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LncRNA HOTAIR regulates the proliferation and apoptosis of vascular smooth muscle cells through targeting miRNA-130b-3p/PPARα axis

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Abstract. – OBJECTIVE: To investigate the role of long non-coding RNA (lncRNA) HOTAIR in influencing the proliferative and apoptotic abilities of vascular smooth muscle cells (VSMCs) by regulating microRNA-130b-3p (miRNA-130b-3p)/peroxisome proliferator-activated receptor alpha (PPARα) axis.

MATERIALS AND METHODS: The expression levels of HOTAIR, miRNA-130b-3p, and PPARα in VSMCs treated with different doses of ox-LDL for different time points were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The subcellular distribution of HOTAIR in VSMCs was examined. The regulatory effects of HOTAIR, miRNA-130b-3p, and PPARα on the viability and apoptosis of ox-LDL-treated with VSMCs were assessed. The interaction among HOTAIR, miRNA-130b-3p, and PPARα was evaluated through Dual-luciferase reporter gene assay and Western blot.

RESULTS: After treatment of different doses of ox-LDL in VSMCs, the levels of HOTAIR and PPARα were gradually downregulated, whereas miRNA-130b-3p was upregulated. The overexpression of HOTAIR reversed the enhanced viability and suppressed apoptosis in ox-LDL-treated VSMCs. HOTAIR was mainly distributed in nucleus of VSMCs. MiRNA-130b-3p was the direct target of HOTAIR and its level was negatively regulated by HOTAIR. Moreover, miRNA-130b-3p could bind to PPARα and could negatively regulate its level. The knockdown of HOTAIR could reverse the regulatory effect of PPARα on the proliferative and apoptotic abilities of VSMCs

CONCLUSIONS: HOTAIR reduces the proliferative ability and stimulates the apoptosis in ox-LDL-treated VSMCs by targeting miRNA-130b-3p/PPARα axis.

Key Words:
VSMCs, HOTAIR, MiRNA-130b-3p, PPARα.

Introduction

Vascular smooth muscle cells (VSMCs) are the main components of the blood vessel walls, and they contribute to maintain the vascular tone. Abnormal proliferation of VSMCs is the common pathological basis of vascular diseases, including atherosclerosis, restenosis after revascularization, and hypertension[1,2]. Under certain stimuli, VSMCs present tumor-like biological characteristics[3]. It is very significant to explore the biological functions of VSMCs.

A large number of RNAs that do not have protein-encoding function are identified in eukaryotes. Among them, long non-coding RNAs (lncRNAs) are those with over 200 nt long. LncRNAs exert regulatory effects on cellular function and structure through chromatin regulation, post-transcriptional regulation, protein complexes, and allosteric regulation[4]. Pian et al[5] have shown that lncRNAs are sensitive to changes in vascular transmural pressure and thus influence vascular function and structure. Moreover, lncRNAs are able to mediate the proliferative and apoptotic abilities of VSMCs[6,7]. LncRNA HOTAIR has a trans-regulatory effect on the HOX gene cluster on different chromosomes[8]. HOTAIR is involved in many tumor diseases and autoimmune diseases[9,10]. Fu et al[11] reported that HOTAIR directly targets VEGFA and stimulates its transcription, thus mediating angiogenesis.

MicroRNAs (miRNAs) are highly conserved endogenous small RNA that block the transcription of target genes by specifically binding to mRNA 3′untranslated region (3′UTR), thereafter influencing a series of life activities[12]. MiRNAs are a key link in lncRNA-induced pathological changes. LncRNA-miRNA network is of impor-

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tance in the disease progression[13]. Wang et al[14] showed that the serum level of miRNA-130b-3p is elevated in obese population, which is positively correlated to BMI, blood triglycerides, and blood cholesterol levels. The role of miRNA-130b-3p in VSMCs, however, is still unclear.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear transcription factors. PPARs are members of the type II nuclear receptor superfamily and have multiple functions. PPARα is a subtype of PPARs, which is abundantly expressed in VSMCs and has an inhibitory effect on the proliferative and migratory abilities of VSMCs[15]. It is reported that miRNA-27 specifically binds to the 3'UTR of PPARγ and thus inhibits its expression, demonstrating that PPARγ is a target gene for miRNA-27[16]. In this paper, we mainly explored the role of HOTAIR/miRNA-130b-3p/PPARα regulatory loop in influencing the proliferative and apoptotic abilities of VSMCs, thus providing a new idea for clinical treatment of vascular diseases.

Materials and Methods

Cell Culture

VSMCs were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 Ul/mL penicillin and 0.1 mg/mL streptomycin, in at 37°C in a 5% CO_2 incubator. Fourth- or fifth-generation 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 *in vitro* hyperlipidemia.

Cell Transfection

The cells were pre-seeded in a 6-well plate with 2×10^5 cells per well and subjected to transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 h later, the complete medium was replaced. The transfected cells for 24-48 h were harvested for *in vitro* studies. Primers of transfection vectors were as follows: si-HOTAIR forward primers: 5′-CTCTCTGTCCCGGCTGGATG-3′, and reverse primers: 5′-CGTCGCCGTCCAGCTCGACCAG-3′.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The extraction of the total RNA in cells or tissues was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was qualified by an ultraviolet spectrophotometer and subjected to reverse transcription. The extracted complementary deoxyribose nucleic acid (cDNA) was applied for PCR using SYBR Green method (TaKaRa, Otsu, Shiga, Japan). The primer sequences were listed as follows: HOTAIR, forward: 5′-GCTTCCTTGCTCTTCTTAT-3′ and reverse: 5′-TGAGATAGAGGTGCTTGG-3′; miRNA-130b-3p, forward: 5'-GGGCAGTGCAATGATGAAA-3' and reverse: 5'-GTGCGTGTCGTGGAGTCG-3'; PPARα, forward: 5'-GGACCGACACAGCCATGTAAA-3' and reverse: 5'-CTCTTCATCCCCAAGCGTAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-CGAGATCCCTCCAAAATCAA-3', and reverse: 5'-TTCACACCCATGACGAACAT-3'; U6, forward: 5'-TCCGATCGTGAAGCGTTC-3', and reverse: 5'-GTGCAGGGTCCGAGGT-3'.

Cell Counting Kit-8 (CCK-8)

VSMCs were seeded in the 96-well plate with 2×10^4 cells per well 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.

Flow Cytometry

VSMCs were washed with Phosphate-Buffered Saline (PBS) twice and digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin. After resuspension and density adjustment to 1×10^6 cells/mL, the 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. The apoptosis was determined within 1 h by flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

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 reference of nucleus and U1 was that of cytoplasm.

Dual-Luciferase Reporter Gene Assay

293T cells were inoculated in a 96-well plate with 1.5×10^4 cells/well. They were co-transfected with miRNA-130b-3p mimics/NC and wild-type/

mutant-type vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 24 h later, the co-transfected cells were harvested for determining the luciferase activity using a Dual-luciferase reporter assay system (Promega, Madison, WI, USA).

Western Blot

The 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) membrane (Roche, Basel, Switzerland). The membranes were blocked in 5% skim milk for 2 h, and subjected to incubation with primary and secondary antibodies. The bands were exposed by electrochemiluminescence (ECL) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 19.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The intergroup differences were analyzed by the *t*-test. $p<0.05$ was considered as statistically significant.

Results

HOTAIR Mediated VSMCs Proliferation and Apoptosis

After treatment of 0, 25, 50, and 100 mg/L ox-LDL in VSMCs, the relative level of HOTAIR was gradually downregulated in a dose-dependent manner (Figure 1A). Besides, HOTAIR was time-dependently downregulated with the prolongation of ox-LDL treatment (Figure 1B). The transfection of pcDNA-HOTAIR greatly upregulated the HOTAIR level in VSMCs, suggesting a sufficient transfection efficacy (Figure 1C). ox-LDL treatment led to viability elevation and apoptosis reduction in VSMCs, which were reversed by the overexpression of HOTAIR (Figures 1D, 1E).

HOTAIR Sponged MiRNA-130b-3p

The subcellular distribution revealed that HOTAIR was mainly expressed in the nucleus of VSMCs (Figure 2A). MiRNA-130b-3p was dose-dependently upregulated in ox-LDL-treated VSMCs (Figure 2B). The potential binding sequences between HOTAIR and miRNA-130b-3p were identified through online website (StarBase 3.0) (Figure 2C). Through Dual-luciferase reporter gene assay, a remarkable decline in the luciferase activity was observed after the co-transfection of HOTAIR-WT and miRNA-130b-3p mimics, verifying that HOTAIR could sponge miRNA-130b-3p (Figure 2D). After transfection of pcDNA-HOTAIR, the relative level of miRNA-130b-3p was markedly downregulated in VSMCs (Figure 2E). In ox-LDL-treated VSMCs, a negative correlation was identified between HOTAIR and miRNA-130b-3p (Figure 2F).

MiRNA-130b-3p Targeted PPARα

Potential binding sequences were searched between miRNA-130b-3p and PPARα on Starbase 3.0 (Figure 3A). The luciferase activity was markedly reduced by the co-transfection of PPARα-WT and miRNA-130b-3p mimics (Figure 3B). PPARα was dose-dependently downregulated in VSMCs treated with ox-LDL (Figure 3C). The protein level of PPARα was upregulated after transfection of miRNA-130b-3p inhibitor in VSMCs (Figures 3D, 3E). Hence, miRNA-130b-3p was determined to target and negatively regulate PPARα.

HOTAIR Regulated VSMCs Proliferation and Apoptosis Via Targeting PPARα

To uncover the role of HOTAIR/miRNA-130b-3p/PPARα axis in influencing the viability and apoptosis of VSMCs, a series of rescue experiments were conducted. It is found that the protein level of PPARα was downregulated after the co-transfection of si-HOTAIR in VSMCs overexpressing PPARα (Figures 4A, 4B). In addition, the enhanced apoptotic rate in VSMCs overexpressing PPARα was reversed by knockdown of HOTAIR (Figure 4C). The overexpression of PPARα reduced the viability in VSMCs, which were reversed by the co-transfection of si-HOTAIR (Figure 4D). It is suggested that HOTAIR influenced the viability and apoptosis in VSMCs by targeting PPARα.

Discussion

Under normal physiological conditions, VSMCs are highly differentiated cells that mainly express contractile-related proteins. VSMCs contribute to contraction perfor-

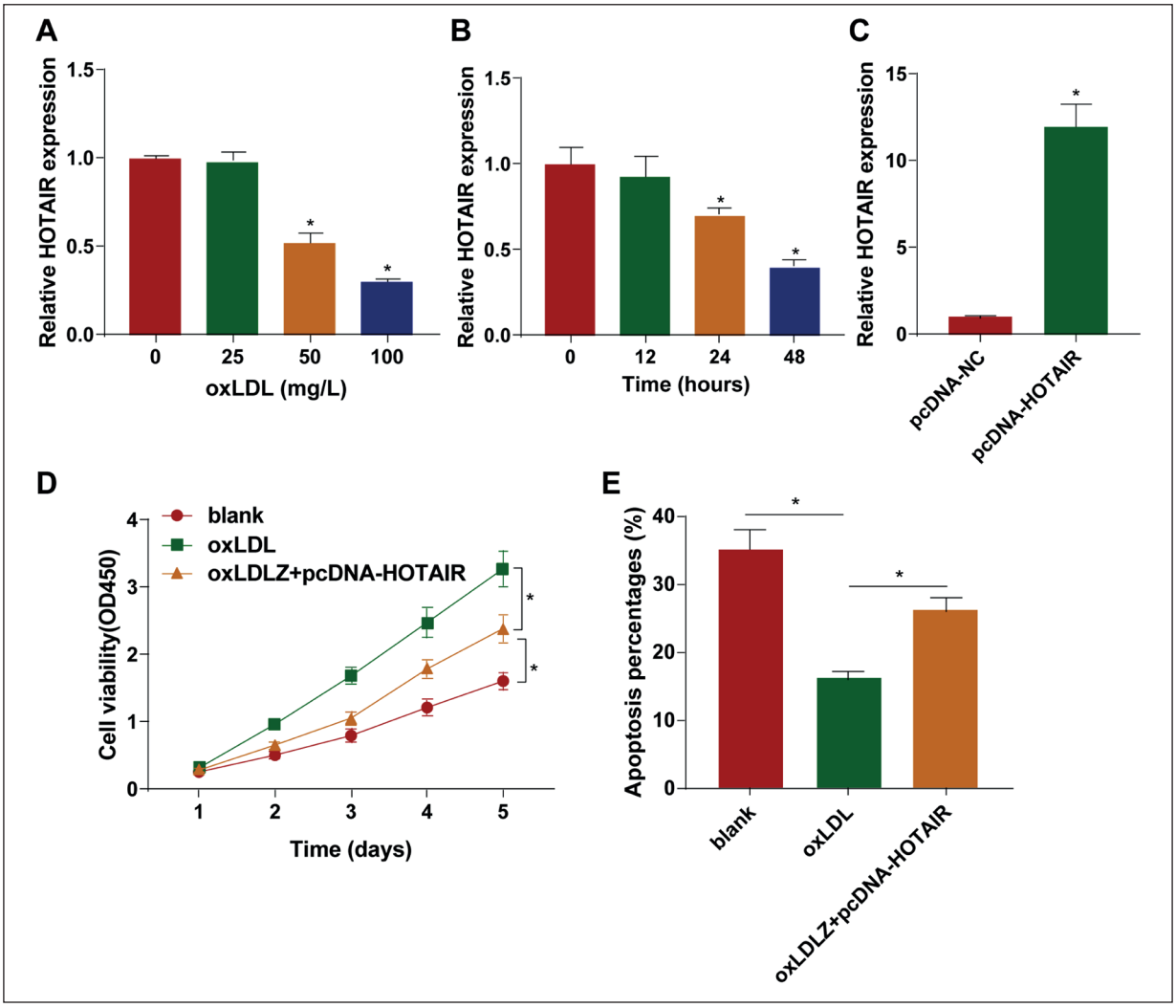

Figure 1. HOTAIR mediated VSMCs proliferation and apoptosis. **A,** The relative level of HOTAIR in VSMCs treated with 0, 25, 50, and 100 mg/L ox-LDL. **B,** The relative level of HOTAIR in VSMCs treated with 100 mg/L ox-LDL for 0, 12, 24, and 48 h. **C,** The transfection efficacy of pcDNA-HOTAIR in VSMCs. **D,** The cell viability in VSMCs treated with blank control, ox-LDL or ox-LDL + pcDNA-HOTAIR. **E,** The apoptotic rate in VSMCs treated with blank control, ox-LDL, or ox-LDL + pcDNA-HOTAIR.

mance, vascular tone regulation, and blood pressure maintenance. Under certain stimuli, the contractile phenotype of VSMCs is transformed into proliferative phenotype, manifesting as suppressed expressions of contractile-related proteins, weakened contractility, and enhanced proliferative ability. The proliferative VSMCs exert anti-injury and repair functions, and are involved in the progression of vascular diseases (i.e., hypertension and atherosclerosis)[17-19].

The initially considered transcriptional noises - miRNAs and lncRNAs - have been identified to participate in cellular behaviors and tumor progression at epigenetic, transcriptional and post-transcriptional levels[20]. For example, the upregulation of lncRNA ROR is positively correlated to susceptibility to myocardial hypertrophy. LncRNA ROR regulates the pathological hypertrophy of cardiomyocytes by downregulating miR-133[21]. Consistently, our study showed that lncRNA HOTAIR influenced the proliferative and apoptotic abilities of ox-LDL-treated VSMCs by targeting miRNA-130b-3p.

PPARs are related to insulin sensitivity, glucose balance, cytokine production, and vascular protection[22]. PPARα agonists inhibit

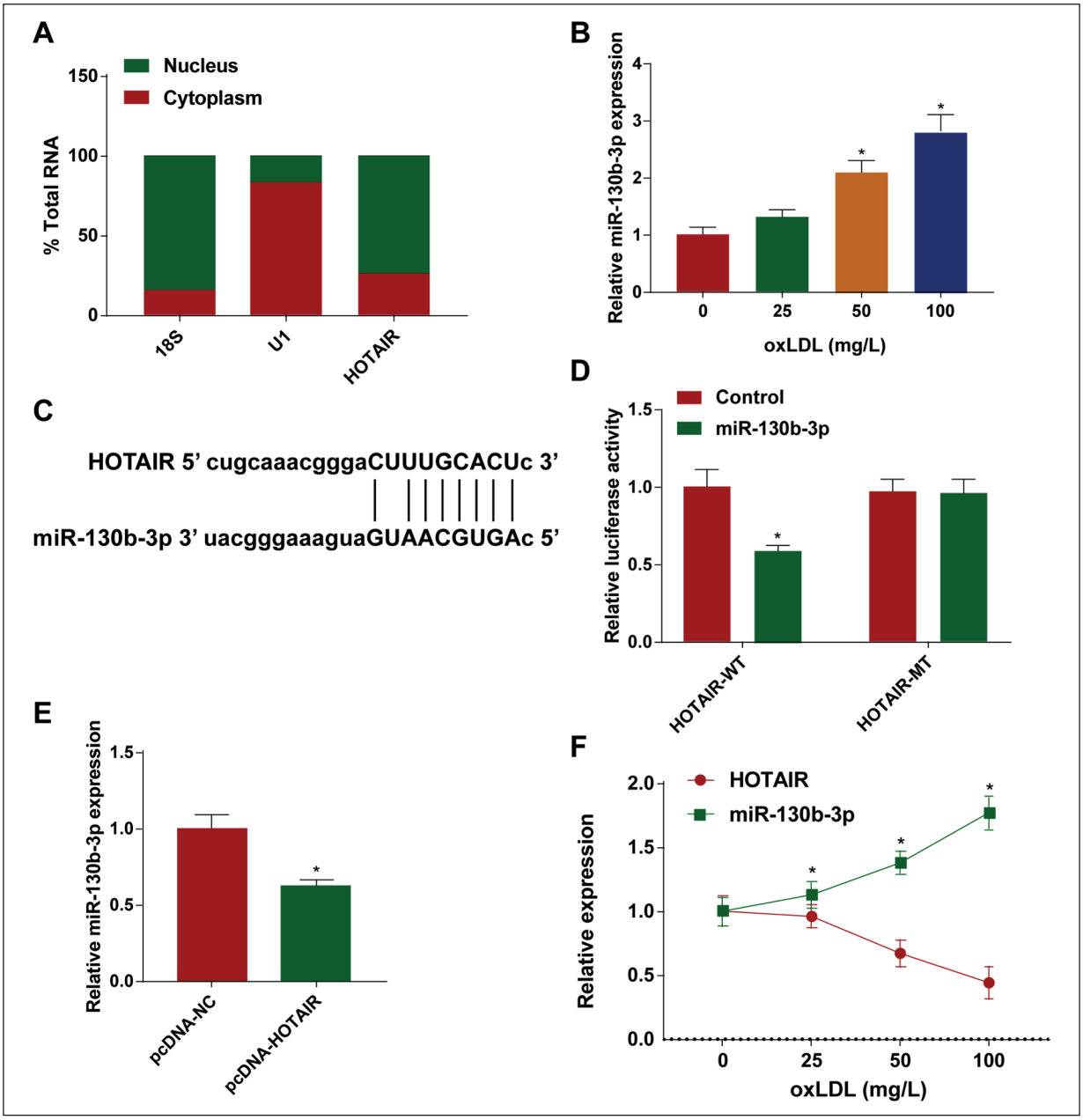

Figure 2. HOTAIR sponged miR-130b-3p. **A,** The subcellular distribution of HOTAIR in nucleus and cytoplasm. 18S and U1 were the internal references for nucleus and cytoplasm, respectively. **B,** The relative level of miR-130b-3p in VSMCs treated with 0, 25, 50, and 100 mg/L ox-LDL. **C,** The potential binding sequences between HOTAIR and miR-130b-3p. **D,** The luciferase activity in VSMCs co-transfected with HOTAIR-WT/HOTAIR-MT and miR-130b-3p mimics/control. **E,** The relative level of miR-130b-3p in VSMCs transfected with pcDNA-NC or pcDNA-HOTAIR. **F,** A negative correlation between HOTAIR and miR-130b-3p in ox-LDL-treated VSMCs.

cytokine expressions (i.e., IL-6, 6-keto-PG-F1α, and COX-2) by negatively regulating NF-κB pathway, thus exerting anti-inflammatory and anti-proliferative effects[16]. Liu et al[23] have shown that lncRNA Gm15290 promotes PPARγ-induced fat deposition and weight gain in mice by adsorbing miR-27b. In this paper, HOTAIR was discovered to affect the proliferative and apoptotic abilities of VSMCs by targeting miRNA-130b-3p/PPARα axis. Our findings provide novel ideas for the explorations on VSMCs.

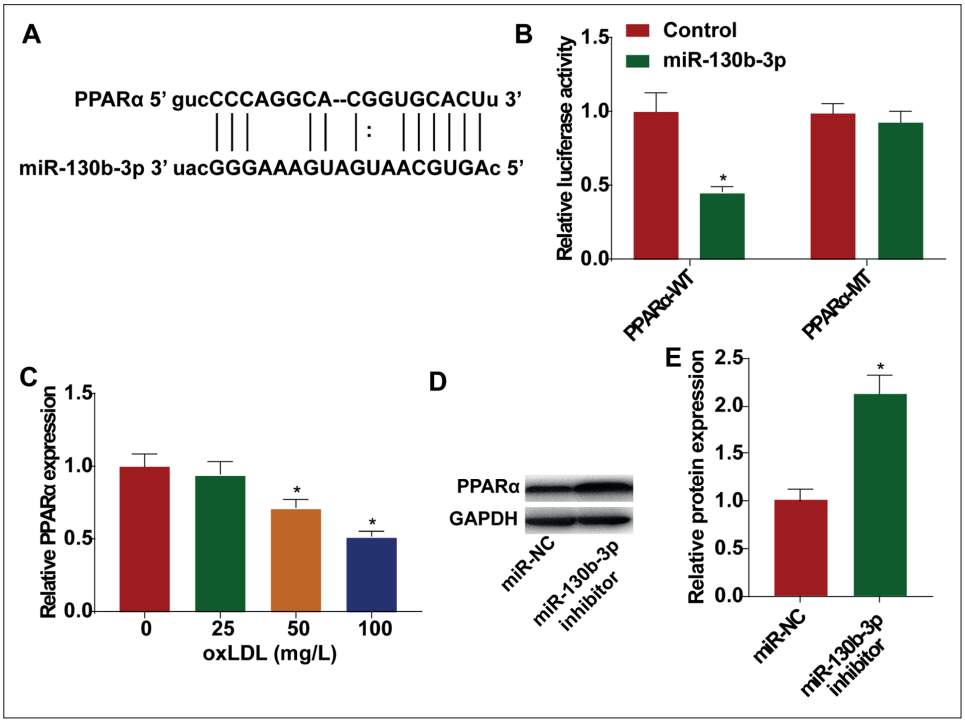

Figure 3. MiR-130b-3p targeted PPARα. **A,** The potential binding sequences between PPARα and miR-130b-3p. **B,** The luciferase activity in VSMCs co-transfected with PPARα-WT/PPARα-MT and miR-130b-3p mimics/control. **C,** The relative level of PPARα in VSMCs treated with 0, 25, 50, and 100 mg/L ox-LDL. **D,** The Western blot analysis of PPARα in VSMCs transfected with miR-NC or miR-130b-3p inhibitor. **E,** The protein expression of PPARα in VSMCs transfected with miR-NC or miR-130b-3p inhibitor.

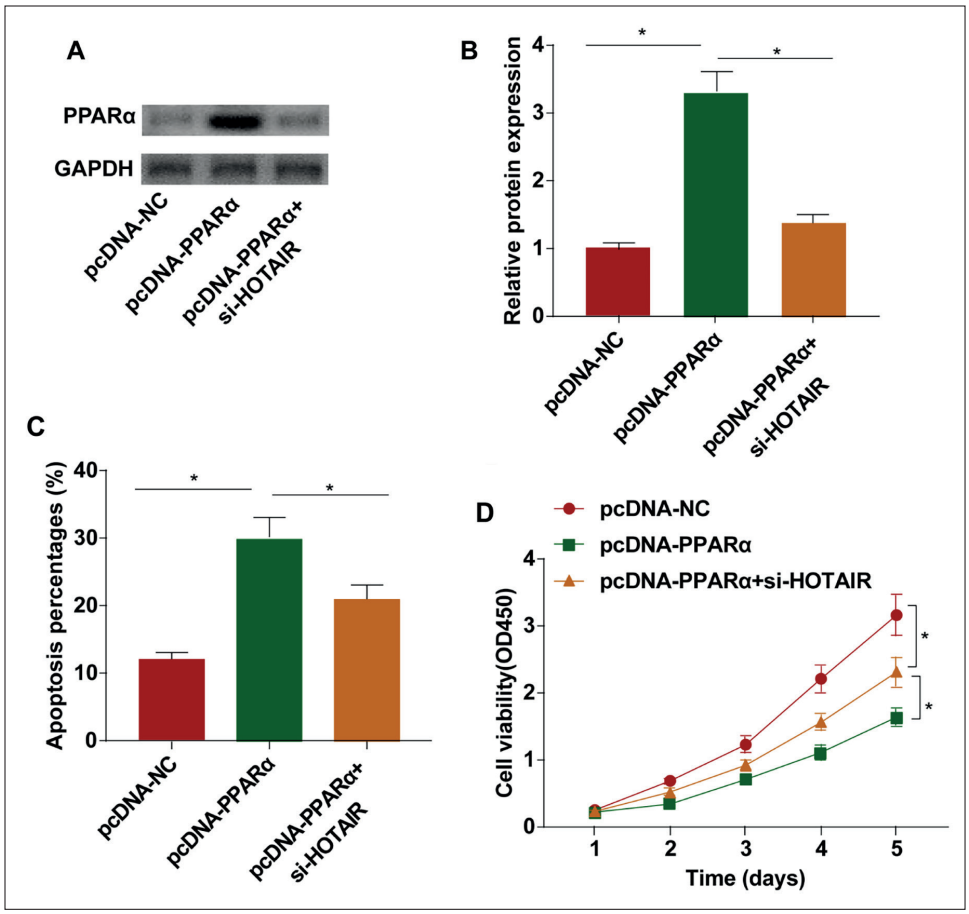

Figure 4. ,HOTAIR regulated VSMCs proliferation and apoptosis via targeting PPARα. **A,** The Western blot analysis of PPARα in VSMCs transfected with pcDNA-NC, pcDNA-PPARα, or pcDNA-PPARα + si-HOTAIR. **B,** The protein expression of PPARα in VSMCs transfected with pcDNA-NC, pcDNA-PPARα, or pcDNA-PPARα + si-HOTAIR. **C,** The apoptotic rate in VSMCs transfected with pcDNA-NC, pcDNA-PPARα, or pcDNA-PPARα + si-HOTAIR. **D,** The cell viability in VSMCs transfected with pcDNA-NC, pcDNA-PPARα, or pcDNA-PPARα + si-HOTAIR.

Conclusions

We demonstrated that HOTAIR reduces the proliferative rate and enhances the apoptotic rate in ox-LDL-treated VSMCs by targeting miRNA-130b-3p/PPARα axis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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