunction, such as leukocyte adhesion, inflammatory reaction, endothelial cell activation, oxidative stress,^[17] platelet aggregation, and vascular smooth-muscle cell proliferation and migration.^[4,18–21]

Statins are a popular and well-recognized lipid-lowering agent family. Atorvastatin works by inhibiting HMG-CoA reductase, an enzyme found in the liver that plays an integral role in the production of cholesterol.^[22] Many studies have shown that atorvastatin improves endothelial dysfunction by ameliorating oxidative stress and normalizing eNOS/iNOS imbalance.^[23] Atorvastatin activates eNOS to release more NO to improve endothelium-dependent vasodilation. In addition, atorvastatin

has antithrombosis and immunomodulatory functions for improving endothelial dysfunction. However, the endothelial dysfunction is too complex to be reversed by the use of atorvastatin alone. Further, some studies have reported several adverse events about atorvastatin, for example, atorvastatin may worsen glycemic control and insulin resistance in the diabetic patients with endothelial dysfunction^[24]; atorvastatin administration has been associated with the occurrence of musculoskeletal adverse events^[25]; and there is evidence demonstrating an increase in cardiovascular risk in women and young people who use atorvastatin.^[26] However, there is insufficient evidence to support these conclusions. Therefore, the endothelial status after atorvastatin administration requires further study. Although several studies have focused on the role of lncRNAs in endothelial dysfunction induced by ox-LDL, an integrated analysis of long noncoding RNA and mRNA in ox-LDL exposure after atorvastatin administration is missing. Our research may shed light on the pleiotropic study of atorvastatin.

In the present study, we detected DE genes in HAECs after ox-LDL exposure. The main GO enrichment terms were the steroid metabolic process, regulation of angiogenesis, cell proliferation and cell migration, microtubule cytoskeleton, oxidoreductase activity, and chemokine activity. KEGG analysis showed that DE mRNAs may lead to endothelial dysfunction via the steroid biosynthesis, PPAR signaling, cell cycle, and p53 signaling pathways, among others. Simultaneously, *cis* and *trans* prediction suggested that DE lncRNAs were enriched in terpenoid backbone biosynthesis, PPAR signaling pathway, glutathione metabolism glycerolipid metabolism, and p53 signaling pathway. These results indicated that DE mRNAs might be regulated by their corresponding DE lncRNAs.

Many proinflammatory cytokines and cell-surface adhesion molecules participate in endothelial dysfunction to initiate the development of cardiovascular events, and disorders of lipid metabolism (including triglycerides and cholesterol) aggravate inflammatory reactions.^[27] In our microarray results, significant expression changes of both the mRNA and their corresponding lncRNAs related to inflammation (IL1B, IL6, THBS1, PTGES, and CCL11) and cell-surface adhesion molecules (ICAM-1, MPZ, CDH4, and VCAN) were detected. The PPAR signaling pathway was one of the most enriched pathways in the KEGG analysis, relating to inflammation and adhesion.^[28–32] Some DE genes associated with lipid metabolism were also DE, such as FDPS, HMGCS1, SQLE, ACAT2, AKR1C1, CYP1B1, AKR1B1,

and PNPLA3. In HAECs treated with ox-LDL for 24 hours, the expression levels of KLF2 and its related lncRNA, lnc-EP515L1-2:1, were both decreased.^[33,34] In the GO analysis, NQO1 and GLA were enriched in nitric oxide biosynthetic process and oxidative stress,^[35] and their corresponding regulators (lnc-PIP5K1C-1:1, NONHSAT061051, lnc-KIAA0240-1:3, and NR_104312) were DE. PPAR α ligands influence the antioxidant enzyme Cu, Zn-superoxide dismutase, and NO generation^[33,36] and therefore play a crucial role in the development of atherosclerosis. In the present study, CPT1A, SCD, and FABP3 participated in the PPAR pathway with degrees of 2, 6, and 41, respectively, in the correlation network. Our results for endothelial dysfunction induced by ox-LDL were consistent with those of Zhang et al.^[37] and Singh et al.^[38]

After atorvastatin administration, endothelial dysfunction was partly improved. There were 324 mRNAs and 179 lncRNAs normalized, although atorvastatin did not affect 167 lncRNAs and 262 mRNAs. The functions of these 262 mRNAs were primarily focused on cell cycle, mitosis, and metabolism. Among them, BIRC5, PLK1, PRC1, KIF20A, CDC25, KIFC, and TACC3 were enriched in the cell cycle and mitotic pathway. A decrease in these genes may lead to dysfunction of the cell cycle, mitosis and microtubule motor inactivity and abnormal microtubule function. Microtubule function and junctional proteins are key determinants of the stability of the endothelial cell structure and regulation of endothelial barrier function.^[1,39] Microtubule dysfunction often lead to changes in cell shape, mitosis, intracellular transport, adhesion, and migration.^[40] In the present study, Kinesin family members, BIRC5, PLK1, PRC1 and KIF20A, CDC25, KIFC, TACC3 were involved in microtubule function. The metabolism of substances and energy is another point: ACLY participated in the TCA cycle; FDPS in terpenoid backbone biosynthesis and metabolism; ADH1A in oxidoreductase activity and alcohol dehydrogenase (NAD) activity; ACAT2 in metabolism and fatty acid metabolism; AKR1C1 in oxidoreductase activity and acting on NAD(P)H; and HMGCS1 in terpenoid backbone biosynthesis and metabolism. These results indicated that atorvastatin did not completely correct the metabolic abnormality. The KEGG analysis of the DE mRNAs was consistent with the analysis of the *cis* and *trans* target genes. Additionally, correlation network analysis showed that the DE mRNAs were coexpressed with their corresponding lncRNAs. From these results, we inferred that after atorvastatin administration, DE mRNAs were mainly regulated by lncRNAs. However, the mechanisms by which lncRNA regulates mRNA in endothelial dysfunction need further study. Moreover, independent of ox-LDL, atorvastatin induced changes in 288 lncRNAs and 358 mRNAs, which suggest that atorvastatin may have vascular benefits other than LDL-C reduction, and our research may provide new insight into the pleiotropic study of atorvastatin. However, more rigorous experiments are required for further study.

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