

LINC01123 Promotes the Proliferation and Migration of Vascular Smooth Muscle Cells and Alleviates Carotid Atherosclerosis by Modulating miR-1277-5p/KLF5

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Research

Keywords: carotid atherosclerosis, VSMCs, LINC01123, miR-1277-5p, KLF5

Posted Date: October 15th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-70002/v1>

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Abstract

Background: The pathophysiological mechanism of carotid atherosclerosis (CAS) involves endothelial cell dysfunction, vascular smooth muscle cells (VSMCs) and macrophage activation, which ultimately leads to fibrosis of the vessel wall. lncRNA works weightily in the formation of CAS, but the function and mechanism of lncRNA LINC01123 in CAS are still equivocal.

Methods: We collected blood samples from 35 CAS patients as well as 33 healthy volunteers. VSMCs treated with oxidized low-density lipoprotein (ox-LDL) were utilized as the CAS cell model. We applied qRT-PCR for detecting LINC01123, miR-1277-5p and KLF5 mRNA expression, CCK-8 method and BrdU test for determining cell proliferation, Transwell test for measuring cell migration, as well as Western blot for assaying KLF5 protein expression. Dual-luciferase reporter experiment was adopted for assessing the interaction between LINC01123 and miR-1277-5p, as well as KLF5 and miR-1277-5p.

Results: LINC01123 and KLF5 expression was dramatically up-regulated, while miR-1277-5p expression was down-regulated in CAS patients and ox-LDL-induced CAS cell models. Overexpressed LINC01123 notably promoted VSMC migration and proliferation. LINC01123 knockdown repressed cell proliferation and migration. Also, LINC01123 targeted miR-1277-5p and down-regulated its expression, while miR-1277-5p could negatively regulate KLF5 expression.

Conclusion: LINC01123 is highly expressed in CAS patients and is capable of being utilized as a latent target for treating CAS.

Introduction

Atherosclerosis (AS), a momentous causation of death in patients, is the common pathological basis of cardiovascular and cerebrovascular diseases [1]. Carotid atherosclerosis (CAS) is a part of systemic atherosclerosis. According to research investigations, the annual incidence of cerebrovascular accidents is 250/100,000, which is 5 times that of coronary heart disease [2]. Considerable studies have evinced that CAS is the staple incentive of ischemic cerebral infarction [3]. The degree of carotid artery stiffness, blood flow and cerebral infarction have a very close link [4]. Currently, the specific regulatory mechanism of the emergence and progress of CAS at the gene and molecular level is still not fully explicit.

Long non-coding RNA (lncRNA) is a class of non-coding RNA transcript over 200 nucleotides and no protein coding capability, which modulates gene expression in the form of RNA at the levels of transcription, post-transcription and epigenetics [5]. Research has authenticated that lncRNA is a crucial modulatory factor engaged in the emergence and progress of cardiovascular and cerebrovascular diseases, and is tightly connected to the formation of CAS [6]. Research has recently uncovered that lncRNA acts vitally in the modulation of AS, and is probably a biomarker for diagnosing cerebrovascular diseases. According to reports, lncRNA p21 can curb VSMC proliferation of ApoE^{-/-} mouse and induce their apoptosis through fortifying the transcriptional activity of p53 [7]. lncRNA H19 is highly expressed in AS patients, fosters VSMC proliferation and represses their apoptosis via modulating the MAPK and NF-

kB signal pathways [8]. LINC01123 belongs to lncRNA family member and exerts significant functions in cancer progression [9–11]. Nevertheless, the function and mechanism of LINC01123 in CAS vascular smooth muscle cells (VSMCs) are still nebulous.

MicroRNA (miRNA) is a type of small single-stranded RNA with a length of about 22 nucleotides, which is highly conservative, sequential and tissue specific. miRNA is engaged in diverse biological processes, including cell differentiation, proliferation, development, tumorigenesis and metastasis, etc. [12]. Evidence has displayed that miRNAs function substantially in the physiological and pathological processes of multiple diseases, especially closely concerned with the emergence and progress of cardiovascular and cerebrovascular diseases [13]. Recent research has exhibited that various miRNAs take part in the emergence and progress of AS lesions. For instance, miR-181b contributes to the emergence of AS via modulating TIMP-3 and elastin expression [14]. miR-181a-5p and miR-181a-3p block NF- κ B activation and vascular inflammation through targeting TAB2 and NEMO separately, thus deferring AS progression [15]. Nonetheless, the function of miR-1277-5p in CAS pathogenesis and the causative role remain cryptic.

Kruppel like factor 5 (KLF5) is a staple member of the KLFs family and a transcription factor intimately associated with cell differentiation, proliferation, development, and apoptosis [16]. Studies have manifested that KLF5 is extensively expressed in prostate, pancreas, kidney, colon, small intestine, skeletal muscle and breast tissues, and is one of the momentous signal transduction systems in the organism [17]. Scholars have corroborated that KLF5 exerts a momentous part in vascular remodeling diseases, particularly in AS, vascular restenosis, and cardiac hypertrophy [18]. Howbeit the causative role of KLF5 in the emergence and progress of CAS has not yet been fully illustrated.

This work aimed to dig into the expression and function of LINC01123 in CAS, and to inquire into its potential mechanism. We disclosed that LINC01123 was highly expressed in the serum of CAS patients and the ox-LDL-induced AS cell model. LINC01123 governed KLF5 expression through sponge miR-1277-5p to foster VSMC migration and proliferation, thus facilitating CAS progress.

1. Methods

1.1 Clinical sample

Serum samples were harvested from 35 CAS patients treated in the Department of Neurology of our institution from September 2018 to December 2019 and 33 healthy volunteers. All participants were informed and signed a consent form. The collected samples were immediately preserved at -80°C . Medical ethics committee had endorsed this research protocol.

1.2 Cell culture and transfection

Our team procured VSMCs and 293T cells from Chinese Academy of Sciences Cell Bank and placed them in DMEM (Invitrogen) encompassing 10% FBS and 1% Penicillin/Streptomycin (HyClone) in a moist

incubator under 5% CO₂, 37°C condition. ox-LDL was acquired from UnionBiol (Beijing, China). Besides, we obtained miR-1277-5p mimic (miR-1277-5p), mimic negative control (miR-con), miR-1277 inhibitor (miR-1277-5p-in), inhibitor negative control (miR-in), KLF5 siRNA (si-KLF5) or KLF5 plasmid from GenePharma. Small interfering RNA LINC01123 (si-LINC01123#1, si-LINC01123#2, si-LINC01123#3), negative control (si-NC), pcDNA3.0 (Vector), and pcDNA-LINC01123 (LINC01123) were prepared by RiboBio. Based on producer's descriptions, lipofectamine 2000 (Invitrogen) was employed for transfecting VSMCs.

1.3 qRT-PCR

We applied TRIzol reagent (Invitrogen) for extracting total RNA from clinical specimens and cells. Our member used NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) for checking RNA concentration, PrimeScrip-RT kit (Takara) and ABI7900 system (Applied Biosystems) for generating cDNA. Next, we performed reverse transcription reaction and used GAPDH or U6 as internal control. The primer sequence were: LINC01123, F: 5'-FACAGTGGCCGCACGCATAGCTG-3', R: 5'-RCTGACGACCGAGGTGACAACGATGA-3'; miR-1277-5p, F: 5'-GCCGAGTATATATATGTACGTAT-3', R: 5'-CTCAACTGGTGTCTGTGGA-3'; KLF5, F: 5'-ACCTCAGCTTCCTCCAGTTC-3', 5'-CGCATGGTCTCTGGGATTG-3'; U6, F: 5'-CTCGCTTCGRCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH, F: 5'-CCTGACCTGCGTGTGGACT-3', R: 5'-GCTGTGGATGGGGAGGTGTC-3'. Using $2^{-\Delta\Delta Ct}$ method, the relative expression of genes was computed. We completed each experiment in triplicate and done each measurement in triplicate too.

1.4 MTT assay

Our crew seeded the transfected VSMCs into a 96-well plate comprising ox-LDL (100 mg/L) at a density of 5×10^3 cells per well, and incubated them at 37°C. After incubating for 12, 24, 48, 72, and 96 h, MTT (Sigma-Aldrich) was added to each well. We incubated the mixture under 37°C and 5% CO₂ condition lasting 4 h, supplemented it with 150 mL DMSO and dissolved it under room temperature lasting 20 min. The absorbance value of each well was assayed at 490 nm via a microplate reader.

1.5 BrdU analysis

After seeding the transfected cells into a 96-well culture plate at a density of 2×10^3 cells/well, we fostered them lasting 24 h or 48 h, and incubated them with a final concentration of 10 μ M BrdU (BD Pharmingen) 2 to 24 h. In the next step, we withdrew the medium, fixed the cells at room temperature lasting 30 min, incubated them with the peroxidase-conjugated anti-BrdU antibody (Sigma-Aldrich) at room temperature lasting 60 min, washed them 3 times applying PBS, and incubated them with the oxidase substrate lasting 30 min. The absorbance value was assayed at 450 nm. Background BrdU immunofluorescence was assayed in cells not exposed to BrdU but dyed with BrdU antibody.

1.6 Transwell assay

The cell migration detection was accomplished making use of a 24-well Transwell chamber (8µm pore size; BD Biosciences). About 1×10^5 cells were resuspended in 100 µL serum-free medium, and DMEM comprising 10% FBS was filled into the lower chamber. After incubating lasting 24 h under 37°C condition, the upper chamber was fixed in anhydrous methanol lasting 20 min, dyed applying 0.1% crystal violet lasting 20 min, then washed and dried. An inverted microscope (Nikon, Japan) was applied for measuring the number of migrating cells.

1.7 Dual Luciferase reporter experiment

The LINC01123 or KLF5 fragment encompassing the forecasted miR-1277-5p binding site, as well as the wild-type or mutant hypothetical sequence of the binding site, was subcloned into the pMiRGLO dual luciferase vector (Promega) to construct report vectors, pMiRGLO-LINC01123-wild type (LINC01123-WT), pMiRGLO-KLF5-wild type (KLF5-WT) or pMiRGLO-LINC01123-mutant (LINC01123-MUT), pMiRGLO-KLF5-mutant (KLF5-MUT). Subsequently, we applied lipofectamine 2000 for co-transfecting SNHG16-WT/KLF5-WT or LINC01123-MUT/KLF5-MUT with miR-1277-5p mimic or miR-con into 293T. After transfecting for 48 h, we measured the luciferase activity making use of dual-luciferase reporter assay system (Promega).

1.8 Western blot analysis

The total cell protein was extracted applying RIPA buffer (Pierce). The protein concentration was determined utilizing Bradford method (Pierce). The same amount of protein (40µg) was disassociated on 10% SDS-PAGE, and then transferred to a PVDF membranes (Millipore). We blocked the PVDF membrane applying 5% bovine serum albumin in TBST for 2 h at 37 °C. Subsequently, the membrane was incubated with anti-KLF5 antibody (Abcam, ab137676, 1:500) or anti-GAPDH antibody (Abcam, ab8245, 1:3000) at 4°C nightlong. We washed the membrane 3 times adopting TBST and incubated it with goat anti-rabbit HRP (IgG) (Abcam, ab6721, 1:2000) at 37°C lasting 1 h. An enhanced chemiluminescence detection system (ECL) was adopted to visualize proteins.

1.9 Statistical Analysis

All the data were expressed as the mean±SD. Each assay was applied at least three independent experiments or replicates. Student's t-test was utilized for analyzing the significance between groups. *P <0.05. All statistical analyses were performed by LSD post-hoc analysis employing SPSS 18.0 and GraphPad Prism 8.0 software.

2. Result

2.1 LINC01123 was up-regulated in the serum of CAS patients and ox-LDL-induced cells

To delve into LINC01123 expression level in CAS, first, we used qRT-PCR for assaying LINC01123 expression in the serum of CAS patients. The results exhibited that compared with the control group,

LINC01123 was noticeably higher in the serum of CAS patients (Figure 1A). Besides, human VSMCs were treated with ox-LDL and detected via qRT-PCR. The results displayed that LINC01123 expression in ox-LDL-treated VSMCs was notably up-regulated compared with the 0 mg/L group (24 hours of induction) or the 0h group (100 mg/L ox-LDL induction) (Figure 1B-C). The above consequences implicated that LINC01123 was up-regulated in the serum of CAS patients and ox-LDL-induced CAS cell lines.

2.2 LINC01123 affected CAS cell migration and proliferation

For probing into the biological functions of LINC01123 in the progress of CAS, we successfully transfected pcDNA-LINC01123 and LINC01123 siRNAs into VSMCs, and established a cell model of LINC01123 overexpression or knockdown (Figure 2A). Then we adopted MTT method, BrdU experiment and Transwell method for determining VSMC migration and proliferation. It was unveiled that compared with the control group (Vector or si-NC), overexpressed LINC01123 noteworthy promoted VSMC migration and proliferation, whereas LINC01123 knockdown suppressed VSMC migration and proliferation (Figure 2B-F). These outcomes revealed that LINC01123 fostered CAS cell migration and proliferation.

2.3 LINC01123 acted as a molecular sponge for miR-1277-5p in CAS cells

We previously corroborated that LINC01123 functioned in promoting CAS cell migration and proliferation. Therefore, it was pretty momentous to inquire into the causative role of LINC01123 modulating the progress of CAS. We used StarBase database and observed that miR-1277-5p was likely one of the functional target miRNAs of LINC01123 (Figure 3A). For further verifying the targeting association between LINC01123 and miR-1277-5p, we conducted dual-luciferase reporter experiment. The results manifested that miR-1277-5p mimics markedly repressed the wild-type LINC01123 luciferase activity, but did not remarkably change the mutant LINC01123 (Figure 3B). Furthermore, we revealed that miR-1277-5p expression was visibly down-regulated in VSMCs overexpressing LINC01123, whereas LINC01123 knockdown up-regulated miR-1277-5p expression in VSMCs (Figure 3C). qRT-PCR evinced that miR-1277-5p was observably lower expressed in the serum of CAS patients and ox-LDL-induced CAS cell lines (Figure 3 D-F). In a word, this study validated that LINC01123 could adsorb miR-1277-5p and negatively modulate its expression.

2.4 miR-1277-5p reversed the role of LINC01123 in CAS

The aforementioned research suggested that LINC01123 could bind to miR-1277-5p. In the next experiment, we aim to inquire into whether LINC01123 can target miR-1277-5p to govern CAS progress. MTT method, BrdU experiment and Transwell experiment evinced that overexpressed miR-1277-5p restrained VSMC migration and proliferation, and knocking down miR-1277-5p fostered VSMC migration and proliferation. On the other hand, the effect of overexpressed LINC01123 on VSMC migration and proliferation was partially impaired by overexpressed miR-1277-5p. miR-1277-5p knockdown greatly reversed the inhibitory influence of LINC01123 knockdown on VSMC migration and proliferation (Figure 4

AF). These outcomes indicated that LINC01123 governed CAS cell migration and proliferation via adsorbing miR-1277-5p.

2.5 KLF5 was the immediate target of miR-1277-5p

For further looking into the potential mechanism of the LINC01123/miR-1277-5p axis in the progression of CAS, we investigated the downstream targets of miR-1277-5p. We screened the potential target genes of miR-1277-5p through StarBase database. It was discovered that KLF5 was one of the potential targets of miR-1277-5p (Figure 5A). Subsequently, we took advantage of dual-luciferase reporter experiment for detecting the targeting relation between miR-1277-5p and KLF5. The results manifested that overexpressed miR-1277-5p strikingly sapped the luciferase activity of KLF5-WT, KLF5-MUT1, KLF5-MUT2 and KLF5-MUT3, whereas the luciferase activity of KLF5-MUT1&2&3 did not change dramatically (Figure 5B). qRT-PCR indicated that KLF5 mRNA was plainly down-regulated in the serum of CAS patients and ox-LDL-induced CAS cell lines (Figure 5 C-E). Moreover, miR-1277-5p overexpression or LINC01123 knockdown restrained KLF5 mRNA and protein expression, while miR-1277-5p knockdown or overexpressed LINC01123 enhanced KLF5 mRNA and protein expression. On the other hand, overexpressed miR-1277-5p debilitated the promotion of overexpressed LINC01123 on KLF5 expression, and the inhibition of LINC01123 knockdown on KLF5 could be greatly reversed by miR-1277-5p inhibitors (Figure 5 F-I). These data implied that KLF5 was the downstream target of miR-1277-5p in CAS cells, and its expression was modulated negatively or positively by miR-1277-5p and LINC01123.

2.6 KLF5 reversed the impact of miR-1277-5p on CAS cell migration and proliferation

Next, we also investigated whether miR-1277-5p modulated CAS progress through KLF5. We transfected miR-1277-5p mimics, miR-1277-5p inhibitors, miR-1277-5p+KLF5, miR-1277-5p-in+si-KLF5 in VSMCs. VSMC migration and proliferation were further detected through MTT method, BrdU experiment and Transwell experiment. The results unveiled that the down-regulation of VSMC migration and proliferation caused by miR-1277-5p mimics could be partially reversed by overexpressed KLF5. KLF5 knockdown could enervate the promotion of miR-1277-5p inhibitors on VSMC migration and proliferation (Figure 6A-F). These data indicated that miR-1277-5p could target KLF5 to govern CAS cell migration and proliferation.

3. Discussion

AS is the most usual and most serious type of arterial pathological changes. It often results in the involvement of critical organs such as the heart and brain, which gravely affects people's health [19, 20]. In recent years, studies have substantiated that lncRNA performs a diversity of biological functions. lncRNA is broadly expressed in biological cells, primarily through epigenetic modification, transcription regulation and post-transcriptional regulation to modulate DNA methylation, histone modification or chromatin remodeling, etc., to silence or activate genes [21]. The imbalance of lncRNA has been corroborated to be concerned with the emergence, progress and prognosis of AS, and it is expected to become a novel target for diagnosing and treating AS [6–8]. According to reports, lncRNA LOC285194 is

down-regulated in aortic atherosclerotic plaques in the ApoE^{-/-} mouse model, and is capable of regulating VSMC apoptosis [22]. LncRNA NEXN-AS1 bates the progression of AS through modulating the actin binding protein NEXN [23]. LINC01123 is a newly discovered lncRNA with cancer-promoting effects, and is engaged in the emergence and progression of multiple tumors. Nevertheless, there is no pertinent report on the specific role of LINC01123 in CAS. In this paper, we substantiated that LINC01123 was patently highly expressed in the serum of CAS patients and ox-LDL induced cells. Also, overexpressed LINC01123 conspicuously promoted VSMC migration and proliferation, whereas LINC01123 knockdown exerted the opposite effect. These findings indicated that LINC01123 likely facilitated the progress of CAS.

Studies have corroborated that miRNAs exert a key part in cell apoptosis, proliferation and differentiation. Evidence has recently verified that miRNA is engaged in the emergence and progress of AS. miR-200c is up-regulated in carotid plaques and is available to be served as a biomarker of AS [24]. miR-126-5p promotes endothelial cell proliferation and curbs the progression of AS through suppressing Dlk1 [25]. Up-regulated miR-330-5p is connected to the stability of carotid plaque via targeting Talin-1 [26]. Additionally, there are reports that lncRNA can act as a molecular sponge of miRNA to adjust human diseases. For example, lncRNA MIAT up-regulates CD47 through sponge miR-149-5p to contribute to AS cell proliferation [27]. Knockdown of lncRNA TUG1 can inhibit the progression of AS via targeting miR-133a to modulate FGF1 [28]. Similarly, the down-regulation of lncRNA HOTTIP suppresses ox-LDL-induced VSMC migration and proliferation through governing the miR-490-3p/HMGB1 axis and PI3K-AKT signal pathway [29]. In this work, we first revealed the interaction mechanism between LINC01123 and miR-1277-3p in CAS. We found that LINC01123 could work as a molecular sponge of miR-1277-5p in CAS, and miR-1277-5p expression was down-regulated in CAS patients and ox-LDL-induced CAS cell lines. Additionally, miR-1277-5p could stifle CAS cell migration and proliferation, and could weaken the promotion of LINC01123 on CAS cell migration and proliferation. These results indicated that LINC01123 regulated the process of CAS through sponge miR-1277-5p.

KLF5 is a multifunctional transcription factor with zinc finger structure. It acts vitally in cardiovascular, cerebrovascular, cancer and other diseases, and takes part in the modulation of cell proliferation, migration and apoptosis [18, 30]. Research evidence has showed that KLF5 is highly expressed in VSMCs and participates in phenotypic transformation of smooth muscle cells and vascular remodeling process [31]. Recent studies have evidenced that KLF5 plays pivotally in the tissue remodeling of cardiovascular diseases, such as atherosclerosis, vascular restenosis, and cardiac hypertrophy [32]. Scholars have previously justified that KLF5 expression is tightly connected to the proliferation of smooth muscle cells in cardiovascular diseases [33]. Moreover, there is evidence that miRNA can affect disease progression by targeting KLF5. For example, miR-145-5p promotes the differentiation of gastric cancer cells by directly targeting KLF5 [34]. miR-152 dampers the malignant progression of AS via down-regulating KLF5 [35]. miR-9 stifles VSMC migration and proliferation through targeting KLF5 [36]. However, the association between KLF5 and miR-1277-5p or LINC01123 has not been elucidated in CAS. In this work, we attested that KLF5 was the downstream target of miR-1277-5p, and LINC01123 modulated KLF5 expression through competitive binding with miR-1277-5p. Additionally, KLF5 could greatly reverse the suppressive

impact of miR-1277-5p on CAS progression. Those indicated that LINC01123 modulated KLF5 expression through sponge miR-1277-5p to promote the progress of CAS.

To sum up, we attested that LINC01123 was overtly up-regulated in CAS patient serum and ox-LDL-induced CAS cells. Overexpressed LINC01123 promoted CAS cell migration and proliferation, and LINC01123 knockdown curbed CAS cell migration and proliferation. Mechanistically, we proved that LINC01123 modulated KLF5 expression through sponge miR-1277-5p to promote the progress of CAS. Our results indicate that the LINC01123miR-1277-5p/KLF5 axis is a vital factor in the progress of CAS and a promising novel target for CAS treatment.

Abbreviations

carotid atherosclerosis (CAS); vascular smooth muscle cells (VSMCs); oxidized low-density lipoprotein (ox-LDL); Atherosclerosis (AS); Long non-coding RNA (lncRNA); Kruppel like factor 5 (KLF5)

Declarations

Ethics statement

The experiment was often approved by the Ethics Committee of the Hainan Provincial Hospital of Chinese Medicine, and all patients participating in this study provided written informed consent in accordance with the “Helsinki Declaration”.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

Natural Science Foundation of Guangdong Province (2017A030313622) and Guangzhou Provincial Program of Science and Technology (201804010446).

Authors' contributions

GW and MG conceived and designed the study. YZ and GZ analyzed the data. GZ and YG contributed to literature review. GW and MG and YG wrote the manuscript. GW and MG reviewed and edited the manuscript. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

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Figures

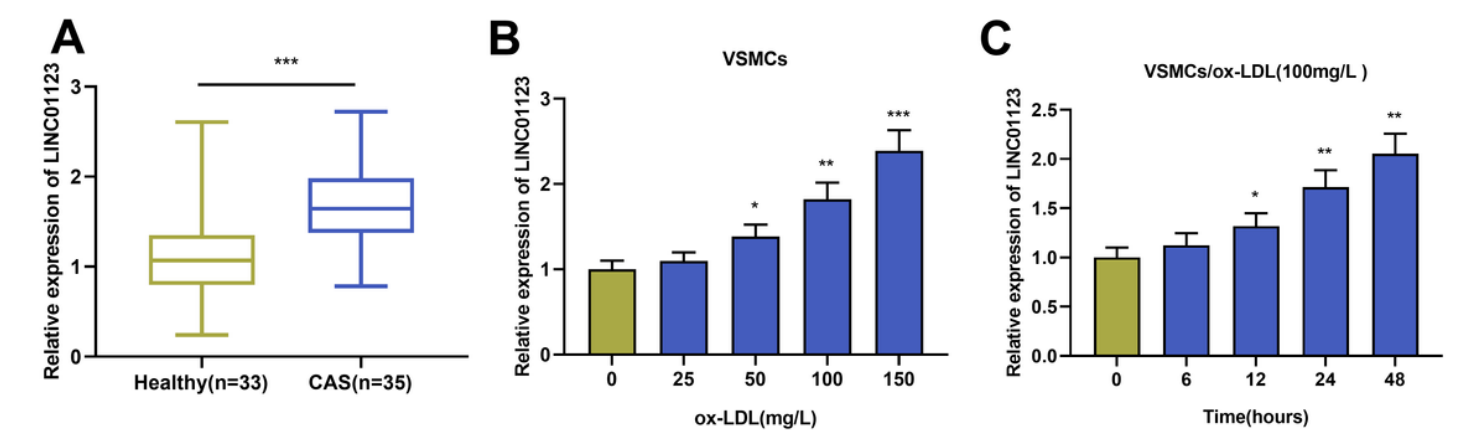


Figure 1

Expression characteristics of LINC01123 in CAS A. qRT-PCR to assay LINC01123 expression in the serum of CAS patients and healthy volunteers. B-C. qRT-PCR to assay LINC01123 expression in VSMCs after ox-LDL treatment. * P<0.05, ** P<0.01, P<0.001.

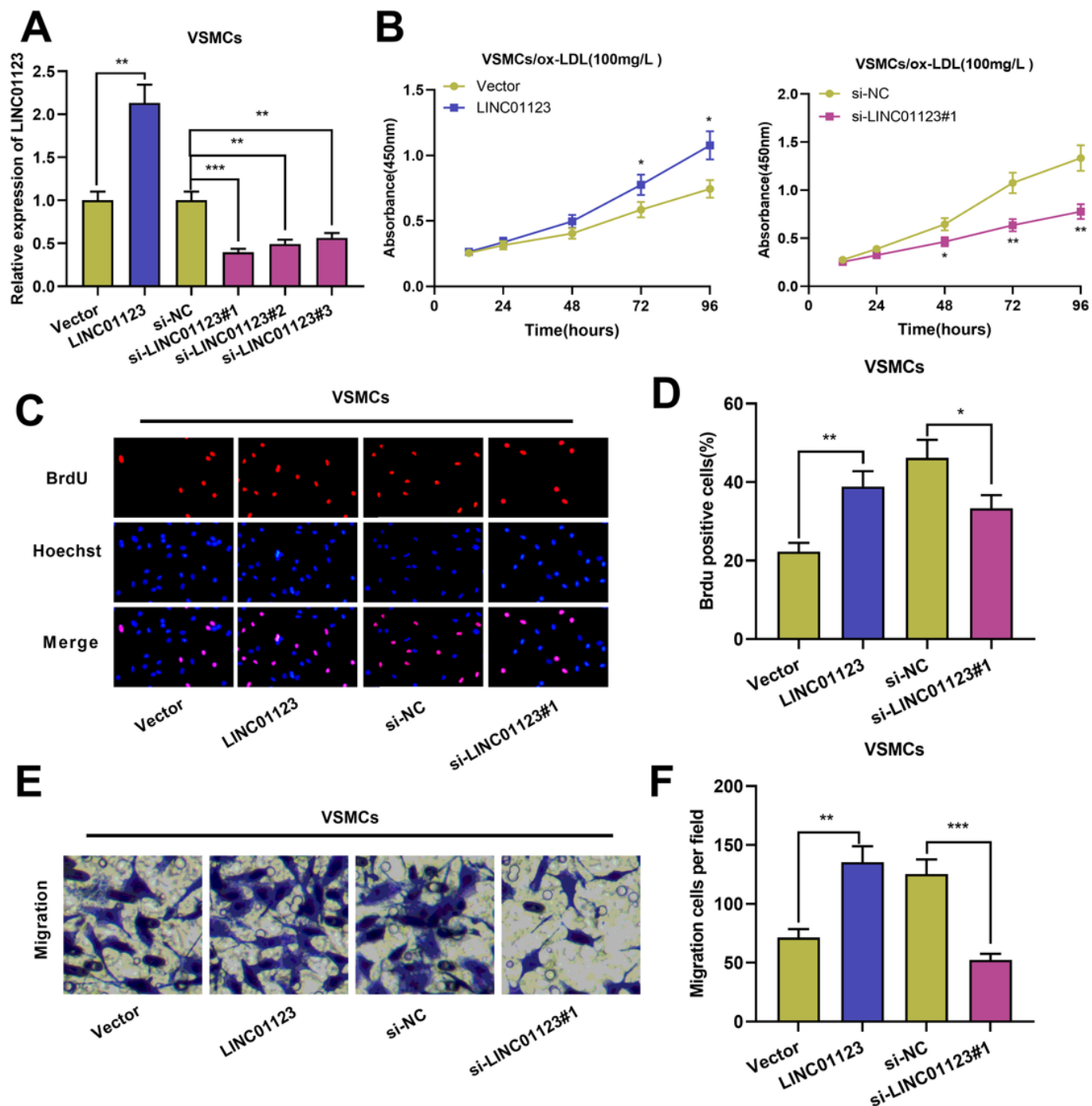


Figure 2

LINC01123 facilitated CAS cell migration and proliferation A. qRT-PCR to detect LINC01123 expression in VSMCs that overexpress or knock down LINC01123. B-D. MTT and BrdU methods to detect VSMC proliferation. E-F. Transwell experiment to assay the migration ability of VSMCs. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$.

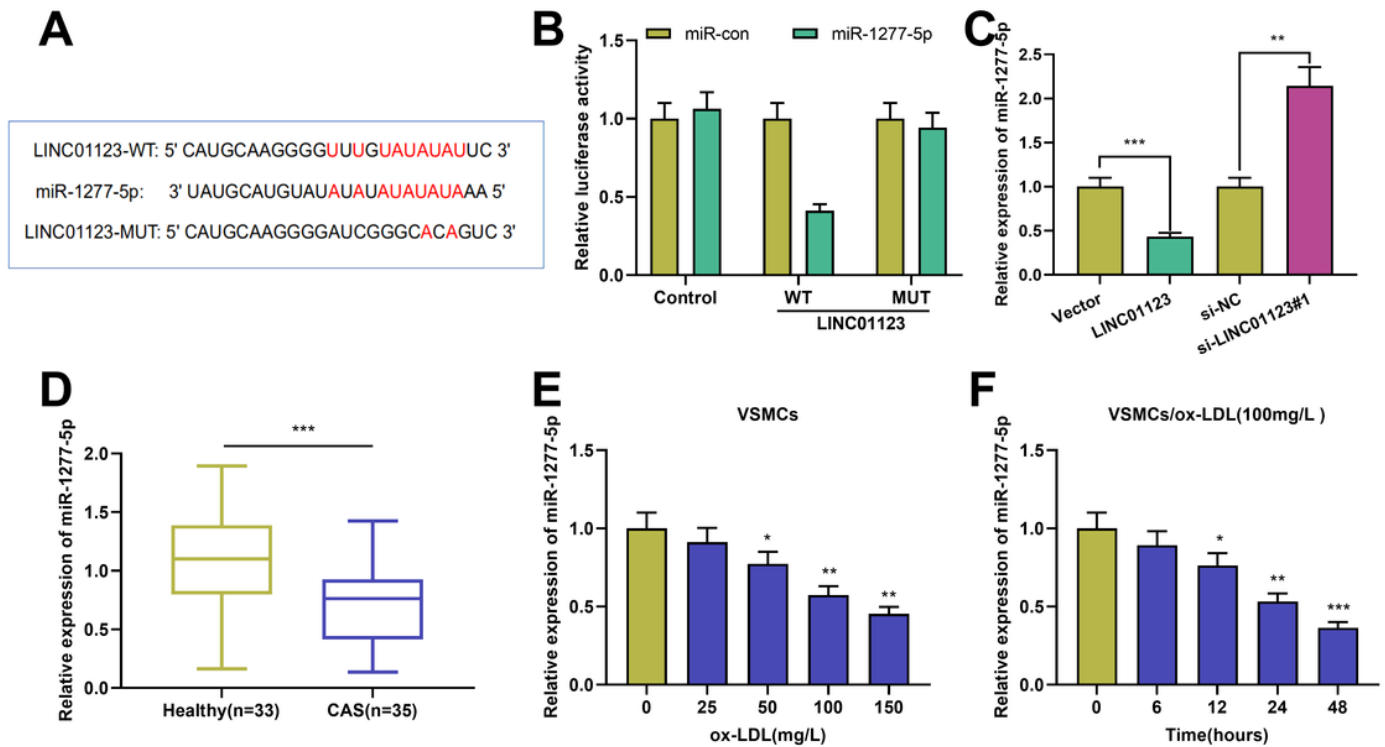


Figure 3

miR-1277-5p was the target of LINC01123 A. Bioinformatics analysis to forecast the binding sequence of miR-1277-5p and LINC01123. B. Dual-luciferase reporter experiment to test the luciferase activity of overexpressed miR-1277-5p combined with clinical 123 wild-type or mutant type. C. qRT-PCR to monitor miR-1277-5p expression in VSMCs overexpressing or knocking down LINC01123. D. qRT-PCR to determine miR-1277-5p expression in the serum of CAS patients and healthy volunteers. E-F. qRT-PCR to assess miR-1277-5p expression in VSMCs after ox-LDL treatment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

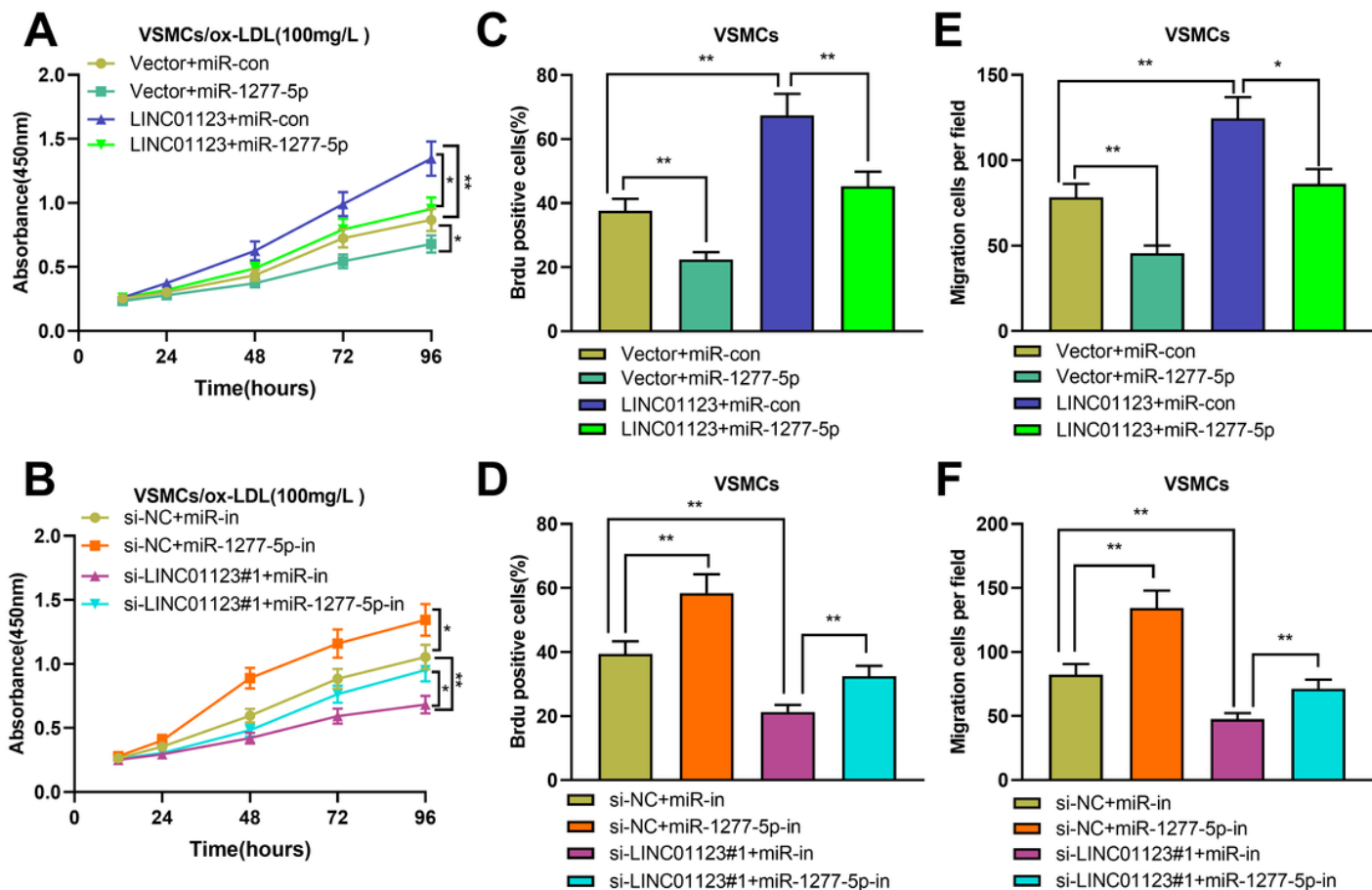


Figure 4

LINC01123/miR-1277-5p axis was engaged in regulating CAS cell migration and proliferation A-D. MTT method and BrdU test to estimate VSMC proliferation. E-F. Transwell experiment to evaluate VSMC migration. * $P < 0.05$, ** $P < 0.01$.

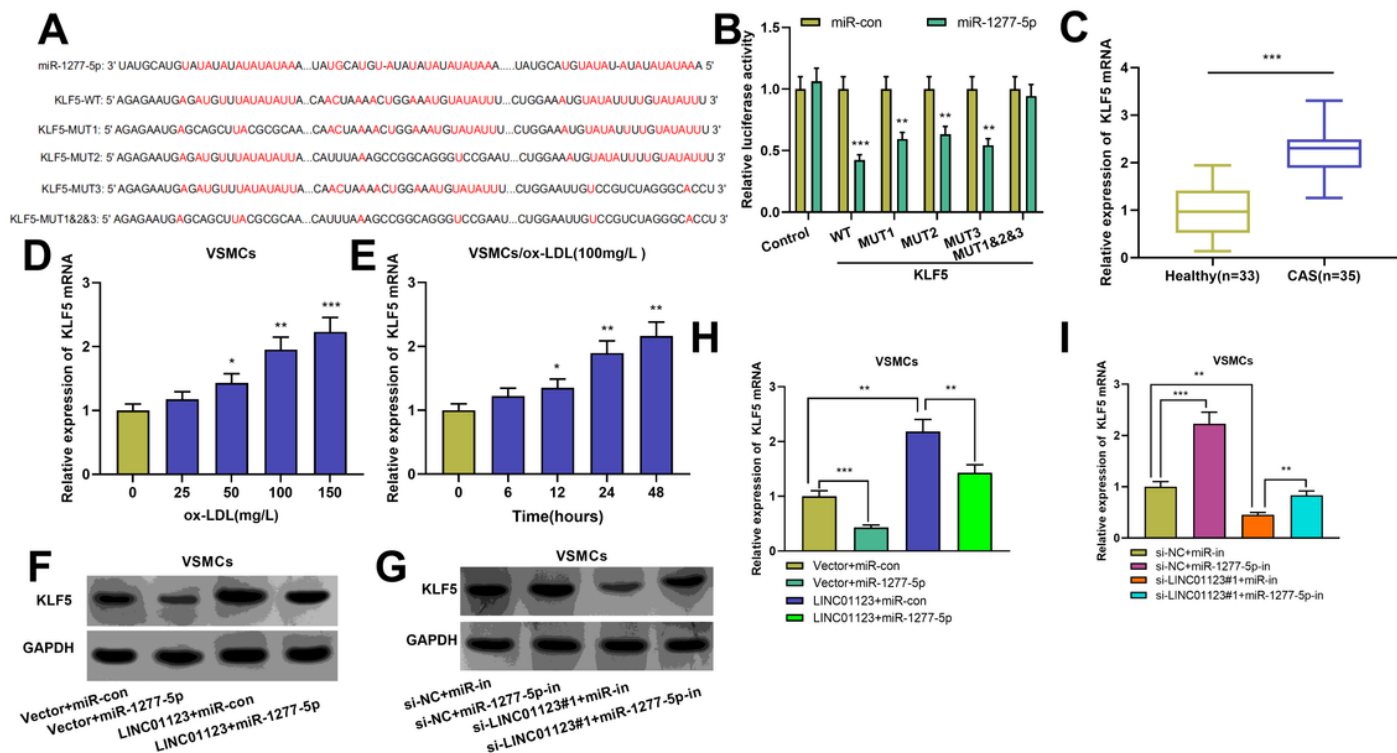


Figure 5

KLF5 was the target of miR-1277-5p A. Bioinformatics analysis to forecast the binding sequence of miR-1277-5p and KLF5. B. Luciferase reporter experiment to observe the luciferase activity of overexpressed miR-1277-5p combined with wild-type or mutant KLF5. C. qRT-PCR to observe KLF5 mRNA expression in the serum of CAS patients and volunteers. D-E. qRT-PCR to investigate KLF5 mRNA expression in VSMCs after ox-LDL treatment. F-I. Western blot and qRT-PCR to explore KLF5 mRNA and protein expression in the transfected CAS cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

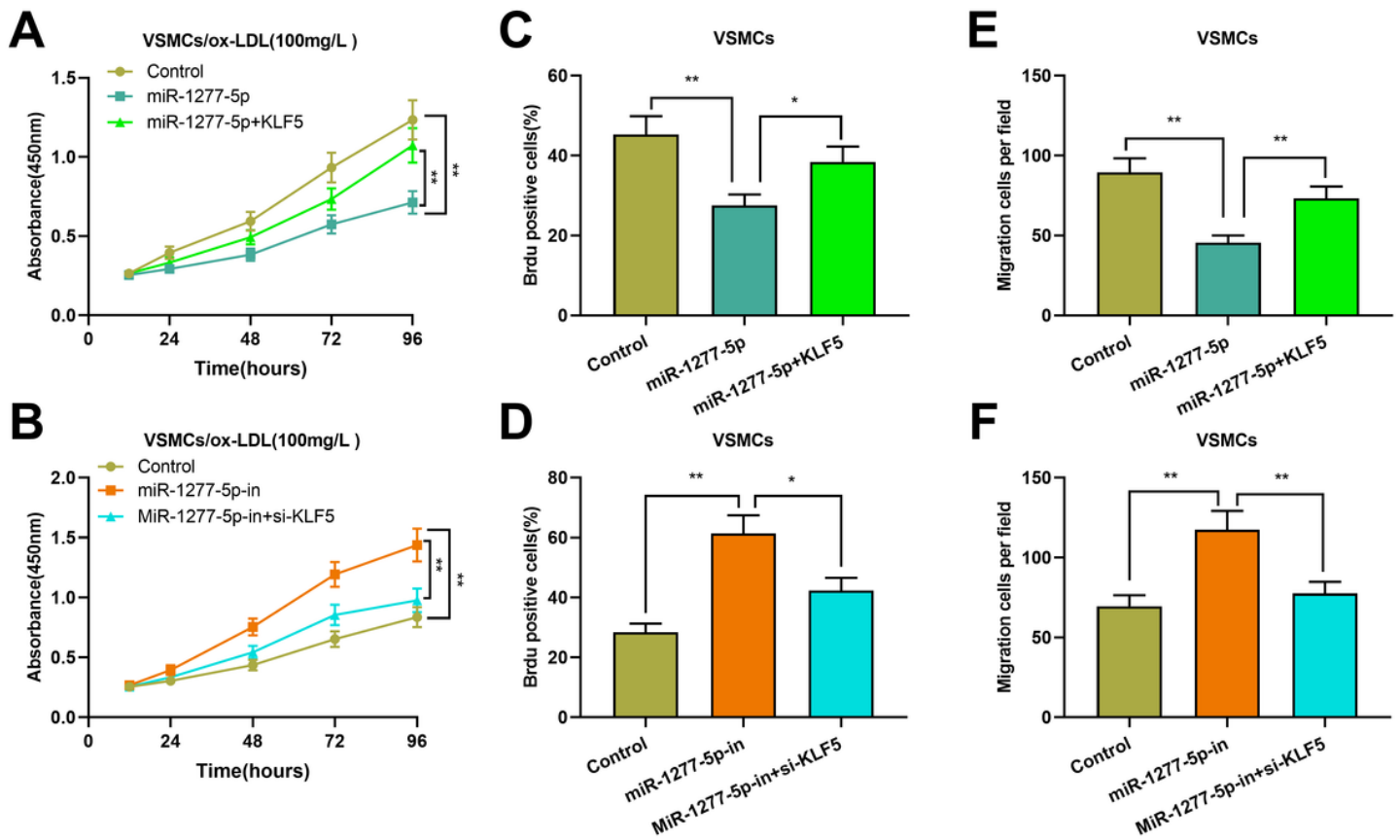


Figure 6

miR-1277-5p targeted KLF5 to modulate CAS cell migration and proliferation A-D. MTT method and BrdU experiment to determine VSMC proliferation after transfection. E-F. Transwell experiment to measure VSMC migration after transfection. * $P < 0.05$, ** $P < 0.01$.