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lincRNA-p21 inhibits foam cell formation and regulates cholesterol uptake and efflux in macrophages through the PKCδ/Akt/Erk and PPARγ/LXRα pathways

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Abstract. Impairment in cholesterol uptake and efflux is the main reason contributing to foam cell formation, which is a marker and key step in the atherosclerotic process. Overexpression of long intergenic non-coding RNA-p21 (lincRNA-p21) was reported to alleviate the development of atherosclerosis. However, whether lincRNA-p21 exerts an antiatherogenic function by alleviating lipid metabolism dysfunction and foam cell formation remains unknown. In our study, human THP-1 monocytes were stimulated for 48 h by phorbol 12-myristate-13-acetate (PMA) to differentiate into macrophages, and THP-1-derived macrophages were further treated for 24 h with oxidized low-density lipoprotein (ox-LDL) to differentiate into foam cells. THP-1 macrophage-derived foam cells were transfected with si-NC/si-lincRNA-p21 or LV-NC/LV-lincRNA-p21 for 48 h to knock down or overexpress lincRNA-p21. lincRNA-p21 expression was reduced in THP-1-derived macrophages after ox-LDL treatment. lincRNA-p21 knockdown promoted lipid uptake and accumulation and inhibited cholesterol efflux in ox-LDL-treated THP-1 macrophages, while lincRNA-p21 overexpression exerted an opposite effect on cholesterol influx and efflux. Importantly, lincRNA-p21 attenuated cholesterol influx by suppressing the PKCδ/Akt/Erk/SR-A/CD36 pathway and enhanced cholesterol efflux by promoting the PPARγ/LXRα/ABCA1/ABCG1 pathway in foam cells derived from THP-1 macrophages.

Key words: lincRNA-p21 — Atherosclerosis — Lipid uptake — Cholesterol efflux — PKCδ — PPARγ

Introduction

Atherosclerosis is a chronic inflammatory vascular disease that is pathologically characterized by blood vessel narrowing and obstruction due to abnormal lipid accumulation in the inner artery wall, being the major pathological basis for serious cardiovascular and cerebrovascular diseases (Markin et al. 2023). In the early stages of atherosclerosis development, monocytes migrate to the endothelium to differentiate into macrophages that are capable of phagocytizing lipids (Moore et al. 2013). The imbalance between lipid efflux and influx is the pivotal reason contributing to the transformation of macrophages into foam cells in the subendothelial space (Achmad et al. 2023). Lipid-rich foam cells are a major component of atherosclerotic plaques and their formation is considered a hallmark and key step in the pathogenesis of atherosclerosis (Galindo et al. 2023). Various genetic and environmental factors disrupt cholesterol homeostasis by inhibiting cholesterol efflux or enhancing its uptake, thereby leading to cholesterol accumulation in arterial macrophages (Wang et al. 2017). Low-density lipoprotein (LDL) is a type of lipoprotein particle that carries cholesterol into peripheral tissue cells, which is readily converted to oxidized LDL (ox-

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LDL) by myeloperoxidase, lipoxygenase, or reactive oxygen species oxidative modification (Khatana et al. 2020). Ox-LDL can only be recognized and phagocytosed by macrophage scavenger receptors (SRs) (Khatana et al. 2020). Macrophages are transformed into foam cells when ox-LDL intake exceeds the capacity of macrophage metabolism, which secrete pro-inflammatory cytokines and chemokines that accelerate the progression of atherosclerosis (Chistiakov et al. 2017). Thus, preventing lipid accumulation in macrophages and foam cell formation by maintaining the homeostasis of cholesterol uptake and efflux reveals a novel therapeutic option for atherogenesis (Maguire et al. 2019).

Macrophages take up ox-LDL through class A type I/II scavenger receptor (SR-A) and cluster of differentiation 36 (CD36) whereas excrete cholesterol through ABC transporters ATP-binding cassette transporter A1 (ABCA1) and G1 (ABCG1) (Govatati et al. 2024). The presence of SR-A and CD36 on macrophages leads to unrestricted cellular uptake of ox-LDL, thus resulting in excess cholesterol accumulation and initiating foam cell formation (Liu et al. 2024). Among classical and novel protein kinase C (PKC) isoforms, PKCδ plays a crucial role in ox-LDL uptake by macrophages and foam cell formation (Szilagyi et al. 2014). PKCδ exerts atheroprotective effects on atherosclerosis by downregulating the expression of SR-A and CD36 (Lin et al. 2012). Both Akt and ERK pathways have been identified as PKCδ-associated downstream signalings that modulate the expression of SRs in primary macrophages and THP-1 macrophages (Lin et al. 2012). Moreover, PKCδ and its downstream molecules (Akt, ERK, and SR-A) are activated or overexpressed in human atherosclerotic tissues compared with normal arterial tissues (Lin et al. 2012). ABCA1 and ABCG1 are transmembrane lipid transport proteins on the membrane surface of macrophages, which are responsible for regulating cholesterol translocation across cellular bilayer membranes, thereby playing an essential role in intracellular cholesterol efflux (Matsuo 2022). Proliferator-activated receptor gamma (PPARγ) and liver X receptor alpha (LXRα) are transcription factors that directly regulate ABCA1 and ABCG1 expression, respectively (Zheng et al. 2021). The PPARγ/LXRα pathway activation in macrophages was reported to alleviate atherosclerosis development by promoting cholesterol efflux and preventing foam cell formation (Jiang et al. 2017).

Long noncoding RNAs (lncRNAs) are RNA molecules that lack protein-coding potential but play important regulatory roles in various biological processes (Gluba-Sagr et al. 2024). lncRNAs make up the largest part of ncRNA and are generally longer than 200 nucleotides (Gluba-Sagr et al. 2024). Multiple lncRNAs are abnormally expressed in different cell types in atherosclerotic lesions, such as macrophages, vascular smooth muscle cells, and endothelial cells, and dysregulated lncRNAs play vital roles in several atherogenic processes, such as inflammatory responses, lipid deposition, and endothelial dysfunction as well as cell types (Ma et al. 2023). For example, lncRNA HOTAIR is downregulated in human macrophage-derived foam cells and the plasma of atherosclerosis patients, and overexpressing HOTAIR effectively reduces lipid uptake, mitigates inflammatory response, and suppresses foam cell formation by decreasing miR-19a-3p expression (Chen et al. 2024). The expression of lncRNA MALAT1 is markedly decreased in atherosclerosis patients and ox-LDL-stimulated THP-1 macrophages, and MALAT1 knockdown promotes cholesterol accumulation in ox-LDL-stimulated macrophages through the miR-17-5p/ABCA1 axis (Liu et al. 2020). Silencing of lncRNA HOXA11-AS not only ameliorates atherosclerosis in mouse models but also alleviates ox-LDL-induced endothelial injury, apoptosis, and oxidative stress *in vitro* by mediating the miR-515-5p/ROCK1 axis (Gao et al. 2022). Long intergenic non-coding RNA-p21 (lincRNA-p21) expression was found to be significantly decreased in coronary artery plaques compared with internal mammary artery without plaque formation (Çekin et al. 2018). The role of lincRNA-p21 in atherosclerosis development has been discussed in diverse types of cells. For example, lincRNA-p21 regulates cell proliferation and apoptosis during atherosclerosis, and its expression is prominently decreased in atherosclerotic plaques of $ApoE^{-/-}$ mice. lincRNA-p21 knockdown promotes proliferation but represses apoptosis of mouse mononuclear macrophage cells and human vascular smooth muscle *in vitro* by attenuating p53 activity (Wu et al. 2014b). However, whether lincRNA-p21 regulates lipid metabolism and foam cell formation during atherosclerosis development remains largely unknown.

In this research, THP-1 macrophages were treated with ox-LDL to construct the macrophage foaming model. lincRNA-p21 expression in foam cells was examined, after which the influence of lincRNA-p21 knockdown and overexpression on cholesterol influx and efflux in foam cells was assessed. Mechanistically, PKCδ and PPARγ were further knocked down in macrophage-derived foam cells after lincRNA-p21 downregulation to confirm the underlying mechanism through which lincRNA-p21 regulates cholesterol accumulation.

Materials and Methods

Cell culture

Human THP-1 monocytes provided by Procell (#CL-0233; Wuhan, China) were incubated in RPMI-1640 medium (#PM150110B; Procell) added with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS) in a humidified atmosphere (37°C, 5% CO_2). THP-1 monocytes were stimulated for 48 h by phorbol 1-myristate-13-acetate (PMA; 160

ng/ml; #LZR5414; Rayzbio, Shanghai, China) to differentiate into macrophages and THP-1-derived macrophages were further exposed for 24 h to ox-LDL (50 μg/ml; #H5009; Warbio, Nanjing, China) to differentiate into foam cells (Zou et al. 2022).

Cell transfection

For cell transfection, THP-1 macrophages after 24 h treatment with ox-LDL were seeded (2×10^6 cells/well) in 24-well plates. For lincRNA-p21, PKCδ, and PPARγ knockdown, the small interfering RNA (siRNA) against lincRNA-p21 (si-lincRNA-p21), PKCδ (si-PKCδ), and PPARγ (si-PPARγ) were purchased from RiboBio (Guangzhou, China), with si-NC as the negative control. For lincRNA-p21 overexpression, a lentiviral overexpression vector (LV-lincRNA-p21) was constructed by GeneChem (Shanghai, China), with LV-NC as the negative control. Cells were transfected with si-lincRNA-p21 (20 μM) or LV-lincRNA-p21 (1×10^{10} TU/ml) using lipofectamine 2000 (Invitrogen) for 48 h. For co-transfection, THP-1-derived foam cells were transfected with si-lincRNA-p21 (20 μM) + si-PKCδ (20 μM) or LV-lincRNA-p21 (1×10^{10} TU/ml) + si-PPARγ (20 μM) for 48 h.

Oil red O staining

THP-1 macrophages were gently rinsed twice with phosphate-buffered solution (PBS; #RY0827; Jisskang, Qingdao, China), and fixed for 10 min with 4% paraformaldehyde solution (#abs9179; Absin, Shanghai, China). Thereafter, cells were stained for 5 min at 37°C protected from light with 0.5% Oil Red O solution (#AWI0570a; Abiowell, Changsha, China) and destained for 15 s with 60% isopropanol (Zhiyuan Chemical, Tianjin, China). After counterstaining with hematoxylin solution, the red intracellular lipid droplets were observed under optical microscopy and quantified by ImageJ software.

Intracellular cholesterol assay

THP-1 macrophages were lysed by sonication in hexane/isopropanol and centrifuged at 14,000×*g* at 4°C for 10 min. The intracellular total cholesterol content in the supernatant was detected using the cholesterol assay kit (#ab133116; Abcam, Shanghai, China) according to the manufacturer's protocol.

Cholesterol uptake assay

The cholesterol uptake capacity of foam cells was measured by performing Dil-ox-LDL uptake assay (Yu et al. 2020). THP-1 macrophages after treatment and transfection were washed twice with PBS and co-incubated for 4 h at 37°C protected from light with Dil-ox-LDL (10 μg/μl; #AWH0348a; Abiowell). Subsequently, 4',6-diamidino-2-phenylindole (DAPI; #CSD4080; Chemstan, Wuhan, China) was added to counterstain the nucleus. An inverted fluorescence microscope (Olympus IX53, Tokyo, Japan) was used to take photographs, with the fluorescence intensity quantified by ImageJ software.

Cholesterol efflux assay

This assay was performed as described elsewhere (Wang G et al. 2021). After treatment and transfection, THP-1 macrophages were cultured for 6 h at 37°C with fluorescently labeled NBD-cholesterol (5 μM; #CY32758; ChemeGen, Shanghai, China), followed by three washes with PBS and 2 h equilibration in RPMI-1640 medium. Then, the lipid acceptor apoA-I (25 μg/ml; #abs01289; Absin) or HDL (50 μg/ml; #20610ES05; Yeason, Shanghai, China) was added to the medium for 4 h at 37°C. Cells were lysed for 15 min using 0.3 M NaOH solution. The fluorescence-labeled cholesterol (green) in the medium and cell lysates was visualized by an inverted fluorescence microscope. ImageJ software was employed to determine fluorescence intensity. NBD-cholesterol efflux mediated by apoA-I or HDL was expressed as the percentage of fluorescence in medium relative to fluorescence in both medium and cell lysates.

RT-qPCR

After extracting total RNA from THP-1 macrophages with TRIzol reagent (#AWR0091a; Abiowell), 1 μg RNA was reverse transcribed to synthesize cDNA using PrimeScript RT Master Mix (#RR047A; TaKaRa, Dalian, China), followed by the qPCR process performed on Exicycler 96 instrument (Bioneer, Daejeon, South Korea) using SYBR Premix Ex Taq II kit (#RR820B; TaKaR) and specific primers listed in

Table 1. Primer sequences used for RT-qPCR

Gene	Primer sequences
p21	F: 5'-GGGTGGCTCACTCTTCTGGC-3'
	R: 5'-TGGCCTTGCCCGGGCTTGTC-3'
ABCA1	F: 5'-TAGCTGGTTCATTAGTAGCCT-3'
	R: 5'-AGCAGGTTTCCTAACTTCAG-3'
ABCG1	F: 5'-ACCTGAACTACTGGTACAGC-3'
	R: 5'-CTGGGAACATGATCTGAAAGG-3'
SR-A	F: 5'-ACATCAGGAAGTATGGAGCAG-3'
	R: 5'-AAATTTCACAGATTCGGAGCAG-3'
CD36	F: 5'-TCCTTATACGTACAGAGTTCGT-3'
	R: 5'-GAAGGTTCGAAGATGGCAC-3'
GAPDH	F: 5'-TCATTTCCTGGTATGACAACGA-3'
	R: 5'-GGTCTTACTCCTTGGAGGC-3'

F, forward; R, reverse.

Table 1. The $2^{-\Delta\Delta Ct}$ method was employed to analyze the relative mRNA level, which was normalized by GAPDH as the reference gene.

Western blotting

After being washed with ice-cold PBS, the whole-cell lysates were prepared using RIPA lysis buffer (#mlsw-3419; mlBio, Shanghai, China) containing phenylmethylsulphonyl fluoride and protease inhibitors and then subjected to centrifugation at 12,000×*g* at 4°C for 30 min. The bicinchoninic acid assay kit (#PC0020; Solarbio, Beijing, China) was utilized to measure the protein concentration. Soluble proteins (30 μg) were loaded onto SDS-PAGE on a 10% Tris-glycine gel for separation and transferred onto a polyvinylidene fluoride membrane, followed by 1 h incubation with 5% skimmed milk to block non-specific binding. After that, primary antibodies against ABCA1 (#PB0490; 1/1000; Boster, Wuhan, China), ABCG1 (#BA3853-2; 1/1000; Boster), SR-A (#A04413-2; 1/1000; Boster), CD36 (#PB9371; 1/1000; Boster), PKCδ (#A00822-1; 1/1000; Boster), p-Akt (ser473; #ab8932; 1/1000; Abcam), Akt (#ab235958; 1/1000; Abcam), p-Erk (thr202/thr185; #ab201015; 1/1000; Abcam), Erk (#ab17942; 1/1000; Abcam), PPARγ (#BA1693-2; 1/1000; Boster), LXRα (#A03331-3; 1/1000; Boster) and GAPDH (#BA2913; 1/1000; Boster) were added to incubate the membrane overnight at 4°C and the secondary HRP-linked antibody (#BA1061; 1/1000; Boster) was added for another 1 h incubation at room temperature. Immuno-reactive bands were detected using an enhanced chemiluminescence substrate kit (#AR1191; Boster), whose gray values were analyzed using ImageJ software and normalized to those of GAPDH.

Statistical analysis

The data obtained from at least three independent experiments were statistically analyzed and graphed using the GraphPad Prism 8.0 software. The data are presented as the mean ± SD. According to a Kolmogorov-Smirnov test, the distribution of the data was not Gaussian. Therefore, the data were analyzed using nonparametric tests, specifically Mann-Whitney U test and Kruskal-Wallis test $p < 0.05$ was considered significant.

Results

LincRNA-p21 inhibits lipid accumulation in ox-LDL-treated THP-1 macrophages

THP-1 monocyte-derived macrophages were first treated with ox-LDL for 24 h, which resulted in a marked reduction in lincRNA-p21 expression (Fig. 1A). Then, untreated or ox-LDL-treated THP-1 macrophages were further transfected with si-lincRNA-p21 and LV-lincRNA-p21 to evaluate whether lincRNA-p21 affects lipid accumulation. As revealed in Figure 1B,C and in Figure S1A,B (in Supplementary material), si-lincRNA-p21 transduction obviously attenuated lincRNA-p21 expression while LV-lincRNA-p21 transfection elevated lincRNA-p21 expression in both untreated and ox-LDL-treated THP-1 macrophages. Oil Red O staining was performed to detect lipid deposition, which showed that lincRNA-p21 knockdown robustly increased while lincRNA-p21 overexpression decreased lipid accumulation in ox-LDL-treated THP-1 macrophages (Fig. 1D,E). Meanwhile, the cholesterol content in ox-LDL-treated THP-1 macrophages was elevated after downregulating lincRNA-p21 but was reduced after overexpressing lincRNA-p21 (Fig. 1F,G). However, neither lincRNA-p21 knockdown nor lincRNA-p21 affected foam cell formation in THP-1 macrophages that were not treated with ox-LDL (Fig. S1C,D).

LincRNA-p21 suppresses lipid uptake and promotes cholesterol efflux in ox-LDL-treated THP-1 macrophages

Next, we examined the influence of lincRNA-p21 upregulation or downregulation on Dil-ox-LDL uptake and NBD-cholesterol efflux in THP-1 macrophages. As depicted in Figure 2A and B, lincRNA-p21 knockdown prominently enhanced while lincRNA-p21 overexpression weakened the uptake of Dil-ox-LDL by ox-LDL-treated THP-1 macrophages. As expected, the uptake of Dil-ox-LDL by untreated THP-1 macrophages was not affected by both lincRNA-p21 knockdown and lincRNA-p21 overexpression (Fig. S1G,H). Furthermore, the apoA-1/HDL-mediated cholesterol efflux in THP-1 macrophages was markedly repressed after downregulating lincRNA-p21 but was promoted after upregulating lincRNA-p21 (Fig. 2C,D).

LincRNA-p21 affects the expression of membrane cholesterol transporters in ox-LDL-treated THP-1 macrophages

To investigate the potential mechanisms by which lincRNA-p21 affects lipid uptake and efflux, the expression of membrane cholesterol transporters after lincRNA-p21 silencing or overexpression was detected. As expected, lincRNA-p21 knockdown substantially reduced ABCA1 and ABCG1 mRNA and protein levels but elevated SR-A and CD36 mRNA and protein levels in ox-LDL-treated THP-1 macrophages (Fig. 3A,B). In contrast, ABCA1 and ABCG1 levels were distinctly upregulated while SR-A and CD36 levels were downregulated upon overexpression of lincRNA-p21 (Fig. 3C,D). Nevertheless, silencing or overexpression of lincRNA-p21 exerted no significant effects on ABCA1, ABCG1, SR-A,

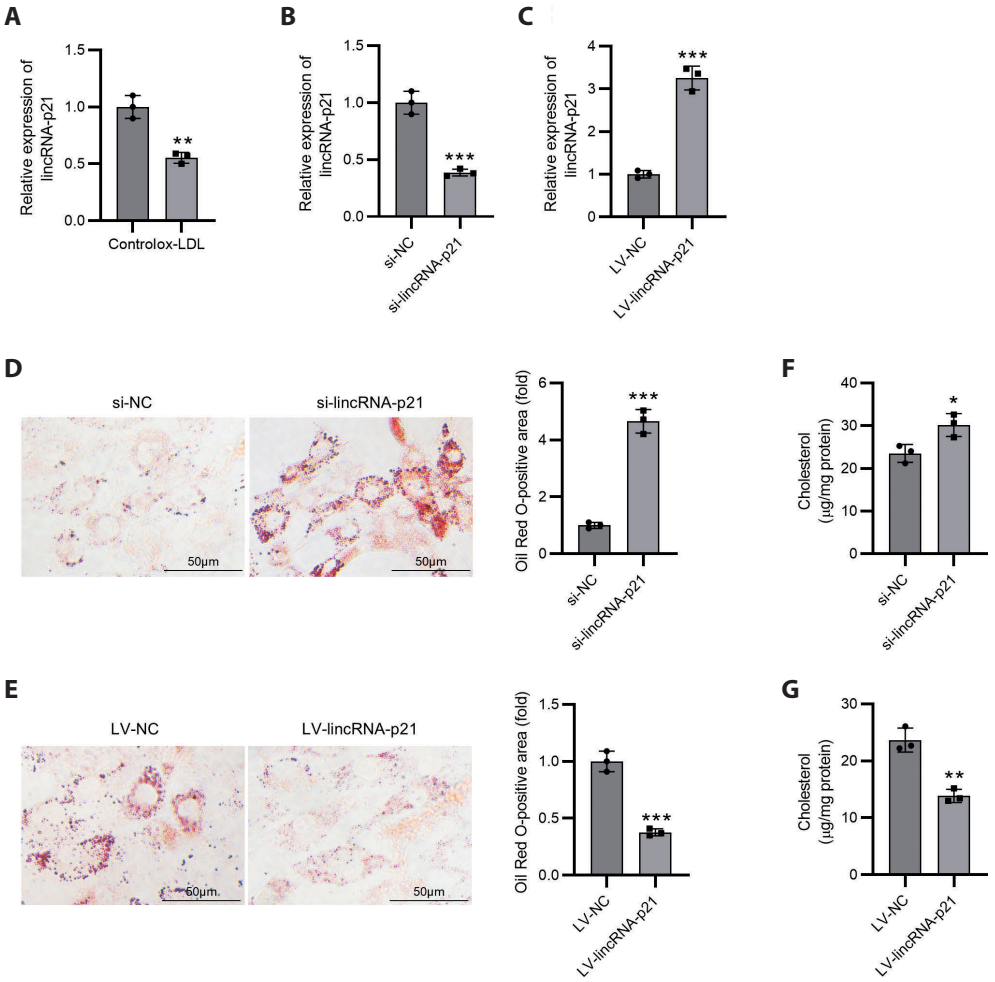


Figure 1. lincRNA-p21 inhibits ox-LDL-induced lipid accumulation in THP-1 macrophages. **A.** The expression of lincRNA-p21 in THP-1 monocyte-derived macrophages after treatment with ox-LDL (50 μg/ml) for 24 h was detected by RT-qPCR. **B,C.** THP-1 macrophages were treated with ox-LDL (50 μg/ml) for 24 h and then transfected with si-NC/si-lincRNA-p21 (20 μM) or LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml) for 48 h, followed by analyses of lincRNA-p21 expression by RT-qPCR. **D,E.** Lipid droplets in THP-1 macrophages after ox-LDL treatment and cell transfection were observed through Oil Red O staining. **F,G.** Measurement of the intracellular cholesterol levels in ox-LDL-treated THP-1 macrophages after knocking down or overexpressing lincRNA-p21. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

and CD36 protein levels in untreated THP-1 macrophages (Fig. S2A,B).

LincRNA-p21 suppresses lipid accumulation and uptake through the PKCδ/Akt/Erk pathway in ox-LDL-treated THP-1 macrophages

The activation of PKCδ and its downstream signaling molecules Akt and Erk is associated with lipid accumulation and uptake. As indicated in Figure 4A, lincRNA-p21 overexpression considerably attenuated PKCδ protein levels as well as Akt phosphorylation at ser473 and Erk phosphorylation at thr202/thr185 in ox-LDL-treated THP-1 macrophages. Depletion of lincRNA-p21 enhanced PKCδ protein levels and Akt and Erk phosphorylation, which was reversed by knockdown of PKCδ (Fig. 4B). According to Oil Red O staining, the increase in lipid deposition in ox-LDL-treated THP-1 macrophages caused by lincRNA-p21 depletion was antagonized by silencing of PKCδ (Fig. 4C). Moreover, RT-qPCR revealed that PKCδ knockdown overturned si-lincRNA-p21-induced increments in SR-A and CD36 mRNA levels in ox-LDL-treated THP-1 macrophages (Fig. 4D). Similarly, in untreated THP-1 macrophages, depletion or overexpression of lincRNA-p21 did not affect PKCδ protein levels as well as Akt and Erk phosphorylation (Fig. S2C,D).

LincRNA-p21 promotes cholesterol efflux through the PPARγ/LXRα pathway in ox-LDL-treated THP-1 macrophages

The activation of the PPARγ-LXRα-ABCA1/ABCG1 pathway contributes to cholesterol efflux. We found that PPARγ and LXRα protein levels were apparently weakened after silencing of lincRNA-p21 in ox-LDL-treated THP-1 macrophages (Fig. 5A). Conversely, upregulating lincRNA-p21 led to a marked increment in PPARγ and LXRα protein levels, which was annulled after downregulating lincRNA-p21 (Fig. 5B). RT-qPCR manifested that LV-lincRNA-p21-induced elevation in ABCA1 and ABCG1 mRNA levels in ox-LDL-treated macrophage was counteracted by si-PPARγ

(Fig. 5C). In addition, PPARγ knockdown reversed the increased of apoA-1/HDL-mediated cholesterol efflux caused by lincRNA-p21 overexpression in ox-LDL-treated THP-1 macrophages (Fig. 5D).

Discussion

Atherosclerosis is a metabolic disease with the character of progressive lipid deposition, fibrous tissue proliferation, and

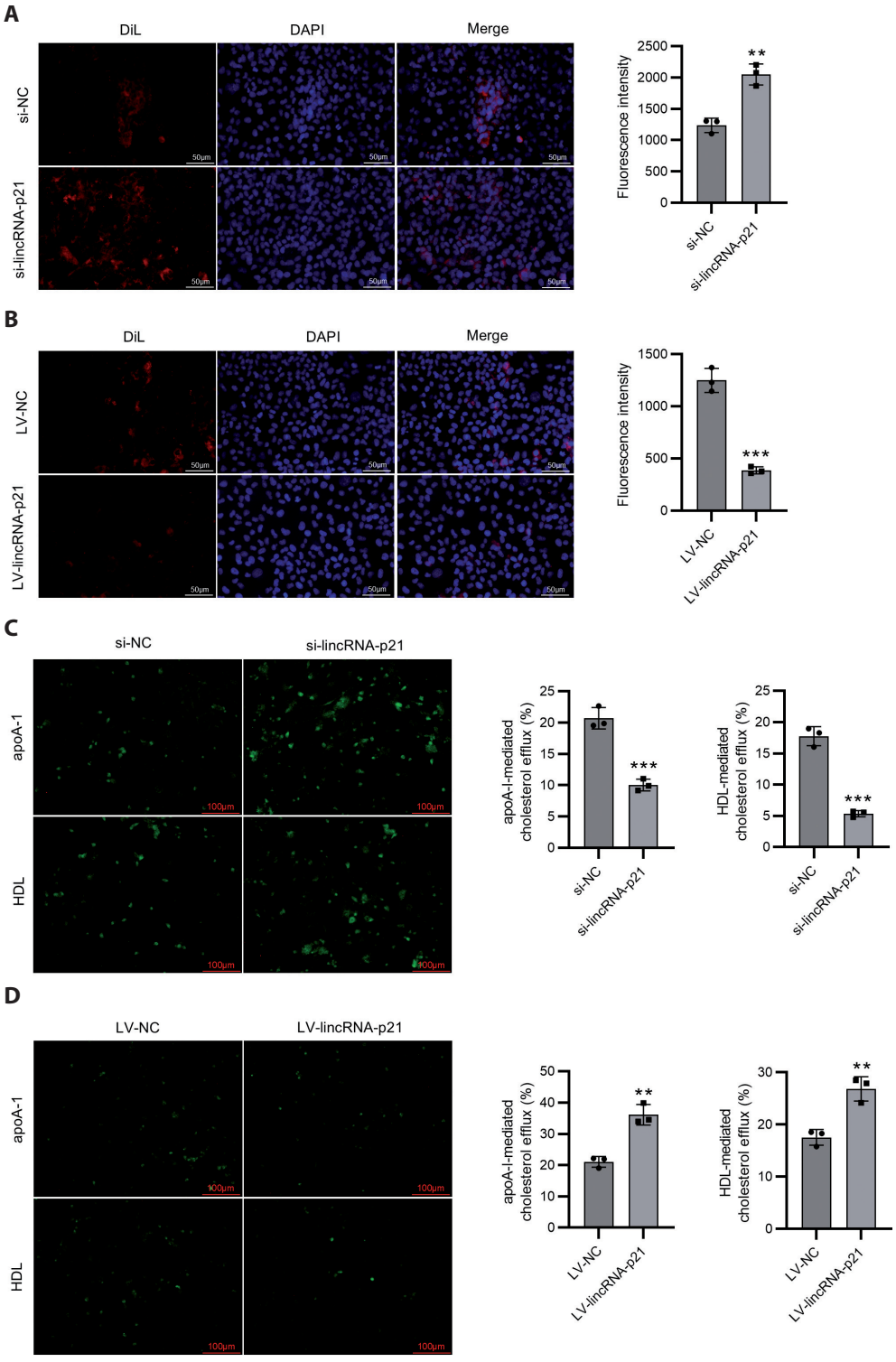


Figure 2. lincRNA-p21 suppresses lipid uptake and promotes cholesterol efflux in THP-1 macrophages. THP-1 macrophages were treated for 24 h with ox-LDL (50 μg/ml), followed by 48 h transfection with si-NC/si-lincRNA-p21 (20 μM) or LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml). **A,B.** Cells were incubated for 4 h with Dil-ox-LDL (10 μg/μl) and observed under a fluorescence microscope. The intensity of red fluorescence is positively associated with Dil-ox-LDL uptake. **C,D.** Cells were loaded for 6 h with NBD-cholesterol (5 μM), followed by incubation for 4 h with apoA-I (25 μg/ml) or HDL (50 μg/ml). The intensity of green fluorescence under a fluorescence microscope is negatively correlated to efflux of NBD-cholesterol mediated by apoA-I or HDL. ** $p < 0.01$, *** $p < 0.001$.

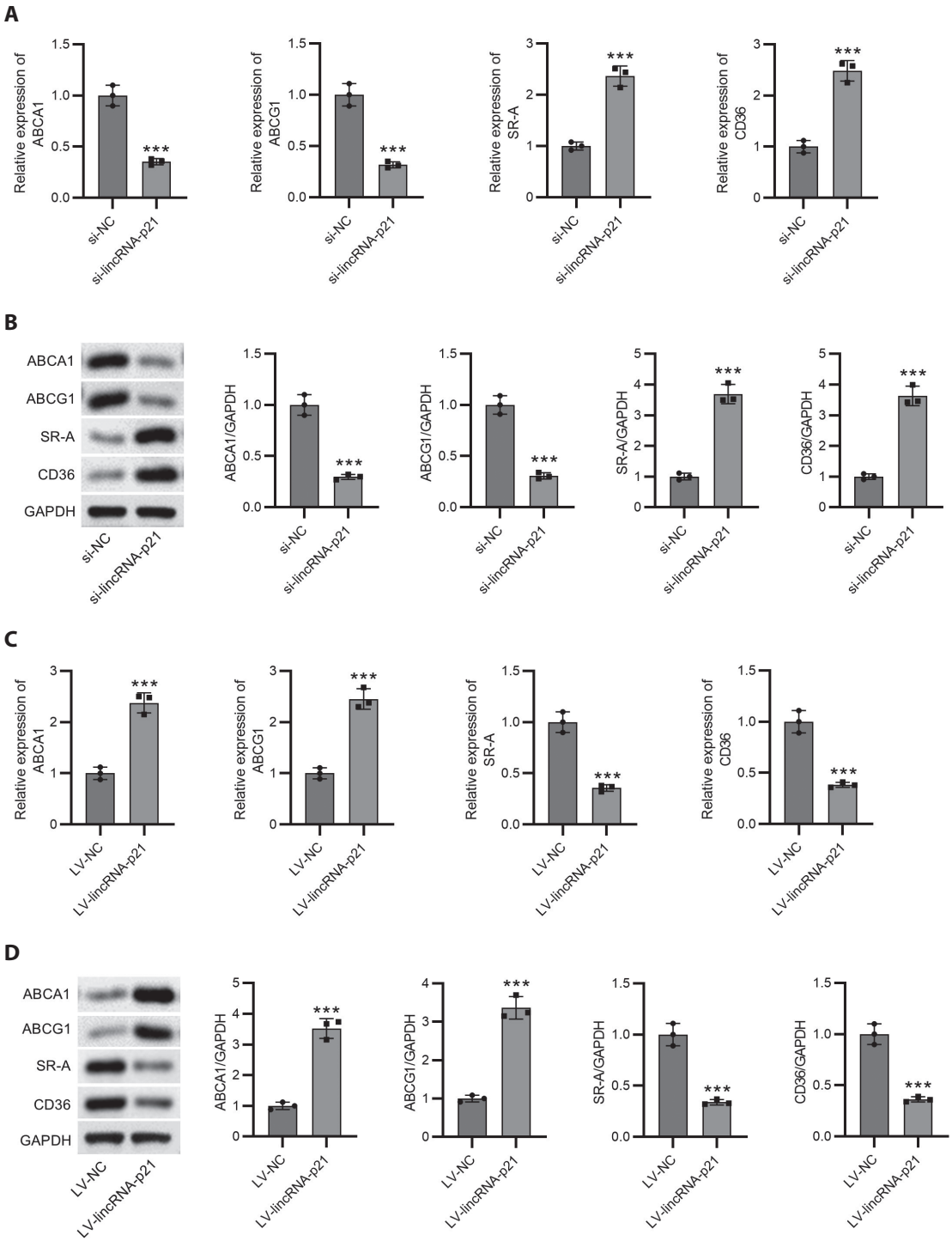


Figure 3. lincRNA-p21 affects the expression of membrane cholesterol transporters in ox-LDL-treated THP-1 macrophages. THP-1 macrophages were treated for 24 h with ox-LDL (50 μg/ml) and transfected for 48 h with si-NC/si-lincRNA-p21 (20 μM) or LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml). **A.** The mRNA levels of ABCA1, ABCG1, SR-A, and CD36 in ox-LDL-treated THP-1 macrophages after silencing of lincRNA-p21 were analyzed by RT-qPCR. **B.** The protein levels of ABCA1, ABCG1, SR-A, and CD36 in ox-LDL-treated THP-1 macrophages after silencing of lincRNA-p21 were determined through Western blotting. **C.** The mRNA levels of ABCA1, ABCG1, SR-A, and CD36 in ox-LDL-treated THP-1 macrophages after overexpression of lincRNA-p21 were examined by RT-qPCR. **D.** The protein levels of ABCA1, ABCG1, SR-A, and CD36 in ox-LDL-treated THP-1 macrophages after lincRNA-p21 overexpression were measured through Western blotting. *** $p < 0.001$.

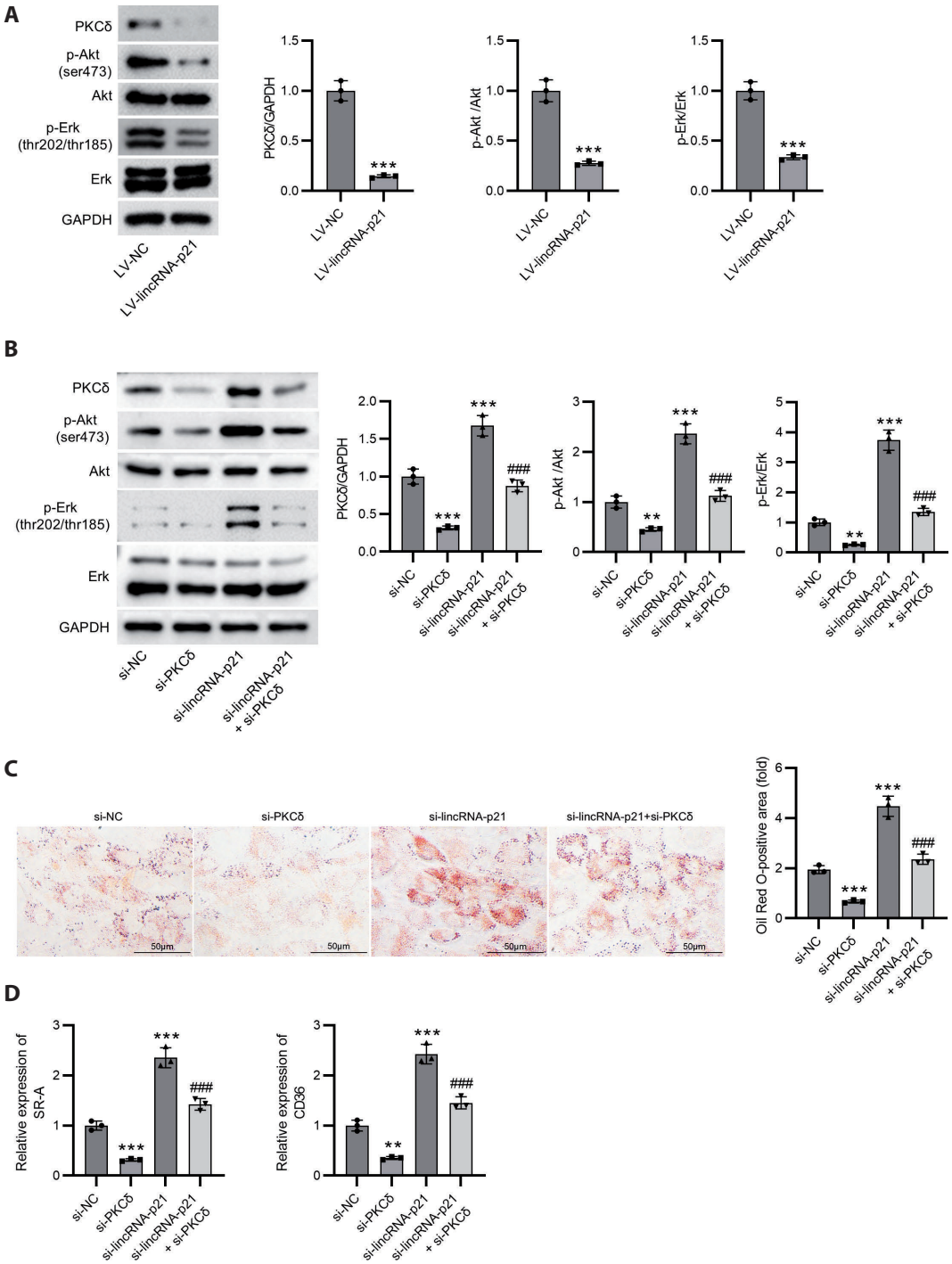


Figure 4. lincRNA-p21 suppresses lipid accumulation and uptake in ox-LDL-treated THP-1 macrophages through the PKCδ/Akt/Erk signaling pathway. **A.** The protein levels of PKCδ, phosphorylated Akt (ser473), total Akt, phosphorylated Erk (thr202/thr185), and total Erk in THP-1 macrophages after 24 h treatment with ox-LDL (50 μg/ml) and 48 h transfection with LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml) were estimated through Western blotting. **B.** The protein levels of PKCδ, phosphorylated Akt (ser473), total Akt, phosphorylated Erk (thr202/thr185), and total Erk in THP-1 macrophages after 24 h treatment with ox-LDL and 48 h transfection with si-NC/si-lincRNA-p21/si-lincRNA-p21+siPKCδ were measured by Western blotting. **C.** Lipid droplets in THP-1 macrophages after ox-LDL treatment and different transfections were observed through Oil Red O staining. **D.** The mRNA levels of SR-A and CD36 in THP-1 macrophages after ox-LDL treatment and different transfections were analyzed by RT-qPCR. ** $p < 0.01$, *** $p < 0.001$ *vs.* LV-NC/si-NC; ### $p < 0.001$ *vs.* si-lincRNA-p21.

inflammatory cell infiltration in vessel walls. The accumulation of cholesterol in macrophages of arterial origin and the subsequent formation and retention of lipid-loaded foam cells in vessel walls accelerate the development of atherosclerotic plaques (Zhu et al. 2017). In the current research, THP-1 monocytes were treated with ox-LDL to establish a foam cell model. LincRNA-p21 expression in THP-1-derived macrophages was reduced after ox-LDL stimulation. LincRNA-p21 knockdown promoted lipid uptake and accumulation and repressed cholesterol efflux in ox-LDL-treated THP-1 macrophages, while lincRNA-p21 overexpression exerted an opposite effect on cholesterol influx and efflux. Additionally, it was validated that the protective effects of lincRNA-p21 overexpression against macrophage lipid accumulation were attributed to the downregulated PKCδ/Akt/Erk/SR-A/CD36 and upregulated PPARγ/LXRα/ABCA1/ABCG1 pathways (Fig. 6).

In recent years, lncRNAs have been found to participate in atherosclerosis occurrence and progression by mediating cellular autophagy, abnormal lipid metabolism, macrophage dysfunction, and endothelial dysfunction in atherosclerotic plaques (Josefs and Boon 2020). The involvement of lincRNA-p21 in human diseases such as cancer, cardiac hypertrophy, cerebral ischemia/reperfusion injury, diabetic nephropathy, and liver diseases (Chen et al. 2017; Zhang et al. 2018; Wang et al. 2019; Wang et al. 2024; Zhao et al. 2024). Importantly, lincRNA-p21 was revealed to be implicated in atherosclerosis development. For example, Zhou and Chen clarified that ox-LDL treatment elevated lincRNA-p21 expression in human coronary artery endothelial cells, and lentiviral overexpression of lincRNA-p21 markedly enhanced whereas lentiviral knockdown of lincRNA-p21 attenuated ox-LDL-induced apoptosis, implying the role of lincRNA-p21 in atherosclerosis-associated endothelial dysfunction (Zhou and Chen 2017). Wang et al. (2021b) suggested that lincRNA-p21 was lowly expressed in the peripheral blood of atherosclerosis patients, and overexpressing lincRNA-p21 facilitated human aortic endothelial cell proliferation, migration, and tube formation but reduced the extent of lipid deposition and the lesion area of atherosclerosis in high-fat diet-fed ApoE$^{-/-}$ mice through modulating the miR-221/SIRT1/Pcsk9 axis. Similarly, lincRNA-p21 was discovered to be positively regulated by p53 to further sponge miR-17-5p and upregulate SIRT7, thereby decreasing the vulnerable plaque and lipid accumulation in atherosclerosis mouse models as well as impairing vascular smooth muscle cell proliferation *in vitro* (Wang et al. 2021a). Herein, we observed that lincRNA-p21 expression was remarkably reduced after ox-LDL stimulation in THP-1 macrophages. LincRNA-p21 knockdown dramatically promoted lipid accumulation and uptake but suppressed cholesterol efflux in ox-LDL-treated THP-1 macrophages. In contrast, lincRNA-p21 overexpression had the opposite effect on lipid influx and efflux.

Ox-LDL, as a key atherogenic molecule, plays a crucial role in damaging vascular endothelial cells, promoting monocyte adhesion, and inducing foam cell and thrombus formation. Uptake of ox-LDL by macrophages is an essential process in atherosclerotic plaque formation, which is regulated many some necessary factors, including SR-A and CD36 (Dong et al. 2024). CD36 is a key driver of foam cell formation, and blocking CD36 expression can result in a 50% inhibition of monocyte-derived macrophages and weaken their ability to bind ox-LDL (Rahaman et al. 2006). It has been reported that inhibition of SR-A and CD36 can remarkably alleviate foam cell formation and reduce atherosclerotic plaques (Zhao et al. 2018). Manning-Tobin et al. (2009) revealed that atherosclerotic lesion complexity was reduced in ApoE $^{-/-}$

mice lacking SR-A and CD36, including reduced macrophage apoptosis, inflammatory gene expression, and atherosclerotic plaque necrosis. Pro-atherogenic mediators ox-LDL and interferon-g have been demonstrated to induce PKCδ, which contributes to the development of cardiovascular diseases through inducing insulin resistance, hepatosteatosis, vascular inflammation, and cell apoptosis in human and experimental animals (Bezy et al. 2011; Wong et al. 2011). There are reports suggesting the beneficial role of PKCδ reduction in protecting against atherosclerosis. For example, Lien et al. (2021) elucidated that knockdown of PKCδ hindered diabetes-associated atherosclerosis by improving hepatic cholesterol metabolism. Vogl et al. (2016) found that silencing of PKCδ distinctly repressed caspase 3/7 activation and reduced cell apoptosis in RAW 264.7 macrophages affected by oxidized phospholipids (oxPl), the cytotoxic component of ox-LDL. Previously, Lin et al. (2012) confirmed that PKCδ was previously confirmed to inhibit foam cell formation in both primary macrophages and THP-1 macrophages by impairing native LDL and ox-LDL uptake and decreasing cholesterol contents through downregulating SR-A and CD36 expression. The PI3K/Akt and ERK signaling pathways were reported to be involved in PKCδ-regulated SR-A and CD36 expression and PKCδ-mediated pro-atherosclerotic events (Lin et al. 2012). Moreover, PKCδ inhibitor rottlerin and shRNA targeting PKCδ can attenuate Akt and ERK1/2 phosphorylation and activation (He et al. 2016). Inhibiting the PI3K/Akt or ERK pathway exhibits atheroprotection effects in ApoE $^{-/-}$ mice (Zhang et al. 2022; Chang et al. 2024). Herein, our findings illustrated that SR-A, CD36, PKCδ, phosphorylated Akt (ser473), and phosphorylated Erk (thr202/thr185) levels in ox-LDL-treated THP-1 macrophages were reduced after lincRNA-p21 knockdown but were elevated after lincRNA-p21 overexpression. Importantly, PKCδ silencing reversed the enhancement in lipid accumulation as well as p-Akt,

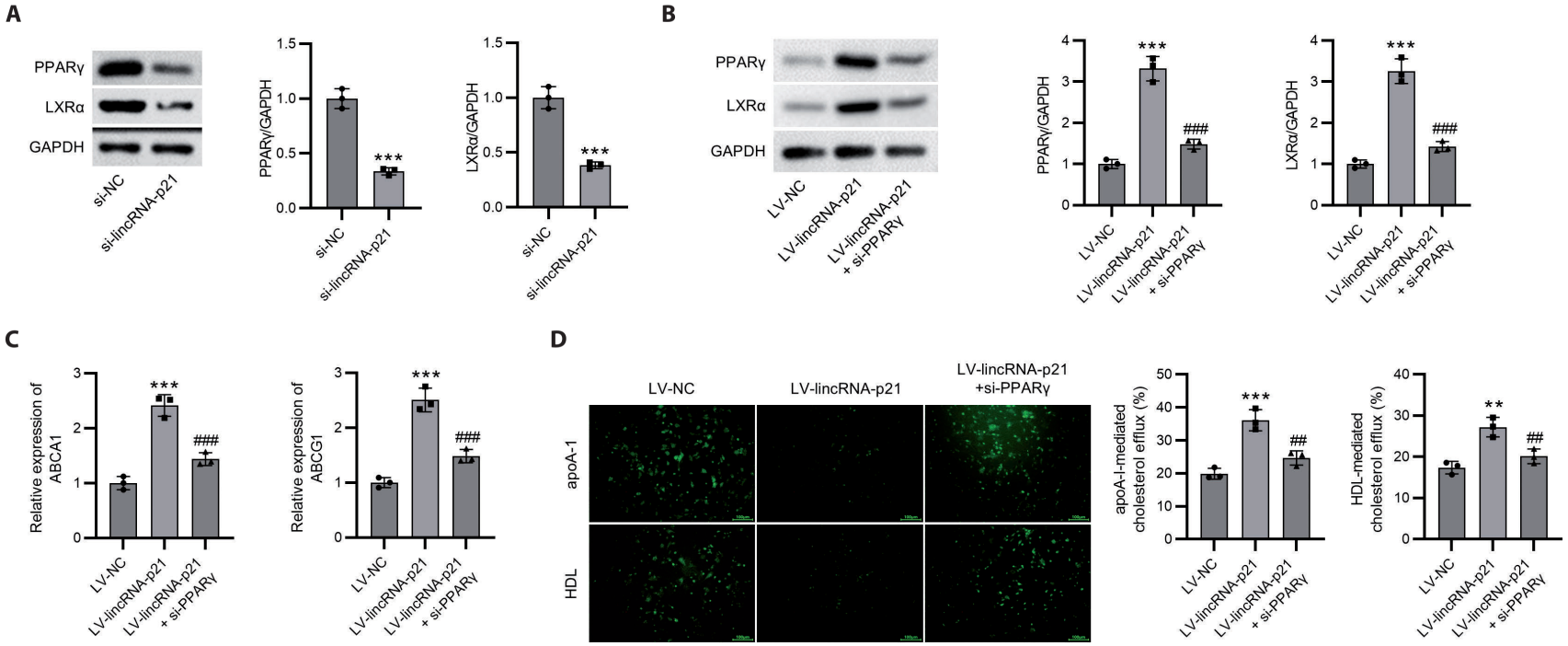


Figure 5. lincRNA-p21 promotes cholesterol efflux in ox-LDL-treated THP-1 macrophages through the PPARγ/LXRα signaling pathway. **A.** The protein levels of PPARγ and LXRα in THP-1 macrophages after 24 h treatment with ox-LDL (50 μg/ml) and 48 h transfection with si-NC/si-lincRNA-p21 (20 μM) were evaluated by Western blotting. **B.** The protein levels of PPARγ and LXRα in THP-1 macrophages after 24 h treatment with ox-LDL and 48 h transfection with LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml) or LV-lincRNA-p21 (1×10^{10} TU/ml) + si-PPARγ (20 μM) were assessed by Western blotting. **C.** The mRNA levels of ABCA1 and ABCG1 in THP-1 macrophages after ox-LDL treatment and different transfections were detected by RT-qPCR. **D.** THP-1 macrophages were treated for 24 h with ox-LDL (50 μg/ml), followed by 48 h transfection with LV-NC/LV-lincRNA-p21/LV-lincRNA-p21+si-PPARγ. Treated cells were loaded for 6 h with NBD-cholesterol (5 μM) and incubated for 4 h with apoA-I (25 μg/ml) or HDL (50 μg/ml). The intensity of green fluorescence is negatively associated with apoA-I- or HDL-mediated efflux of NBD-cholesterol. ** $p<0.01$, *** $p<0.001$ *vs.* LV-NC/si-NC; ## $p<0.01$, ### $p<0.001$ *vs.* LV-lincRNA-p21.

p-Erk, SR-A, and CD36 levels caused by lincRNA-p21 knockdown.

Promoting cholesterol efflux is another major pathway to suppress foam cell formation, which requires the involvement of ABCA1 and ABCG1 (Kim et al. 2024). ABCA1 mainly facilitates the transport of intracellular free cholesterol and phospholipids to extracellular lipid-poor apolipoproteins, such as apolipoprotein A1 (apoA1), to form nascent high-density lipoprotein (HDL) particles (Yu and Tang 2022). Anascent HDL forms mature HDL by the action oflecithin-cholesterol acyltransferase and continues to receive ABCG1-transported cholesterol (Yu and Tang 2022). ABCA1 and ABCG1 are respectively responsible for 50% and 20% of cholesterol efflux from cholesterol-loaded mouse peritoneal macrophages to human serum (Adorni et al. 2007). Previously, pregnancy-associated plasma protein-A or angiopoietin-1 was reported to decrease ABCA1 and ABCG1 expression, thereby curbing cholesterol efflux and inducing lipid deposition in THP-1 macrophage-derived foam cells and aggravating atherosclerosis development (Tang et al. 2019; Ou et al. 2020). In contrast, increasing ABCA1- and ABCG1-dependent cholesterol release is beneficial for reducing atherosclerotic lesion areas in ApoE$^{-/-}$ mice and alleviating lipid accumulation in THP-1 macrophages (Zhang et al. 2022b). Reverse cholesterol transport refers to a cholesterol-cleaning mechanism in foam cells, which is responsible for the transportation of the cholesterol in foam cells to the circulating blood, the liver, and then the small intestine. The PPARγ/LXRα/ABCA1/ABCG1 pathway plays an essential role in reverse cholesterol transport by facilitating cholesterol transport from intracellular to extracellular. PPAR-γ is a transcription factor that induces ABCA1 and ABCG1 expression by upregulating LXRα. Previous studies have revealed that activating the PPARγ/LXRα/ABCA1/ABCG1 pathway can mitigate atherosclerosis through restraining lipid accumulation and inflammatory response (Wu et al. 2014; Song et al. 2021). In our work, we found that lincRNA-p21 depletion weakened while lincRNA-p21 overexpression enhanced ABCA1, ABCG1, PPARγ, and LXRα levels in ox-LDL-treated THP-1 macrophages. Rescue experiments verified that PPARγ knockdown abrogated the promotive effects of lincRNA-p21 overexpression on ABCA1, ABCG1, PPARγ, and LXRα levels and cholesterol efflux in THP-1 macrophages.

To sum up, our study uncovered the function of lincRNA-p21 on foam cell formation and lipid metabolism dysfunction in atherosclerosis. Overexpression of lincRNA-p21 decreased cholesterol uptake by restraining the PKCδ/Akt/Erk/SR-A/CD36 pathway and increased cholesterol efflux by facilitating the PPARγ/LXRα/ABCA1/ABCG1 pathway in ox-LDL-treated THP-1 macrophages. These findings clarify the detailed mechanisms through which lincRNA-p21 modulates lipid accumulation and foam cell formation in atherosclerosis.

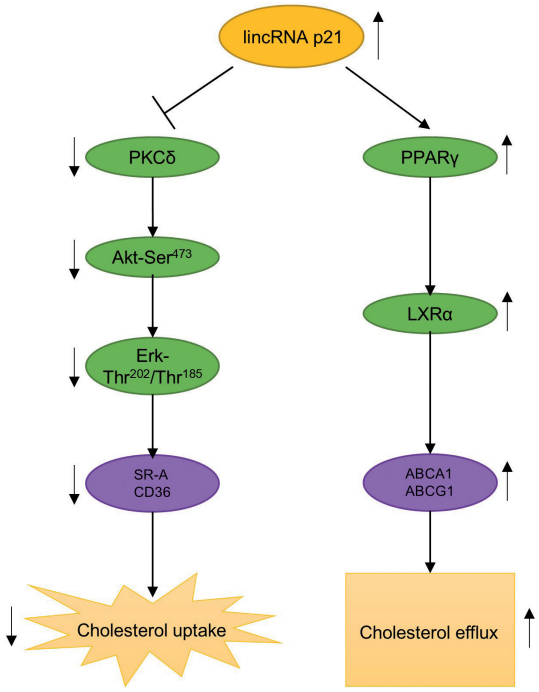


Figure 6. Schematic diagram showing the mechanisms beneath the regulation of lincRNA-p21 on cholesterol uptake and efflux in ox-LDL-treated THP-1 macrophages.

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Conflict of interests. The authors declare that they have no competing interests.

Authors' contributions. ChH, WY: conceived and designed the experiments, ChH, WY, FZ, LT, HW, ZG, JD, JZ, JY, SL: analyzed the data and drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

Data availability. The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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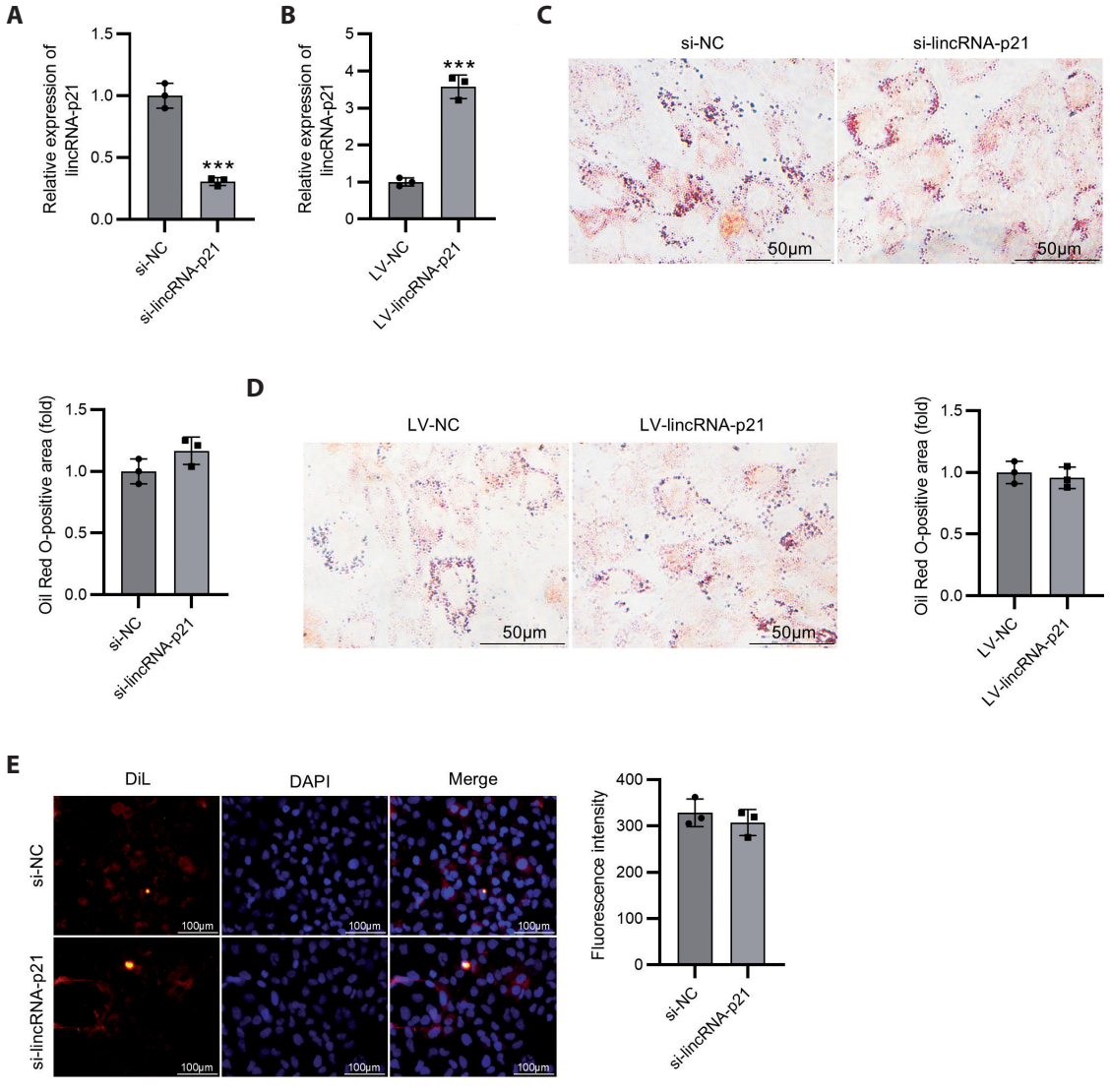
Supplementary Material

lincRNA-p21 inhibits foam cell formation and regulates cholesterol uptake and efflux in macrophages through the PKCδ/Akt/Erk and PPARγ/LXRα pathways

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Supplementary Figures



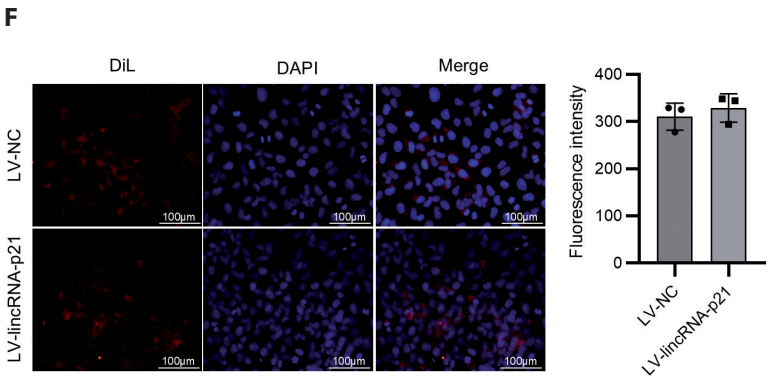
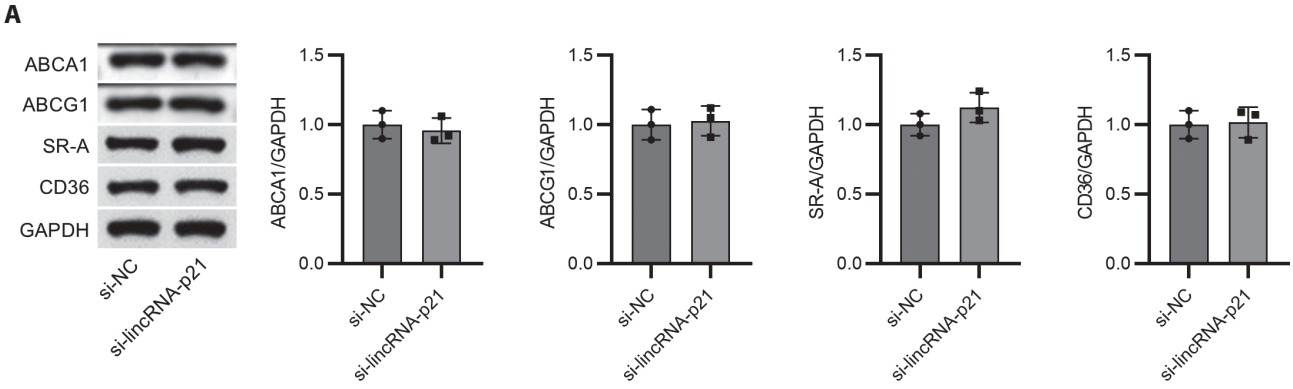
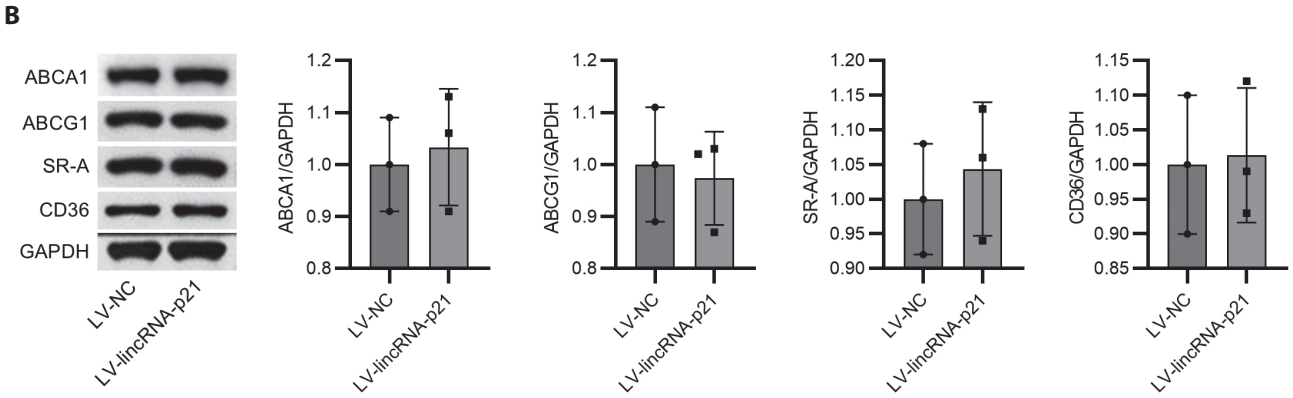
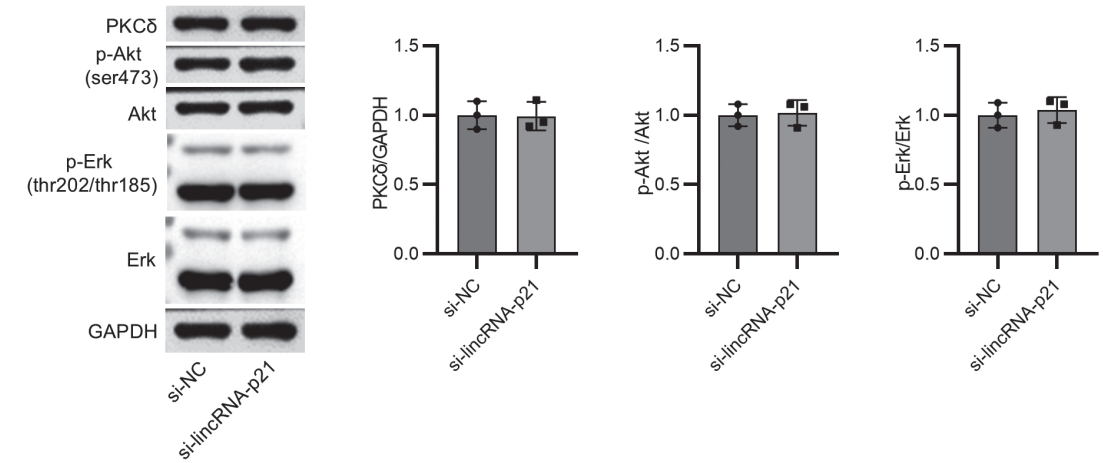


Figure S1. LincRNA-p21 does not affect lipid accumulation and uptake in untreated THP-1 macrophages. THP-1 macrophages without ox-LDL treatment were transfected with si-NC/si-lincRNA-p21 (20 μM) or LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml) for 48 h. **A,B.** LincRNA-p21 expression in transfected THP-1 macrophages was detected by RT-qPCR. **C,D.** Foam cell formation in transfected THP-1 macrophages were observed through Oil Red O staining. **E,F.** Transfected THP-1 macrophages were incubated for 4 h with Dil-ox-LDL (10 μg/μl) and observed under a fluorescence microscope. The intensity of red fluorescence is positively associated with Dil-ox-LDL uptake. *** $p < 0.001$.







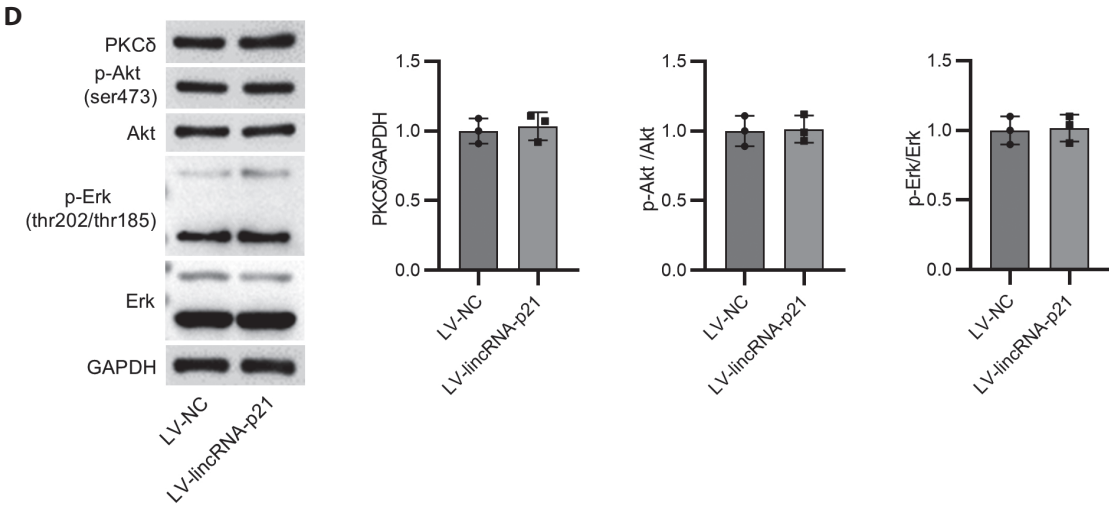


Figure S2. LincRNA-p21 exerts no significant effects on the expression of membrane cholesterol transporters and the PKCδ/Akt/Erk pathway in untreated THP-1 macrophages. THP-1 macrophages without ox-LDL treatment were transfected with si-NC/si-lincRNA-p21 (20 μM) or LV-NC/LV-lincRNA-p21 (1×10^{10} TU/ml) for 48 h. **A,B.** The protein levels of ABCA1, ABCG1, SR-A, and CD36 in transfected THP-1 macrophages were determined by Western blotting. **C,D.** The protein levels of PKCδ, phosphorylated Akt (ser473), total Akt, phosphorylated Erk (thr202/thr185), and total Erk in transfected THP-1 macrophages were examined through Western blotting.