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LncRNA MALAT1 regulates proliferation and apoptosis of vascular smooth muscle cells by targeting miRNA-124-3p/PPARα axis

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Abstract. – OBJECTIVE: To uncover the involvement of long non-coding RNA (lncRNA) MALAT1 in the proliferation and apoptosis of vascular smooth muscle cells (VSMCs), and the underlying mechanism.

MATERIALS AND METHODS: Relative levels of MALAT1, microRNA-124-3p (miRNA-124-3p) and peroxisome proliferator-activated receptor alpha (PPARα) in VSMCs treated with different doses of oxidized low-density lipoprotein (ox-LDL) for different time points were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Proliferative and apoptotic changes of VSMCs overexpressing MALAT1 were assessed. Subcellular distribution of MALAT1 was analyzed. The potential binding among MALAT1, miRNA-124-3p and PPARα was determined by dual-luciferase reporter gene assay, and their interaction was determined as well. Finally, the influences of MALAT1/miRNA-124-3p/PPARα regulatory loop on the proliferative and apoptotic abilities of VSMCs were examined.

RESULTS: MALAT1 and PPARα were dose-dependently downregulated in ox-LDL-treated VSMCs, whereas miRNA-124-3p was gradually upregulated. Overexpression of MALAT1 attenuated viability and induced apoptosis in ox-LDL-treated VSMCs. Moreover, MALAT1 was mainly distributed in the nucleus. Dual-luciferase reporter gene assay verified that MALAT1 could sponge miRNA-124-3p, and moreover, PPARα was the direct target of miRNA-124-3p. MALAT1 negatively regulated miRNA-124-3p level and miRNA-124-3p negatively regulated PPARα level as well. Finally, MALAT1/miRNA-124-3p/PPARα regulatory loop was identified to regulate the viability and apoptosis of ox-LDL-treated VSMCs.

CONCLUSIONS: LncRNA MALAT1 mediates proliferation and apoptosis of VSMCs by sponging miRNA-124-3p to positively regulate PPARα level.

Key Words:
MALAT1, MiRNA-124-3p, PPARα, VSMCs, Proliferation, Apoptosis.

Introduction

Atherosclerosis (AS) is a major cause of coronary heart disease, cerebral infarction, peripheral vascular disease, cardiovascular and cerebrovascular diseases[1]. Lipid metabolism disorder is the basis of atherosclerotic lesions, which is characterized by arterial lesions in the intima, thickening of the arterial wall and narrowing of the vascular lumen[2]. AS is named due to the deposition of yellow atheroma-like lipids in the intima[3]. Apoptosis of vascular smooth muscle cells (VSMCs) leads to the formation of atherosclerotic plaques[4]. Vascular injuries, such as myocardial infarction and cerebral infarction, can stimulate the proliferation and differentiation of VSMCs. During the progression of atherosclerotic disease, the specific molecular mechanism underlying the proliferative and apoptotic VSMCs is poorly understood.

Long non-coding RNA (LncRNA) is a non-coding RNA with a length greater than 200 nucleotides[5]. LncRNA exerts a crucial function in life activities, such as epigenetics, cell cycle progression, and cell differentiation, which has been well concerned in genetic researches[6]. Besides, lncRNA is capable of influencing the occurrence and progression of tumors at transcriptional or post-transcriptional level[7]. In atherosclerotic disease, cellular behaviors of VSMCs could be mediated by certain lncRNAs[8]. For example, lncRNA UCA1 mediates the migratory and proliferative

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abilities of VSMCs by sponging microRNA-26a (miR-26a)[9]. LncRNA MALAT1 suppresses oxidized low-density lipoprotein (ox-LDL)-induced release of inflammatory cytokines and apoptosis of HCAECs by targeting the microRNA-155 / SOCS1 axis[10].

MiRNAs are a class of non-coding, single-stranded RNAs encoded by endogenous genes with approximately 22 nucleotides in length. They are involved in post-transcriptional regulation of gene expressions in plants and animals[11]. A single miRNA can have multiple target genes, and several miRNAs can also regulate the same gene[12]. About one-third of human genes are regulated by miRNAs. Initially, a pri-miRNA is processed to be a pre-miRNA, and the latter is further digested by Dicer to form the mature miRNA[13]. Functionally, mature miRNAs recognize target mRNAs in the manner of base complementary pairing, and subsequently, they degrade mRNAs or inhibit their translation[14]. Loyer et al[15] have demonstrated the role of miRNAs in mediating development, organ formation and cellular behaviors.

In this paper, we focused on exploring the role of lncRNA MALAT1 in the progression of AS. Cellular behaviors of VSMCs influenced by MALAT1/miRNA-124-3p/PPARα regulatory loop was specifically investigated.

Materials and Methods

Cell Culture and Ox-LDL Treatment

VSMCs were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). VSMCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 UI penicillin and 0.1 mg/mL streptomycin, in a 37°C, 5% CO_2 incubator. Until 60% confluence, VSMCs were treated with different doses of ox-LDL (0, 25, 50, and 100 mg/L) and for different time points (0, 12, 24, and 48 h) to mimic an *in vitro* environment of hyperlipidemia.

Cell Transfection

Cells were cultured until 60% confluence and subjected to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 h later, complete medium was replaced. Transfected cells for 24-48 h were harvested for *in vitro* experiments.

RNA Extraction and Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

Total RNA in cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), quantified by an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan) and preserved at -80°C. Subsequently, RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) and subjected to PCR using the SYBR Green method (TaKaRa, Otsu, Shiga, Japan). PCR reaction conditions were: Pre-denaturation at 94°C for 5 min, and 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. The relative expression level of the target gene was expressed by $2^{-\Delta\Delta Ct}$. Primer sequences used in this study were as follows: MALAT1, F: 5'-CGAGGAAGCTCCATAACTC-3', R: 5'-CATAGAGGATGTAGTCCGCAGCA-3'; microRNA-124-3p, F: 5'-GCTGTCACATTCAATCGAACTG-3', R: 5'-GATTGCCTGTCGATGGAGCCG-3'; PPARα, F: 5'-GCAGCATTTGGAGCAGAACAA-3', R: 5'-CCGAAGCGGTGACAGTATTCAT-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) and quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Membranes were blocked in 5% skim milk for 2 h, and subjected to incubation with primary and secondary antibodies. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Cell Counting Kit-8 (CCK-8)

Viability determination was performed as previously described[16]. 5×10^3 VSMCs per well were inoculated in a 96-well plate and cultured overnight. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curves.

Determination of Subcellular Distribution

Cytoplasmic and nuclear RNAs were extracted using the PARIS kit (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR. 18S was the

internal references of the nucleus and U1 was that of cytoplasm.

Dual-Luciferase Reporter Gene Assay

VSMCs were inoculated in a 24-well plate. They were co-transfected with miRNA-877-3p mimics/NC and wild-type/mutant-type vectors using Lipofectamine 2000, respectively. 24 h later, co-transfected cells were harvested for determining luciferase activity using a dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Apoptosis Determination

VSMCs were washed with phosphate-buffered saline (PBS) twice and digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin. After resuspension and adjustment to 1×10^6 cells/mL, cells were transferred to a flow cytometry tube, incubated with buffer and 1.25 μL of fluorescein isothiocyanate (FITC) annexin V/Propidium Iodide (PI) for 15 min in dark. Apoptosis was determined within 1 hour by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) was used for data analyses. Figure editing was performed using GraphPad Prism 6.0 (La Jolla, CA, USA). Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

Downregulated MALAT1 in Ox-LDL-VSMCs and Its Influence on VSMCs

VSMCs were treated with different doses of ox-LDL to mimic an *in vitro* condition of atherosclerotic hyperlipidemia. It is shown that MALAT1 level dose-dependently decreased by 0, 25, 50 or 100 mg/L ox-LDL treatment (Figure 1A). Moreover, MALAT1 was time-dependently downregulated after 100 mg/L ox-LDL treatment for 0, 12, 24 or 48 h (Figure 1B). Transfec-

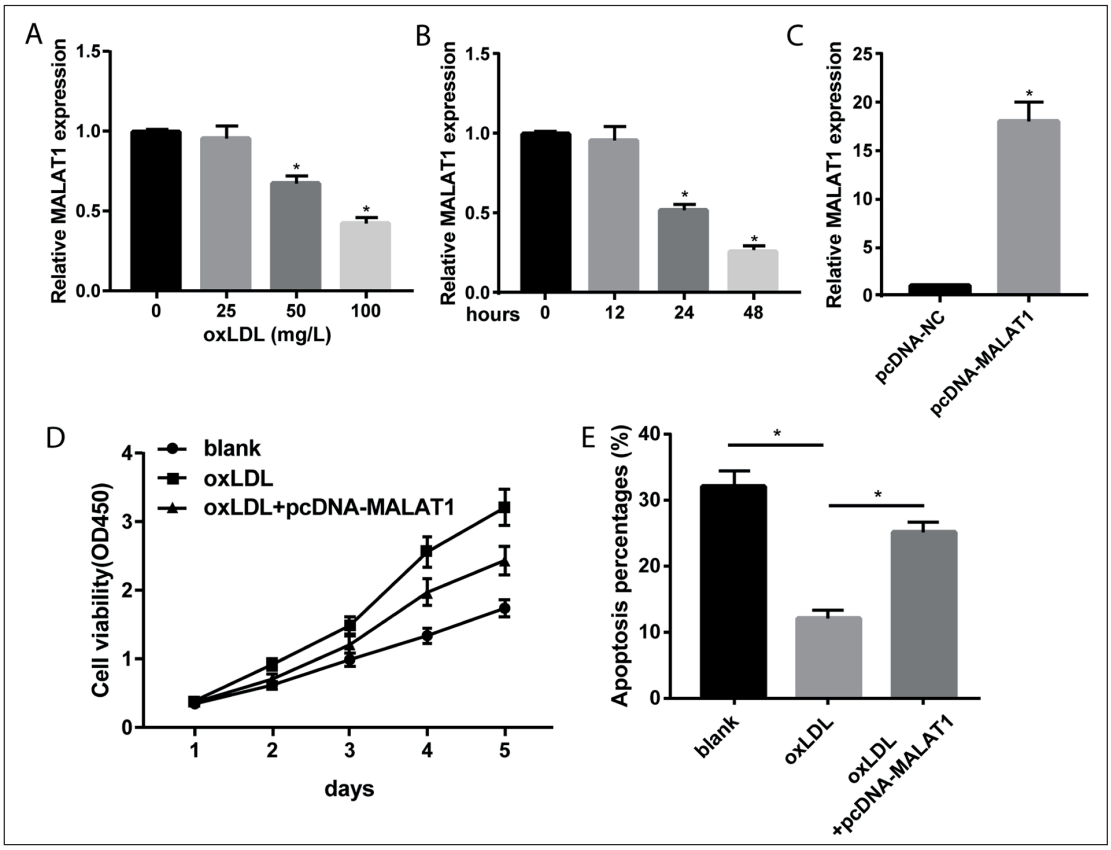

Figure 1. Downregulated MALAT1 in ox-LDL-treated VSMCs. **A,** Relative level of MALAT1 in VSMCs treated with 0, 25, 50 and 100 mg/L ox-LDL for 48 h. **B,** Relative level of MALAT1 in VSMCs treated with 100 mg/L ox-LDL for 0, 12, 24 and 48 h. **C,** Transfection efficacy of pcDNA-MALAT1 in VSMCs. **D,** Cell viability in VSMCs with blank control, ox-LDL treatment and ox-LDL + pcDNA-MALAT1. **E,** Apoptotic rate in VSMCs with blank control, ox-LDL treatment and ox-LDL + pcDNA-MALAT1.

tion of pcDNA-MALAT1 in VSMCs markedly upregulated MALAT1 level, showing an effective transfection efficacy (Figure 1C). As viability curves revealed, ox-LDL treatment markedly enhanced the viability of VSMCs, which was reduced in VSMCs overexpressing MALAT1 (Figure 1D). The apoptotic rate was suppressed by ox-LDL treatment, and it was partially reversed by transfection of pcDNA-MALAT1 in VSMCs (Figure 1E).

MALAT1 Sponged MiRNA-124-3p

Subcellular distribution analysis indicated a higher abundance of MALAT1 in nuclear fraction than that of the cytoplasmic part (Figure 2A). With the increased doses of ox-LDL treatment, miRNA-124-3p was gradually upregulated in VSMCs (Figure 2B). Potential binding sequences between MALAT1 and miRNA-124-3p were searched from an online bioinformatics website (Figure 2C). Subsequently, dual-lucifer-

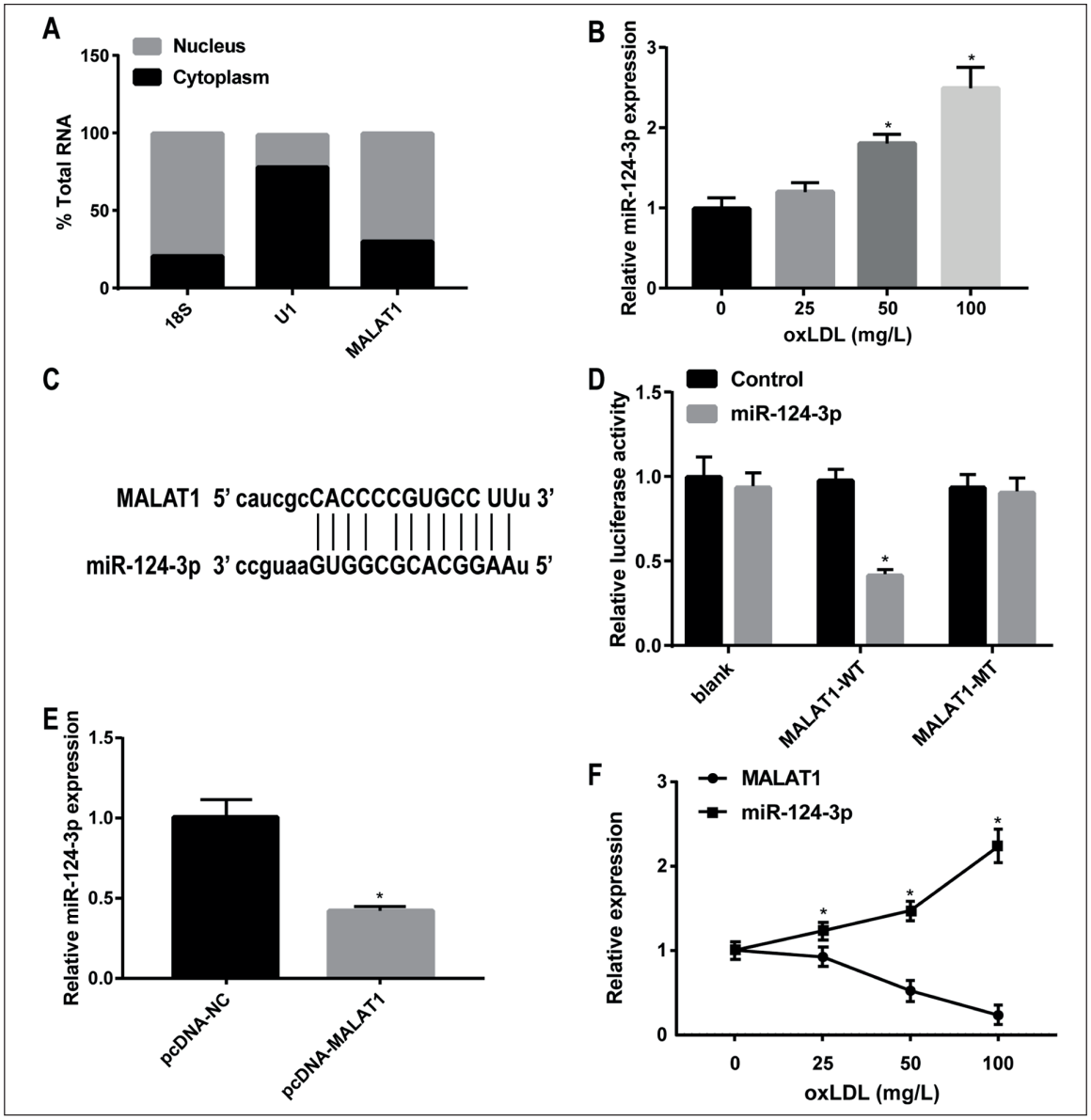

Figure 2. MALAT1 sponged miR-124-3p. **A,** Subcellular distribution of MALAT1 in nuclear and cytoplasmic fractions of VSMCs. U1 and 18S were internal reference for cytoplasm and nucleus, respectively. **B,** Relative level of miR-124-3p in VSMCs treated with 0, 25, 50, and 100 mg/L ox-LDL for 48 h. **C,** Potential binding sequences between MALAT1 and miR-124-3p. **D,** Relative luciferase activity in VSMCs co-transfected with control/miR-124-3p mimics and MALAT1-WT/MALAT1-MT. **E,** Relative level of miR-124-3p in VSMCs transfected with pcDNA-NC or pcDNA-MALAT1. **F,** Relative levels of miR-124-3p and MALAT1 in VSMCs treated with 0, 25, 50, and 100 mg/L ox-LDL for 48 h.

ase reporter gene assay illustrated a remarkable decline in luciferase activity after co-transfection with MALAT1-WT and miRNA-124-3p mimics, confirming their binding relationship (Figure 2D). Moreover, transfection of pcDNA-MALAT1 in VSMCs downregulated miRNA-124-3p level (Figure 2E). In VSMCs treated with the increased doses of ox-LDL, a negative correlation was identified between expression levels of MALAT1 and miRNA-124-3p (Figure 2F). It is demonstrated that MALAT1 could absorb miRNA-124-3p and negatively regulate its level.

MiRNA-124-3p Targeted PPARα and Negatively Regulated its Level

Through searching in TargetScan, potential binding sequences between miRNA-124-3p and PPARα were identified (Figure 3A). In the same way, decreased luciferase activity after co-transfection of PPARα-WT and miRNA-124-3p mimics confirmed their binding relationship (Figure 3B). PPARα level was dose-dependently downregulated in VSMCs after ox-LDL treatment (Figure 3C). Besides, transfection of miRNA-124-3p inhibitor in VSMCs markedly enhanced PPARα level, showing a negative relationship (Figures 3D, 3E).

MALAT1 Influenced VSMCs Behaviors by Targeting MiRNA-124-3p/PPARα Regulatory Loop

Transfection of pcDNA-PPARα could markedly upregulate PPARα level in VSMCs, which was downregulated by co-transfection of si-MALAT1 (Figures 4A, 4B). Moreover, the elevated apoptotic rate was observed in VSMCs overexpressing PPARα, and it was slightly reduced after silence of MALAT1 (Figure 4C). Transfection

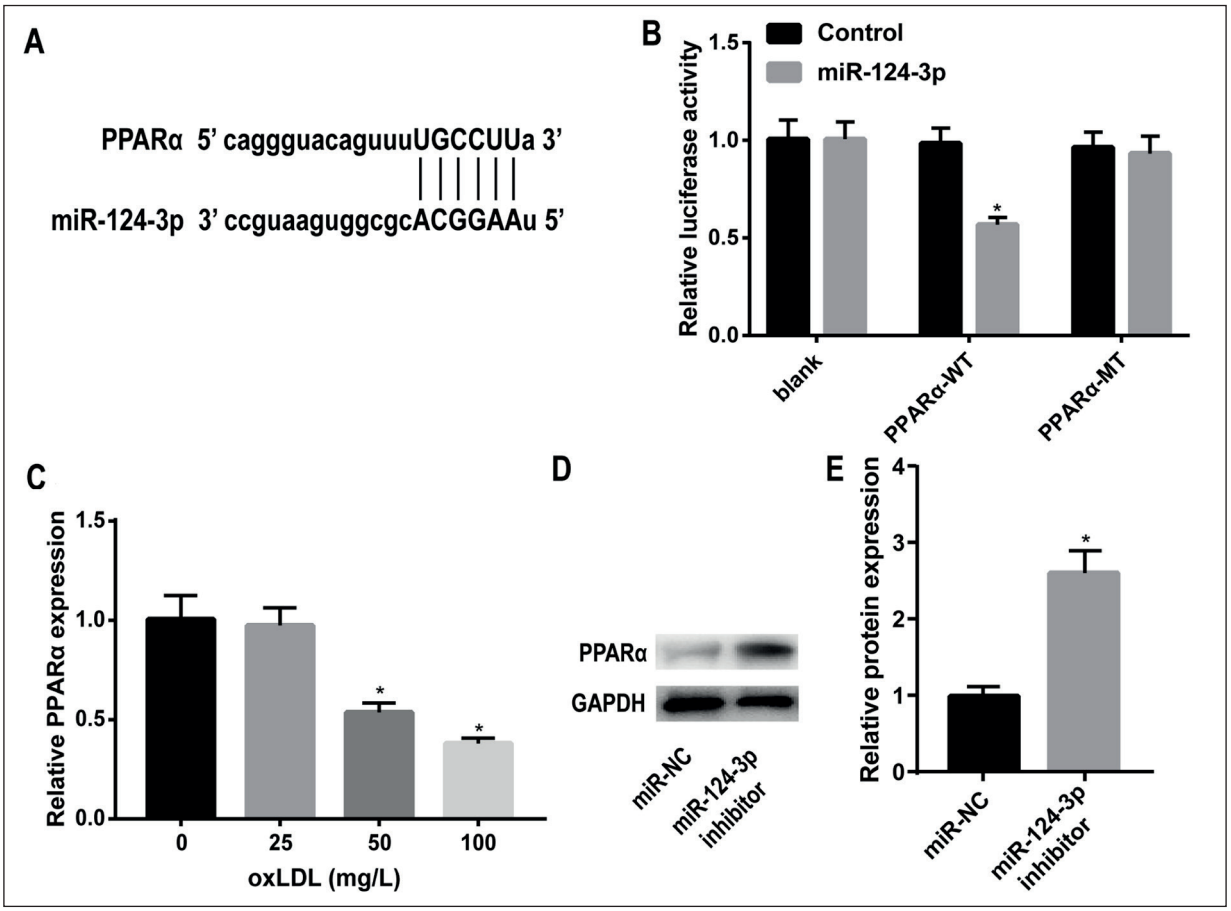

Figure 3. MiR-124-3p targeted PPARα and negatively regulated its level. **A,** Potential binding sequences between miR-124-3p and PPARα. **B,** Relative luciferase activity in VSMCs co-transfected with control/miR-124-3p mimics and PPARα-WT/PPARα-MT. **C,** Relative level of PPARα in VSMCs treated with 0, 25, 50 and 100 mg/L ox-LDL for 48 h. **D,** Protein level of PPARα in VSMCs transfected with miR-NC or miR-124-3p inhibitor. **E,** Grey values of PPARα in VSMCs transfected with miR-NC or miR-124-3p inhibitor.

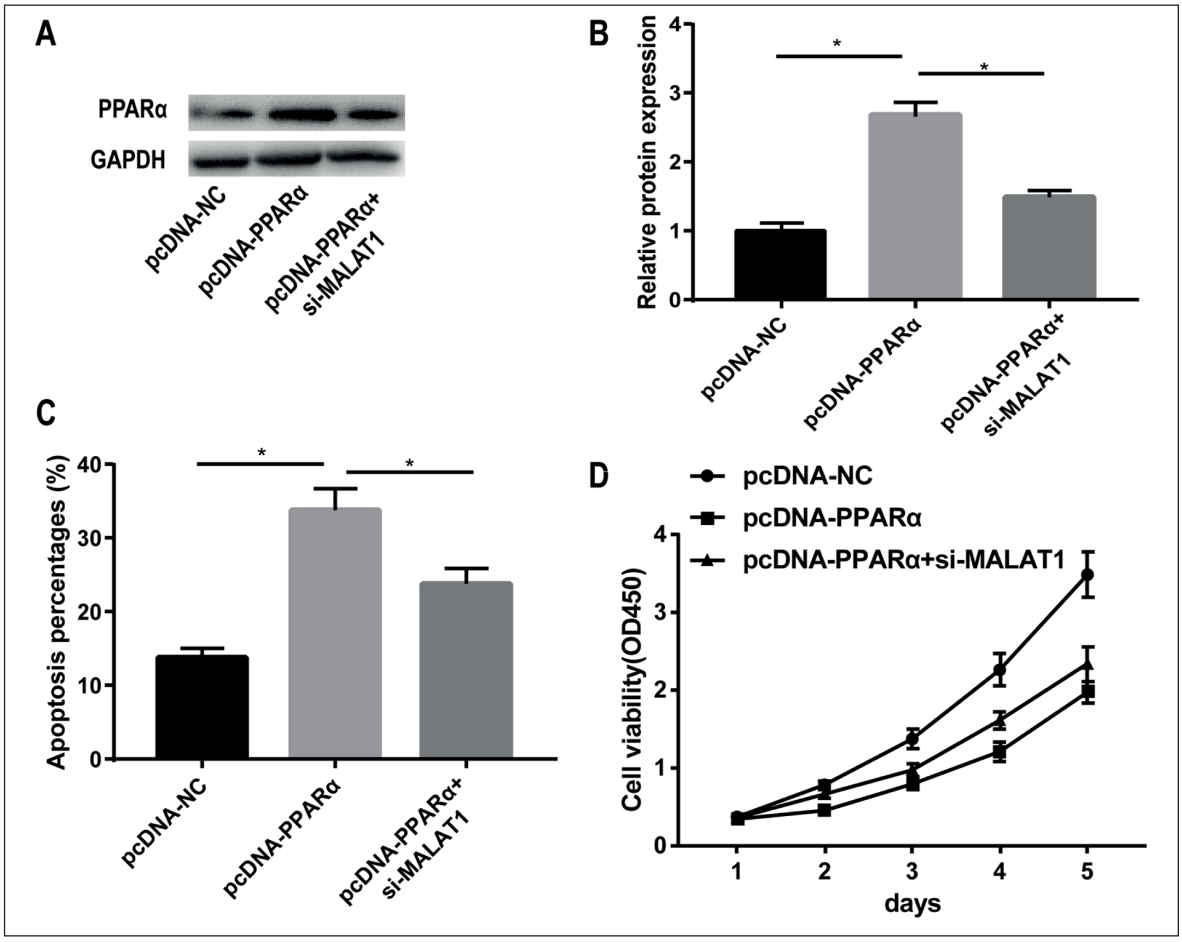

Figure 4. MALAT1 influenced VSMCs behaviors by targeting miR-124-3p/PPARα regulatory loop. **A,** Protein level of PPARα in VSMCs transfected with pcDNA-NC, pcDNA-PPARα or pcDNA-PPARα + si-MALAT1. **B,** Grey values of PPARα in VSMCs transfected with pcDNA-NC, pcDNA-PPARα or pcDNA-PPARα + si-MALAT1. **C,** Apoptotic rate in VSMCs transfected with pcDNA-NC, pcDNA-PPARα or pcDNA-PPARα + si-MALAT1. **D,** Viability in VSMCs transfected with pcDNA-NC, pcDNA-PPARα or pcDNA-PPARα + si-MALAT1.

of pcDNA-PPARα reduced viability in VSMCs, but it was then reversed by co-transfection of si-MALAT1 (Figure 4D). Therefore, we believed that MALAT1 influenced apoptosis and viability of VSMCs by absorbing miRNA-124-3p to positively regulate PPARα level.

Discussion

Cardiovascular disease (CVD) is highly prevalent in modern society. AS is the leading cause of high mortality in CVD populations[17]. As an inflammatory disease, AS affects the arterial wall by the accumulation of lipids and inflammatory cells in the intima of the aorta[18]. Oxidized low-density lipoprotein (ox-LDL) particles infiltrate and accumulate in the extracellular matrix (ECM). It stimulates proliferative and migratory rates of VSMCs, thus promoting the progression of atherosclerotic plaque[19]. The formation of immature blood vessels and decreased number of VSMCs enhance the rupture susceptibility to atherosclerotic lesions, leading to acute myocardial infarction or sudden death[20]. Risk factors of AS include high levels of total cholesterol and LDL-C. In addition, hypertension, smoking, obesity, and sedentary lifestyle are believed to be related to AS[21].

In the epigenetic process, lncRNAs mediate functional modifications, histone modification, and chromosome remodeling[22,23]. Lv et al[24] have shown that lncRNAs participate in the regulation of CVD by absorbing miRNAs. It is reported that

KLF4 protects brain microvascular endothelial cells from ischemic stroke-induced apoptosis by activating MALAT1 at the transcriptional level[25]. MALAT1 could regulate endothelial cell function and vascular growth[26]. Through absorbing miR-204, MALAT1 promotes osteogenic differentiation in human aortic valve stromal cells by upregulating Smad4[27]. In addition, MALAT1-derived genes are involved in cardiovascular innate immunity[28]. These studies revealed that MALAT1 may be involved in the pathological process of CVD.

Proliferation and apoptosis of VSMCs are involved in the pathogenesis of AS. A relevant study[29] suggested that lncRNA XR007793 mediates proliferative and migratory abilities of VSMCs by downregulating miR-23b. LncRNA UCA1 indirectly regulates PETN level by absorbing miR-26a, thus influencing cellular performances of VSMCs[30]. PPARα is a member of the peroxisome proliferator-activated receptor (PPAR) family[31]. PPARα inhibits cell cycle progression from G1 to S phase, proliferative and migratory trends of VSMCs, and induces apoptosis[32]. It is believed that VSMCs injury following AS is extremely important and responsible for aggravating CVD[33,34]. In this paper, MALAT1 and PPARα were gradually downregulated with the prolongation of ox-LDL treatment, whereas miRNA-124-3p was upregulated. Overexpression of MALAT1 attenuated viability and induced apoptosis in ox-LDL-treated VSMCs. More importantly, MALAT1 served as a miRNA sponge that absorbed miRNA-124-3p to further influence the expression level of PPARα. Collectively, this study verified the biological role of MALAT1/miRNA-124-3p/PPARα regulatory loop in mediating proliferative and apoptotic abilities of VSMCs.

Conclusions

In this report it has been demonstrated that lncRNA MALAT1 mediates proliferation and apoptosis of VSMCs by sponging miRNA-124-3p to positively regulate PPARα level.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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