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Expression of Long Noncoding RNA LIPCAR Promotes Cell Proliferation, Cell Migration, and Change in Phenotype of Vascular Smooth Muscle Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: The long noncoding RNA LIPCAR is a type of transcription product (>200 nucleotides long). Recent studies demonstrated that LIPCAR is a potential biomarker in cardiovascular disease and can predict survival in patients with cardiovascular disease. Therefore, the present study explored the role of LIPCAR in the regulation of proliferation, migration, and change in phenotype of vascular smooth muscle cells.

Material/Methods: Human vascular smooth muscle cells (VSMCs) were treated with 20 g/mL oxidatively modified low-density lipoprotein (ox-LDL) or 20 ng/ml platelet-derived growth factor BB (PDGF-BB) for 24 h, then the expression levels of LIPCAR were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. LIPCAR-overexpressing plasmids were transfected into VSMCs. After transfection, cell proliferation and migration were measured using the Cell Counting Kit-8 (CCK-8) and Transwell assays, respectively. The levels of α-smooth muscle actin (α-SMA) a molecular marker of the contractile VSMC phenotype, were measured using Western blot and immunofluorescence assays. Protein levels of cyclin-dependent kinase-2 (CDK2), proliferating cell nuclear antigen (PCNA), matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), vascular endothelial growth factor A (VEGF-A), and angiopoietin-2 (Ang-2) were assessed by Western blot. The level of tissue factor (TF) was measured by enzyme-linked immunosorbent assay (ELISA).

Results: Treatment with PDGF-BB or ox-LDL significantly increased levels of LIPCAR in VSMCs. Overexpression of LIPCAR markedly promoted cell proliferation and migration. Further, upregulation of LIPCAR increased CDK2, p21, PCNA, MMP2, MMP9, VEGF-A, Ang-2, and TF expression and decreased p21 expression. In addition, LIPCAR significantly decreased α-SAM expression.

Conclusions: Together, our data suggest that overexpression of LIPCAR promotes cell proliferation, migration, and phenotypic switch of vascular smooth muscle cells.

MeSH Keywords: Atherosclerosis • Cell Migration Assays • Cell Proliferation

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Background

Phenotypic switch of vascular smooth muscle cells (VSMCs) is crucial in the development of cardiovascular diseases [1,2]. Unlike terminally differentiated cells such as skeletal muscle cells and cardiomyocytes, VSMC is characterized by its phenotypic transformation ability in response to internal and external environmental stimuli [3,4]. VSMC phenotype switching triggers migration to the intima and promotes cell proliferation, ultimately resulting in intimal hyperplasia, arterial wall degeneration, and postangioplasty restenosis [1]. Accumulating evidence shows that proliferation and migration of VSMCs serve key roles in vascular remodeling, and the imbalance of those functions contributes to initial atherosclerotic plaque formation, which plays an important role in arteriosclerosis [5,6]. Therefore, exploring the novel therapeutic targets modulating the VSMCs proliferation, migration and phenotypic switch could improve treatment of atherosclerosis, thus reducing the incidence and mortality of cardiovascular diseases.

Long noncoding RNAs (lncRNAs) are a type of transcription product that are longer than 200 nucleotides. Although lacking the function of encoding proteins, lncRNAs modulate gene expression via a multilevel-regulated pathway [7,8]. Emerging evidence demonstrates that lncRNAs can act as positive or negative regulators in variety of major cellular functions, such as proliferation, migration, and apoptosis [9]. For example, Shi et al. demonstrate that lncRNA TUG1 activates the Wnt/β-catenin pathway via directly targeting miR-145-5p, thus promoting the proliferation and migration of VSMCs in hypertensive state [10]. Similarly, an *in vitro* study by Yao et al. showed that the level of lnc00113 is significantly upregulated in atherosclerosis patients, and downregulation of lnc00113 markedly inhibits VSMCs and human umbilical vein endothelial cells (HUVECs) proliferation, survival, and migration via activating the PI3K/Akt/mTOR signaling pathway [11]. lncRNAs are emerging as important regulators of a variety of VSMCs responses and developmental processes of vascular diseases [12].

Importantly, recent studies demonstrated that long noncoding RNA LIPCAR is a potential biomarker in cardiovascular disease [13,14] and overexpression of LIPCAR in plasma may be a warning sign for the diagnosis of ST-segment elevation myocardial infraction (STEMI) [15]. However, the underlying mechanism of LIPCAR in atherosclerosis is poorly understood.

Here, we explored the role of LIPCAR in the regulation of proliferation, migration, and change in phenotype of vascular smooth muscle cells. We found that the expression of LIPCAR was significantly increased in platelet-derived growth factor BB (PDGF-BB)-treated and oxidatively modified low-density lipoprotein (ox-LDL)-treated VSMCs. We also demonstrated that overexpression of LIPCAR promotes cell proliferation, migration, and phenotypic switch of VSMCs.

Material and Methods

Cell culture and treatment

Human VSMCs obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were human aorta smooth muscle. These VSMCs were originated from an 11-month-old white female. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), incubated at 37°CC in a humidified atmosphere with 5% CO_2. Cells were treated with ox-LDL (20 μg/ml) or PDGF-BB (20 ng/ml) for 24 h. The overexpression plasmid pcDNA-LIPCAR and negative control plasmid pcDNA-control were designed and purchased from Hanbio (Shanghai, China). Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) was used to transfect plasmids into VSMCs. Cells transfected for 48 h were used for further research.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA from VSMCs was isolated with TRIzol reagent (Invitrogen, USA), and the TaqMan Reverse Transcription Kit (Takara, Dalian, China) was used to obtain the cDNA. Then quantitative PCR was conducted with the QuantiTect SYBR Green PCR Kit (Bio-Rad, USA) performed on an ABI 7500 thermocycler (Thermo Fisher Scientific, Inc., USA). The expression levels of LIPCAR relative to U6 were determined using the $2^{-\Delta\Delta Ct}$ method. The primer sequence used was LIPCAR:
Forward 5'-TAAAGGATGCGTAGGGATGG-3',
Reverse 5'-TTCATGATCACGCCCTCATA-3';
U6: Forward 5'-GCTTCGGCAGCACATATACTAAAAT-3',
Reverse 5'-CGCTTCACGAATTTGCGTGTCAT-3'.

Immunofluorescence

At 48 h after transfection, the level of α-smooth muscle actin (α-SMA) was measured using immunofluorescence assay. Briefly, after fixing in 4% formaldehyde (CAS#: 50-00-0, Sigma-Aldrich, USA) for 15 min and permeabilizing with 0.1% Triton™ X-100 (CAS#: 9002-93-1, Sigma-Aldrich, USA) for another 15 min at room temperature, cells were blocked with 5% normal goat serum (#5425, cell signaling technology) at room temperature for 1 h. Subsequently, cells were probed overnight at 4°C with α-SMA (1: 50, #34105, cell signaling technology), followed by incubation with fluorescein-conjugated secondary antibodies (1: 1000, #4409, Cell Signaling Technology) for 1 h at room temperature. Finally, after staining with DAPI (D9542, Sigma-Aldrich, USA) for 5 min, samples were imaged using a fluorescence microscope (IX73-A12FL/PH; Olympus, Japan) at 200× magnification.

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Cell Counting Kit-8 (CCK-8)

At 24 h after transfection, VSMCs (2×10^3 cells/well) were plated into 96-well plates. At 24, 48, and 72 h after incubation, 10 µl CCK-8 reagent (Beyotime, Beijing, China) was added and the cells were incubated for 2 h at 37°C. Absorbance was determined at 450 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

Transwell assay

Cell migration was determined by Transwell assay (Corning Incorporated, Corning, NY, USA). At 24 h after transfection, VSMCs (5×10^3 cells/ml) were placed into the upper chambers, and 500 µl DMEM containing 10% FBS was placed into the lower chamber. After 24 h, the migrated cells in the lower chamber were fixed with 4% formaldehyde and stained with 0.1% crystal violet. The numbers of migrated cells were determined with a light microscope (magnification, ×200; Nikon Corporation, Tokyo, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

At 48 h after transfection, the supernatant is collected. The level of tissue factor (TF) in the supernatant was determined use of the Human Tissue Factor ELISA Kit (ab220653, Abcam, USA) followed the manufacturer's protocols. The absorbance (OD value) of each hole at the wavelength of 450 nm was determined by BioTek Synergy 2 within 20 min.

Western blot

Cells transfected for 48 h were collected, and total proteins were extracted from cells using RIPA lysis buffer (Beyotime, Shanghai, China). Then, protein samples (25 ug/lane) were resolved with 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Wet transfer was then performed and protein blotting was transferred onto polyvinylidene fluoride membranes (PVDF, Millipore, USA). We used 5% non-fat milk to block the non-specific binding sites, and membranes were probed at 4°C overnight with primary antibodies: p21 Waf1/Cip1 (1: 500, #2946S, Cell Signaling Technologies), cyclin-dependent kinase-2 (CDK2) (1: 1000, #2546S, Cell Signaling Technologies), proliferating cell nuclear antigen (PCNA) (1: 1000, #13110, Cell Signaling Technologies), matrix metalloproteinase-2 (MMP-2) (1: 500, #87809, Cell Signaling Technologies), matrix metalloproteinase-9 (MMP-9) (1: 500, #13667, Cell Signaling Technologies), vascular endothelial growth factor A (VEGF-A) (1: 1000, ab46154, Abcam), angiopoietin 2 (Ang2) (1: 1000, ab8452, Abcam) GAPDH (1: 2000, Santa Cruz Biotechnology), and α-SAM (1: 1000, A5228, Sigma). Membranes were then incubated with HRP-conjugated secondary antibodies and immunoblots were visualized using an ECL Plus chemiluminescence kit (Amersham Bioscience, USA).

Statistical analysis

Data were expressed as mean ±SD and were analyzed with SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated ≥3 times. Statistical analysis was conducted with one-way analysis of variance, and Bonferroni's multiple comparison test was used for pairwise comparison. $P<0.05$ was defined as significant.

Results

Upregulation of LIPCAR in PDGF-BB or ox-LDL treated VSMCs

As shown in Figure 1A, the expression of LIPCAR was significantly increased in the PDGF-BB and ox-LDL groups. To investigate the effect of LIPCAR in VSMCs, the pcDNA-LIPCAR and pcDNA-control plasmids were transfected into cells. At 48 h after transfection, the transfection efficiency was detected by RT-qPCR assay. The results suggested that the expression level of LIPCAR was markedly increased in the pcDNA-LIPCAR transfected group compared with the control and NC groups (Figure 1B).

Overexpression of LIPCAR promoted proliferation and migration of VSMCs

The results of CCK-8 assay suggested that overexpression of LIPCAR significantly promoted cell proliferation at 24, 48, and 72 h after transfection (Figure 2A). Moreover, LIPCAR upregulation markedly increased CDK2 and PCNA expression and decreased p21 expression (Figure 2B, 2C). In addition, LIPCAR upregulation markedly increased migration, as determined by Transwell assay (Figure 3A, 3B). Further, the expression of matrix metalloproteinases-2 (MMP2) MMP2 and matrix metalloproteinases-2 (MMP9) were significantly increased by LIPCAR upregulation (Figure 3C).

Overexpression of LIPCAR contributed to change in phenotype of VSMCs

To investigate the role of LIPCAR in human VSMCs phenotype switch, the levels of contractile state marker gene α-SAM were detected by immunofluorescence and Western blot assays. As expected, overexpression of LIPCAR in VSMCs significantly inhibited α-SAM at the protein level in VSMCs (Figure 4A, 4B). In addition, the expression levels of TF, VEGF-A, and Ang-2 were markedly increased with the upregulation of LIPCAR (Figure 5).

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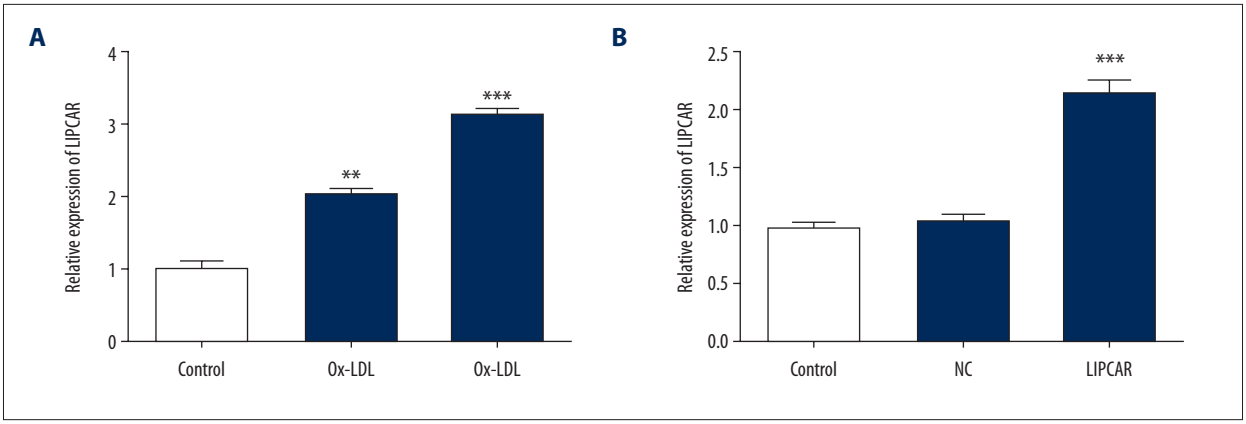

Figure 1. LIPCAR is significantly upregulated in proliferative Human vascular smooth muscle cells (VSMCs). (**A**) Human VSMCs were treated with 20 g/mL of oxidatively modified low-density lipoprotein (ox-LDL) or 20 ng/ml of platelet-derived growth factor BB (PDGF-BB) for 24 h, and the LIPCAR levels were detected using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. (**B**) The overexpression plasmid pcDNA-LIPCAR and negative control plasmid pcDNA-control were transfected into VSMCs, and the transfection efficiency was detected by RT-qPCR assay at 48 h after transfection. ** p<0.01, *** p<0.001 *vs.* control and NC group.

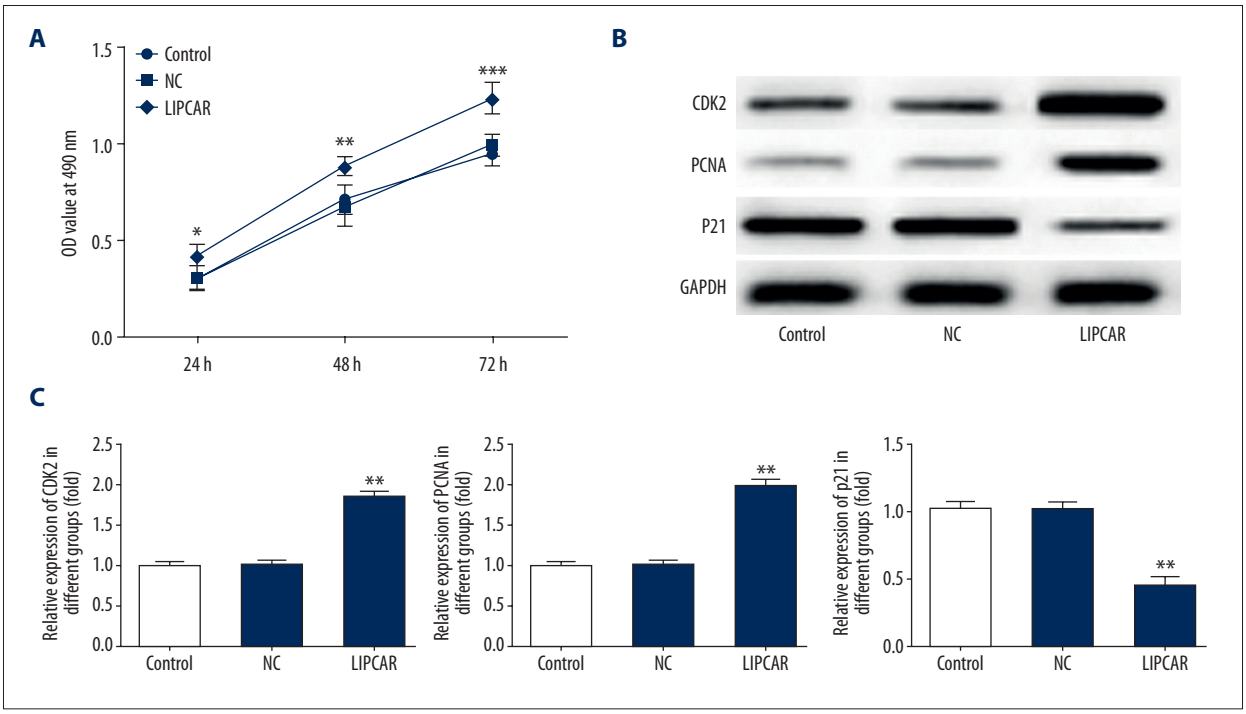

Figure 2. Overexpression of LIPCAR promotes proliferation of VSMCs. (**A**) At 24, 48, and 72 h after transfection, cell proliferation was measured by Cell Counting Kit-8 (CCK-8) assay. (**B**) At 48 h after transfection, the protein levels of cyclin-dependent kinase-2 (CDK2), p21, and proliferating cell nuclear antigen (PCNA) were detected by Western blot. (**C**) Densitometric analysis of CDK2, p21 and PCNA protein levels as measured by Western blot. * p<0.05, ** p<0.01, *** p<0.001 *vs.* control and NC group.

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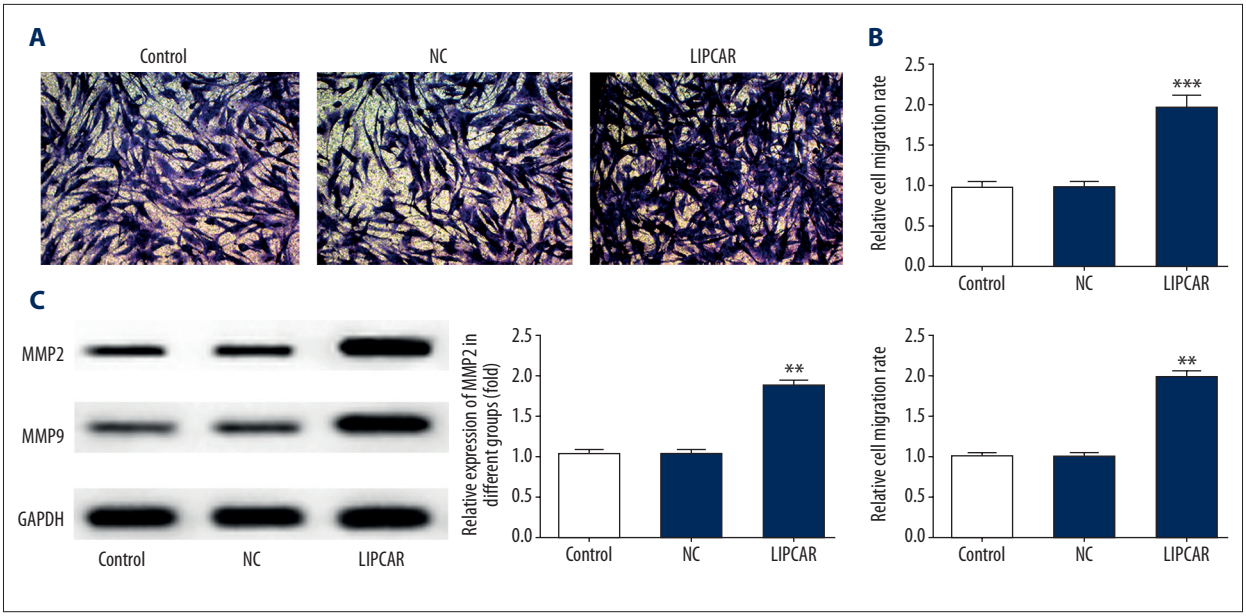

Figure 3. Overexpression of LIPCAR promotes migration of VSMCs. At 48 h after transfection, (**A**) cell migration was observed by a light microscope, (**B**) cell migration rate was measured by Transwell assay, and (**C**) the protein levels of matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9) were detected by Western blot. ** p<0.01, *** p<0.001 *vs.* control and NC group.

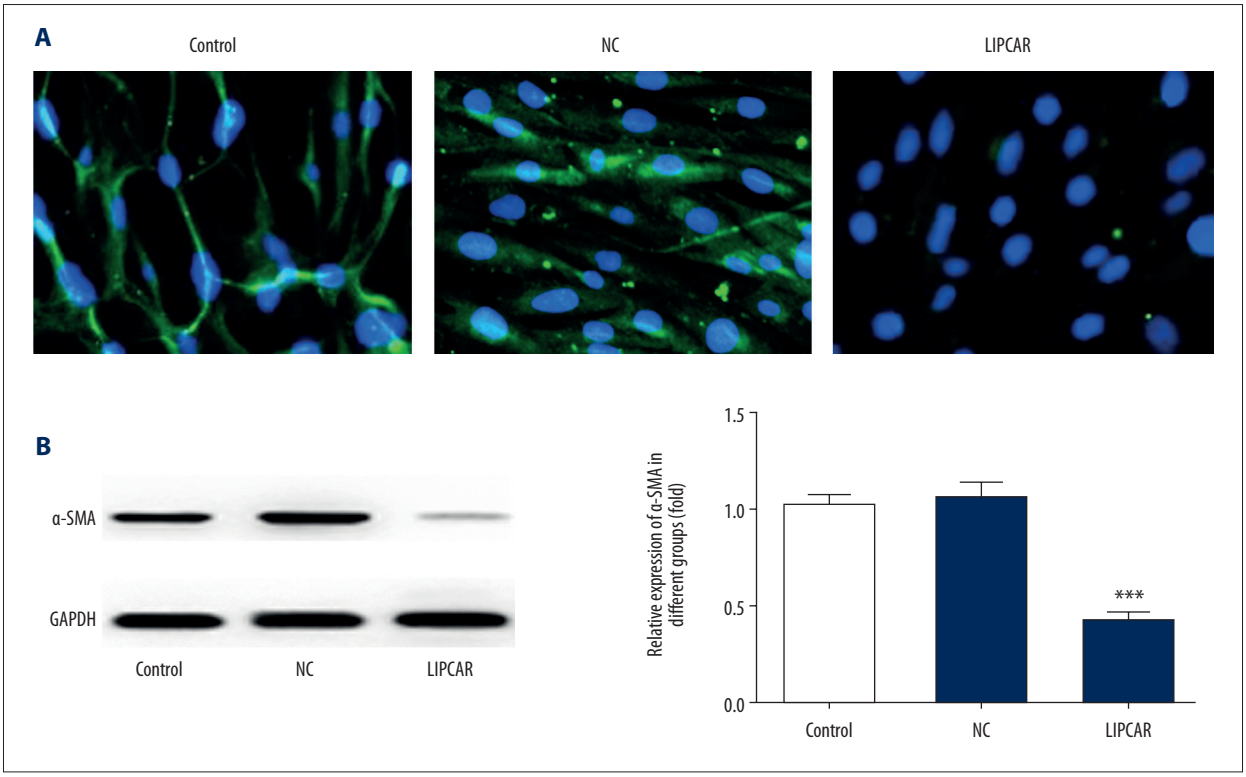

Figure 4. Overexpression of LIPCAR contributes to phenotypic switch of VSMCs. At 48 h after transfection, the levels of α-smooth muscle actin (α-SAM) were measured using immunofluorescence (**A**) and Western blot (**B**) assays. *** p<0.001 *vs.* control and NC group.

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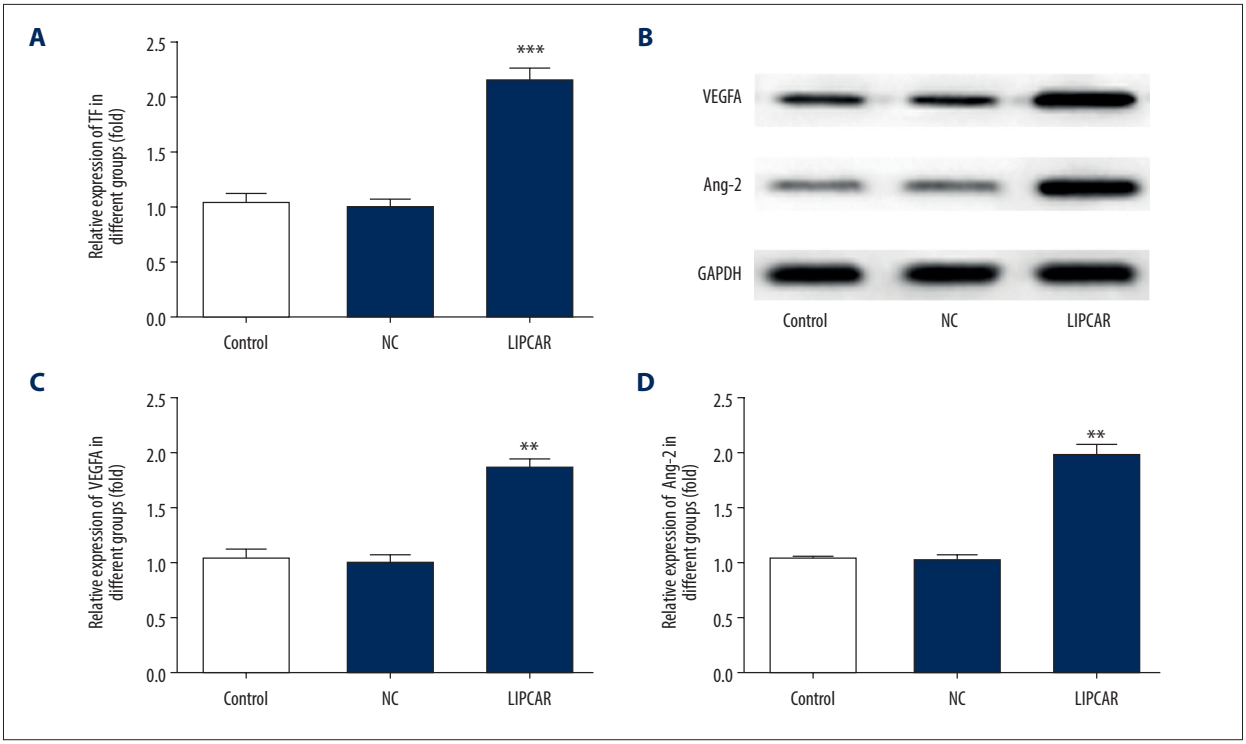

Figure 5. Overexpression of LIPCAR increases expression of vascular endothelial growth factor A (VEGF-A), angiopoietin-2 (Ang-2) and tissue factor (TF). At 48 h after transfection, (**A**) the level of TF was measured by ELISA, and (**B–D**) the protein levels of VEGF-A and Ang-2 were detected by Western blot. ** p<0.01, *** p<0.001 *vs.* control and NC group.

Discussion

In response to a wide variety of stimuli, VSMCs switch from a quiet contractile state to an active synthetic state. The aberrant phenotypic switch, accompanied by increased proliferation and migration, of VSMCs is crucial in the development of cardiovascular diseases, including atherosclerosis [1,2]. In the present study, we identified lncRNA LIPCAR as a novel modulator involved in human VSMCs proliferation, migration, and phenotype transition. We found that the expression of LIPCAR was markedly increased in PDGF-BB- and ox-LDL-treated human VSMCs. Upregulation of LIPCAR significantly increased the expression of the contractile state marker gene α-SAM. Furthermore, upregulation of LIPCAR promoted proliferation and migration of VSMCs. These experiments suggest that LIPCAR plays a key role in VSMCs phenotype switch, proliferation, and migration.

P21 is a well-known anti-proliferation gene, and PCNA and cyclin D2 are reported to exert an pro-proliferative effect in VSMCs [16,17]. Our results showed that LIPCAR markedly increased CDK2 and PCNA expression and decreased p21 expression, suggesting that LIPCAR accelerates the cell cycle by inhibiting P21 and activating CDK2 expression, thus promoting proliferation of VSMCs. In addition, the expression of MMP2 and MMP9 [18,19], which are important regulators of VSMC migration, was significantly increased by LIPCAR upregulation. These results suggest that LIPCAR regulates VSMCs function via multiple pathways.

As a novel class of gene expression regulators, lncRNAs is involved in many pathophysiological processes [20,21]. Recent studies have shown that lncRNA LIPCAR plays an important role in cardiovascular disease and may be a potential biomarker in cardiovascular disease. For example, Regalla et al. demonstrated that LIPCAR is an independent predictor of cardiac remodeling and predicts poor prognosis in patients with heart failure [15]. Moreover, a recent trial involving 300 patients with coronary heart disease (CAD) and 180 healthy control subjects showed a significant increase of LIPCAR expression in CAD patients, and LIPCAR could be a novel biomarker of coronary heart disease [13]. The above studies indicate that LIPCAR plays an important role in cardiovascular diseases. However, little is known about its underlying mechanism. Herein, we explored the effect of LIPCAR on human VSMCs, and our results demonstrated that LIPCAR contributes to differentiation, proliferation, and migration of VSMCs. Research has revealed that VSMCs switching is an essential step in accelerating proliferation and migration, and increased VSMC proliferation and migration ultimately result in intimal hyperplasia and atherosclerotic plaque formation [6,22].

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Conclusions

Taken together, our results reveal that lncRNA LIPCAR is a novel modulator in promoting human VSMCs proliferation, migration, and phenotype transition. As VMSC phenotypic switch accompanied by increased proliferation and migration is one of the key mechanisms involved in the initiation and development of atherosclerosis, LIPCAR could be a potential therapeutic target for the treatment of atherosclerosis. However, the present study has some limitations. The results of this study depended on cellular level using a single cell line. Animal experiments and clinical trials are needed to verify the conclusions of our study.

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