Long non-coding RNA SNHG16 contributes to progression of carotid atherosclerosis by regulating miR-30c-5p/ADAM10 axis

Type

Research paper

Keywords

Carotid atherosclerosis, miR-30c-5p, Lnc-SNHG16, ADAM10

Abstract

Introduction
Carotid atherosclerosis (CAS) is one of the main causes of cerebral infarction in the ageing population. Long non-coding RNA small nucleolar RNA host gene 16 (lnc-SNHG16) could promote the development of atherosclerosis. However, the mechanism of lnc-SNHG16 in CAS remains vague.

Material and methods
The expression levels of lnc-SNHG16, microRNA-30c-5p (miR-30c-5p) and disintegrin and metalloproteinase 10 (ADAM10) were detected by real-time quantitative polymerase chain reaction (RT-qPCR). Cell viability and migration were detected by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) and transwell assays, severally. The levels of interleukin-6 (IL-6), IL-β and tumor necrosis factor-α (TNF-α) were assessed by enzyme-linked immunosorbent assay (ELISA). Protein levels of spinal muscular atrophy (SMA), calponin and ADAM10 were examined by western blot assay. The binding relationship between miR-30c-5p and lnc-SNHG16 or ADAM10 was predicted by Starbase, then verified by the dual-luciferase reporter assay.

Results
Lnc-SNHG16 and ADAM10 were increased, and miR-30c-5p was decreased in CAS patient and oxidized low-density lipoprotein (ox-LDL)-treated human aortic smooth muscle cells (hASMCs). Lnc-SNHG16 silencing repressed cell viability, migration, inflammation, facilitated differentiation in ox-LDL-treated hASMCs. Moreover, mechanical analysis proved that lnc-SNHG16 improved ADAM10 expression by sponging miR-30c-5p.

Conclusions
Our data indicated that lnc-SNHG16 could regulate the progression of ox-LDL induced CAS model by the miR-30c-5p/ADAM10 axis, implying a potential therapeutic strategy for CAS

Long non-coding RNA SNHG16 contributes to progression of carotid atherosclerosis by regulating miR-30c-5p/ADAM10 axis

Running title: Role of lnc-SNHG16/miR-30c-5p/ADAM10 in carotid atherosclerosis

List of abbreviations

CAS: Carotid atherosclerosis

lnc-SNHG16: Long non-coding RNA small nucleolar RNA host gene 16

RT-qPCR: real-time quantitative polymerase chain reaction

SMA: spinal muscular atrophy

ox-LDL: oxidized low-density lipoprotein

hASMCs: human aortic smooth muscle cells

ABSTRACT

Introduction: Carotid atherosclerosis (CAS) is one of the main causes of cerebral infarction in the ageing population. Long non-coding RNA small nucleolar RNA host gene 16 (lnc-SNHG16) could promote the development of atherosclerosis. However, the mechanism of lnc-SNHG16 in CAS remains vague.

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Key words: Lnc-SNHG16, miR-30c-5p, ADAM10, carotid atherosclerosis

Introduction

As a manifestation of systemic atherosclerosis in the carotid artery, carotid atherosclerosis (CAS) has become a leading cause of cerebral infarction in the aging population [1]. Currently, carotid intima–media thickness (cIMT) and carotid plaque

(CP) were the proposed biomarkers of subclinical atherosclerosis associated with stroke risk [2, 3]. Moreover, the vulnerable plaque of CAS can lead to the occurrence of cerebral infarction [4]. Interestingly, as an important component of vascular structure, excessive proliferation and inappropriate migration of vascular smooth muscle cells are key factors in the formation of atherosclerotic plaque [5]. Recent studies have shown that oxidized low-density lipoprotein (ox-LDL) can be used to simulate atherosclerosis through exciting vascular smooth muscle cells [6]. Hence, repairing the dysfunction of vascular smooth muscle cells might be an effective way to mitigate atherosclerosis progression.

In recent years, long non-coding RNAs (lncRNAs), a class of transcripts with about 200 nucleotides (nts), have been reported to play essential regulatory roles in multiple biological and pathological activities by regulating diverse molecules, such as DNA, RNA and proteins [7]. In fact, accumulating evidence suggested that abnormal expression of lncRNAs is involved in the development and progression of numerous diseases, including atherosclerosis [8, 9]. As Cai *et al*. reported, that abundance of lncRNA TNK2-AS1 contributed to cell proliferation and migration of human aortic smooth muscle cells (hASMCs) through inhibiting miR-150-5p and enhancing VEGFA and FGF1 expression in atherosclerosis [10]. Analogously, Ji *et al*. confirmed that excess of linc-ROR aggravated the malignancy of atherosclerosis by increasing hASMCs proliferation and migration through regulating miR-195-5p/FGF2 axis [11]. LncRNA small nucleolar RNA host gene 16 (lnc-SNHG16) has been confirmed to work as an oncogene in a variety of cancers, such as bladder cancer [12], gastric cancer [13] and hepatocellular carcinoma [14]. Moreover, a recent literature manifested that lnc-SNHG16 could accelerate proliferation and inflammatory response of macrophages by interacting with miR17-5p to activate NF-κB signaling in atherosclerosis [15]. However, the underlying mechanism of lnc-SNHG16 in CAS is still unclear.

During the past decades, microRNAs (miRNAs), endogenous non-coding RNAs with 19-25 nts, have been shown to negatively regulate gene expression at post-transcriptional levels [16]. An extensive body of recent studies has proved that miRNAs could exert the regulating role in the progression of atherosclerosis [17, 18]. MicroRNA-30c-5p (miR-30c-5p) has been identified as a tumor suppressor by modulating target genes in gastric cancer [19], clear cell renal cell carcinoma [20] and multiple myeloma [21]. Furthermore, relevant studies have indicated that the low

expression of miR-30c-5p, as an underlying predictive biomarker, was related to the development of carotid intima-media thickness and early plaque in atherosclerosis [22, 23], implying that miR-30c-5p played a crucial function in the development of atherosclerosis.

A disintegrin and metalloproteinase 10 (ADAM10), a member of the metalloprotease family, takes part in the incision of diverse cells surface molecules, containing cytokines, growth factors and adhesion molecules [24]. Simultaneously, some research have confirmed that the knockdown of ADAM10 could weaken atherosclerosis progression by suppressing inflammatory response and boosting cholesterol efflux [25]. Yet, the specific role of ADAM10 in CAS remains unknown.

Herein, our results presented that lnc-SNHG16 was upregulated in CAS patient and ox-LDL-treated hASMCs. Lnc-SNHG16 silencing hindered cell viability, migration, inflammation, facilitated differentiation in ox-LDL-treated hASMCs. Moreover, bioinformatics analysis suggested that miR-30c-5p had some sequences with lnc-SNHG16. Therefore, we aimed to investigate whether lnc-SNHG16 might regulate ox-LDL- induced hASMCs damage through the miR-30c-5p/ADAM10 axis in CAS.

Materials and Methods

Tissue samples and cell culture

This research obtained the approval of Ethics Committee of The Third People's Hospital of Heze, and written informed consent was signed by each participant from The Third People's Hospital of Heze. Serum samples of the CAS patient (n=30) were collected from patients with ischaemic stroke in the internal carotid artery underwent magnetic resonance angiography within 1 week of symptom onset while the central plaque and the interior layer of the outer plaque are relatively stable, and healthy volunteers (n=30) were acquired from healthy volunteers. All the patients had no cardioembolic stroke, haemorrhagic stroke, radiation therapy of the neck.

Human aortic smooth muscle cells (hASMCs) were provided by ScienCell (Carlsbad, CA, USA), and were maintained in 5% CO_2 at 37˚C under moist atmosphere with Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, Beijing, China).

Noteworthily, 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin/streptomycin as the supplement were added the culture medium. Besides, hASMCs were treated with 0 ng/mL, 25 ng/mL, 50 ng/mL, 75 ng/mL and 100 ng/mL ox-LDL (Sigma-Aldrich, St.Louis, MO, USA) for 24 h before measurement.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from human serum and cells referring to the supplier's direction of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) [26]. Extracted RNA was quantified by using a NanoDrop spectrometer (NanoDrop, Wilmington, DE, USA) at 260 nm. Then, a PrimeScript™ RT Reagent Kit (Takara, Dalian, China) was applied to synthesize complementary DNA (cDNA) from total RNA (2-3 μg) following the operation manual. Subsequently, the expression of lnc-SNHG16, miR-30c-5p and ADAM10 was analyzed on an HT7900 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR Kit (Takara). The results were calculated by using the $2^{-\Delta\Delta Ct}$ method [27], normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for lncRNA and mRNA, and U6 small nuclear RNA (snRNA) for miRNA. The primers used were showed as below:

Lnc-SNHG16: 5'-GCAGAATGCCATGGTTTCCC-3' (sense), 5'-GGACAGCTGGCAAGAGACTT-3' (antisense);

miR-30c-5p: 5'-GCCGCTGTAAACATCCTACACT-3' (sense), 5'-GTGCAGGGTCCGAGGT-3' (antisense);

ADAM10: 5'-AAGAAGCTTCCCACAAGGCA-3' (sense), 5'-TGTGTACGCAGAGTATCTAACTGG-3' (antisense);

U6: 5'-CTCGCTTCGGCAGCACA-3' (sense), 5'-AACGCTTCACGAATTTGCGT-3' (antisense);

GAPDH: 5'-GTCAACGGATTTGGTCTGTATT-3' (sense), 5'-AGTCTTCTGGGTGGCAGTGAT-3' (antisense).

Cell transfection

Lnc-SNHG16 small interference RNA (si-lnc-SNHG16) and its negative control (si-NC), miR-30c-5p inhibitor (anti-miR-30c-5p) and its negative control (anti-miR-NC), miR-135a-5p mimics (miR-135a-5p) and its negative control (miR-NC) were acquired from RiboBio (Guangzhou, China). The pcDNA3.1 vector (pcDNA, Addgene, Cambridge, MA, USA) was applied to construct lnc-SNHG16 and ADAM10 overexpression vector, termed as pcDNA-lnc-SNHG16 (lnc-SNHG16) and pcDNA-ADAM10 (ADAM10). With the help of Lipofectamine 3000 reagent (Invitrogen), these oligonucleotides and plasmids were transfected into hASMCs. After 24 h of transfection, transfected cells were harvested and used for the following experiments.

Cell viability assay

Cell viability of hASMCs was detected by using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT, Sigma-Aldrich) referring to the supplier's direction. In brief, hASMCs were seeded on 96-well plates (5×10^3 cells/well). After treatment with ox-LDL for 24 h, 20 μL MTT (Sigma-Aldrich) was added into each well at indicated time points (0 h, 24 h, 48 h and 72 h), followed by incubation for another 4 h at 37˚C. Whereafter, the supernatant was discarded, 150 μL of dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to terminate the reaction. At last, with the help of microplate reader (Thermo Electron Corporation, Vantaa, Finland), cell absorbance was read at 490 nm.

Cell migration assay

Migration ability of hASMCs was evaluated in accordance with the instructions of Transwell chambers (Corning Incorporated, Corning, NY, USA). Generally, the treated hASMCs (1×10^5) in serum-free medium were introduced into the upper chamber of transwell, and the medium with 10% FBS (Hyclone) was added in the lower chamber. After incubation for 24 h, cells remaining on the upper surface of membranes were scraped with cotton swabs, while cells migrated to the lower surface of membranes were fixed in methanol and stained by crystal violet. Finally, an inverted microscope was used to count the number of stained cells.

Enzyme-linked immunosorbent assay (ELISA)

In this assay, hASMCs were incubated in 24-well plates. After treatment with ox-LDL for 24 h, the medium was collected, followed by detection the levels of interleukin-6 (IL-6), IL-β and tumor necrosis factor-α (TNF-α) with an ELISA (Beyotime, Shanghai, China) kits based on the user's guidebook. A SpectraMaxM2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was applied for the measurement of the absorbance in plates.

Western blot assay

In brief, total protein from serums and cells was extracted by using RIPA buffer with protease and phosphates inhibitions (Sigma-Aldrich), and the concentrations were quantified with BCA Protein Assay Kit (Beyotime, China). After separation with a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein samples were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), followed by blocking with 5% skim milk. After incubation for 2 h, the primary antibodies were incubated with the membranes at 4˚C. The next day, the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were further cultured in the membranes. Finally, an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Pittsburg, PA, Sweden) was conducted to analyze these protein signals. Primary antibodies were as followed: anti-spinal muscular atrophy (anti-SMA; 1:1000, ab5694, Abcam, Cambridge, MA, USA), anti-calponin (1:1000, ab46794, Abcam), anti-ADAM10 (1:1000, ab124695, Abcam) and anti-GAPDH (1:1000, ab8227, Abcam).

Dual-luciferase reporter assay

Lnc-SNHG16 wild-type reporter vector (lnc-SNHG16 WT) containing the binding sites for miR-30c-5p and its mutant-type in complementary sites (lnc-SNHG16 MUT), ADAM10 3' un-translated region (3'UTR) wild-type reporter vector (WT ADAM10 3'UTR) possessing the binding sequences with miR-30c-5p and its mutant-type in seed

region (MUT ADAM10 3'UTR) were amplified and then sub-cloned into pMIR-GLO™ vector (Promega, Madison, WI, USA). Subsequently, according to the standard method of Lipofectamine 3000 (Invitrogen), hASMCs in 48-well plates were co-transfected with the constructed reporter plasmids and miR-135a-5p or miR-NC. Luciferase activities at 48 h after transfection were detected under a dual-luciferase reporter assay kit (Promega).

Statistical analysis

Statistical analysis was conducted with GraphPad Prism7 software, and was shown as the mean ± standard deviation (SD). Differences between two groups were analyzed by using Student's t-test. Differences between more than two groups were identified using one-way analysis of variance (ANOVA) followed by Tukey's tests. All assays were carried out at least in triplicate. If P value < 0.05, it was regarded as statistically significant.

Results

Lnc-SNHG16 expression was increased and miR-30c-5p expression was decreased in CAS patient and ox-LDL-treated HASMCs

At first, to investigate the function of lnc-SNHG16 and miR-30c-5p in CAS, their expression levels were detected through RT-qPCR assay. Compared with the serums of healthy volunteers (n=30), lnc-SNHG16 was highly expressed and miR-30c-5p was lowly expressed in CAS patient serums (n=30) (Figure 1A and 1C). Importantly, we found that lnc-SNHG16 expression was improved, and miR-30c-5p expression was reduced in hASMCs with the increase of ox-LDL concentration, when compared to cells un-treated with ox-LDL (Figure 1B and 1D), suggesting that the involvement of lnc-SNHG16 and miR-30c-5p in the ox-LDL induced CAS model. Particularly in cells treated with 50 ng/mL ox-LDL, hence, we chose 50 ng/mL ox-LDL 24 h for subsequent experiments. Furthermore, lnc-SNHG16 level was inversely related to miR-30c-5p expression in CAS patient serums (Figure 1E). In a word, the dysregulation of lnc-SNHG16 and miR-30c-5p might be correlated with CAS.

Lnc-SNHG16 knockdown repressed cell viability, migration, inflammation, facilitated differentiation in ox-LDL-treated hASMCs

Then, to explore the role of lnc-SNHG16 in CAS, we knocked down lnc-SNHG16 expression in ox-LDL treated hASMCs. As displayed in Figure 2A, in the treatment of hASMCs, lnc-SNHG16 level was markedly declined in si-lnc-SNHG16-transfected relative to cells transfected with si-NC. Functional analysis showed that the promotion of cell viability and migration caused by ox-LDL was obviously attenuated through lnc-SNHG16 downregulation (Figure 2B and 2C). Synchronously, introduction of si-lnc-SNHG16 evidently diminished the enhancement of ox-LDL on inflammatory factors (IL-6, IL-β and TNF-α) level (Figure 2D), indicating that lnc-SNHG16 silencing retarded the inflammation of ox-LDL induced CAS model. Apart from that, lnc-SNHG16 deletion abrogated ox-LDL-triggered decrease in the protein levels of differentiation-related factors (SMA and Calponin) in hASMCs (Figure 2E), proving that lnc-SNHG16 deficiency accelerated the differentiation of ox-LDL induced CAS model. Collectively, these results suggested that the knockdown of lnc-SNHG16 impeded cell viability, migration, inflammation, and expedited differentiation in ox-LDL-treated hASMCs.

MiR-30c-5p was direct target of lnc-SNHG16

Given that there was a negative correlation between lnc-SNHG16 and miR-30c-5p in CAS patient serum. We used to the bioinformatics software starBase to further analyze the underlying relationship between lnc-SNHG16 and miR-30c-5p. As presented in Figure 3A, miR-30c-5p was found to harbor some common complementary sequences with lnc-SNHG16. To verify the predicted results, a dual-luciferase reporter assay was conducted in hASMCs. Data showed that the overexpression of miR-30c-5p reduced the luciferase activity of WT-lnc-SNHG16 reporter vector, but not that of MUT-lnc-SNHG16 reporter vector (Figure 3B). Moreover, the transfection efficiency of pcDNA-lnc-SNHG16 was detected and exhibited in Figure 3C. And lnc-SNHG16 knockdown contributed to miR-30c-5p level, and lnc-SNHG16 overexpression suppressed miR-30c-5p level in ox-LDL-treated

hASMCs (Figure 3D). Notably, re-introduction of anti-miR-30c-5p abolished the facilitation of lnc-SNHG16 knockdown on miR-30c-5p level in ox-LDL-treated hASMCs (Figure 3E). Functionally, deletion of miR-30c-5p notably abated the inhibitory effect of lnc-SNHG16 downregulation on cell viability and migration in ox-LDL-induced hASMCs (Figure 3F and 3G). Besides, the reduction of IL-6, IL-β and TNF-α levels due to lnc-SNHG16 deficiency was undermined by miR-30c-5p knockdown in ox-LDL-stimulated hASMCs (Figure 3H), implying that silencing of miR-30c-5p overturned the suppression effect of lnc-SNHG16 downregulation on inflammatory response in ox-LDL-excited hASMCs. Western blot assay confirmed that anti-miR-30c-5p reversed the positive effect of lnc-SNHG16 knockdown on differentiation in ox-LDL-provoked hASMCs, showing that the protein levels of SMA and Calponin increased by si-lnc-SNHG16 was repressed through miR-30c-5p downregulation in ox-LDL-treated hASMCs (Figure 3I). All these results unveiled that miR-30c-5p, as a target of lnc-SNHG16, partially abolished the effects of lnc-SNHG16 on cell viability, migration, inflammation, and differentiation in ox-LDL-trigger hASMCs.

ADAM10 worked as the target of miR-30c-5p

As widely believed, miRNAs could exert the function by binding to the 3'untranslated regions (3'UTR) of mRNAs [28]. Thus, to further explore the mechanism of miR-30c-5p, we searched the latent target genes of miR-30c-5p by using the bioinformatics tool (StarBase). Results presented that miR-30c-5p contained a motif with sites complementary to ADAM10 3'UTR (Figure 4A). Then, we preformed the dual-luciferase reporter assay to further confirm the direct interaction between miR-30c-5p and ADAM10 3'UTR. As showed in Figure 4B, miR-30c-5p upregulation led to an overt decline in luciferase activity of WT-ADAM10 3'UTR, while had little effect on MUT-ADAM10 3'UTR in hASMCs. In addition, ADAM10 was expressed at the high level in both CAS patient serum (Figure 4C and 4D) and ox-LDL-treated hASMCs (Figure 4E and 4F) in comparison with their respective control groups. And transfection efficiency of anti-miR-30c-5p and miR-30c-5p mimics were examined and shown in Figure 4H. Intriguingly, we viewed that there was an inverse relationship between miR-30c-5p and ADAM10 in CAS patient serum (Figure 4G). MiR-30c-5p downregulation

improved the expression level of ADAM10, whereas miR-30c-5p upregulation declined ADAM10 level in ox-LDL-stimulated hASMCs (Figure 4I and 4J). Taken together, these findings suggested that miR-30c-5p directly bound with ADAM10.

MiR-30c-5p-mediated cell viability, migration, inflammation and differentiation were reversed by ADAM10 in ox-LDL-treated hASMCs

As mentioned above, ADAM10 was a direct target of miR-30c-5p in ox-LDL-treated hASMCs. Meanwhile, ADAM10 as a carcinogenic factor has been testified by previous studies [29, 30]. We inferred that miR-30c-5p could exert the suppressive effect on ox-LDL induced CAS model by interacting with ADAM10. Firstly, the transfection efficiency of pcDNA-ADAM10 was detected by RT-qPCR and western blot assays in ox-LDL-induced hASMCs. As illustrated in Figure 5A and 5B, ADAM10 level was augmented in ADAM10-transfected hASMCs versus cells transfected with pcDNA. Importantly, rescue assays rendered that ADAM10 overexpression overturned the inhibiting effect of miR-30c-5p upregulation on cell viability and migration in ox-LDL-stimulated hASMCs (Figure 5C and 5D). Consistently, re-transfection of pcDNA-ADAM10 evidently abrogated miR-30c-5p mimics-caused decrease in the levels of IL-6, IL-β and TNF-α in ox-LDL-treated hASMCs, supporting that ADAM10 upregulation partly reversed the adverse effect of miR-30c-5p overexpression on inflammatory response in ox-LDL-induced hASMCs (Figure 5E). Additionally, reduced SMA and Calponin protein levels further confirmed that upregulation of ADAM10 could effectively abolished the positive effect of miR-30c-5p mimics on differentiation in ox-LDL-triggered hASMCs (Figure 5F). Overall, these results suggested that miR-30c-5p could regulate cell viability, migration, inflammation, and differentiation of ox-LDL-trigger hASMCs by targeting ADAM10.

Verification of lnc-SNHG16/miR-30c-5p/ADAM10 regulatory axis in ox-LDL-treated hASMCs

Based on the above results, we conjectured that lnc-SNHG16 could exert its regulatory role by the miR-30c-5p/ADAM10 axis in ox-LDL-treated hASMCs. To testify the guess, we implemented the rescue assays to further verify whether lnc-

SNHG16 affect ADAM10 expression through miR-30c-5p. As shown in Figure 6, anti-miR-30c-5p improved the protein level of ADAM10, while lnc-SNHG16 knockdown could relieve miR-30c-5p silencing-mediated promotion effect on ADAM10 protein level in ox-LDL-induced hASMCs. Together, the results unveiled that lnc-SNHG16 could perform as a molecular sponge of miR-30c-5p to affect ADAM10 expression.

Discussion

Increasing evidence exhibits that lncRNAs can be used as prognostic biomarkers in multiple diseases, including atherosclerosis [31]. Indeed, as the research moves along, lncRNAs have become necessary regulators in the development and progression of atherosclerosis [32-35]. Lnc-SNHG16, located at 17q25.1 gene, was identified as an oncogenic lncRNA in various types of cancer [36, 37]. Importantly, in a recent publication, lnc-SNHG16 was verified to be abnormally increased and exacerbated the progression of atherosclerosis through triggering hASMCs proliferation and migration [38]. However, the underlying mechanism of lnc-SNHG16 in CAS still needs further clarification. In this study, the different concentration of ox-LDL induced the aortic smooth muscle cells (hASMCs) in an Atherosclerosis simulation environment was used for further research. Lnc-SNHG16 was upregulated in CAS patient serum samples and ox-LDL-treated hASMCs cells, suggesting that lnc-SNHG16 might involve in CAS progression. Functionally, lnc-SNHG16 deficiency constrained ox-LDL-induced proliferation, migration, the expression of inflammatory factor and differentiation of hASMCs, demonstrating that lnc-SNHG16 mitigated the progression of CAS.

MiR-30c-5p, a tumor suppressor, has been pointed out to exert the suppressive action in atherosclerosis development [39]. In this manuscript, our data showed that miR-30c-5p was decreased in CAS patient and ox-LDL-treated hASMCs. Intriguingly, there was an inverse relation between miR-30c-5p and lnc-SNHG16 in CAS. Previous studies have suggested that lncRNAs could exert the function through the interaction with miRNAs [40]. Our results first confirmed that miR-30c-5p was a direct target of lnc-SNHG16, and miR-30c-5p downregulation could relieve the negatively effect of lnc-SNHG16 deletion on ox-LDL-excited hASMCs damage. The inhibitory action of miR-30c-5p on ox-LDL-caused cell injury of atherosclerosis was also testified in prior report [41].

It is widely accepted that lncRNAs could impact mRNA expression by sponging miRNA [42]. ADAM10 as the target of miR-30c-5p was first validated in hASMCs. Apart from that, some reports have presented that ADAM10 was closely linked to the composition of atherosclerosis plaque [43, 44]. In this research, ADAM10 was upregulated in CAS patient and ox-LDL-treated hASMCs, and partly reversed the suppression effect of miR-30c-5p on ox-LDL-caused hASMCs damage. Consistent with our data, ADAM10 could facilitate the inflammatory response in atherosclerosis [25]. Additionally, mechanistic analysis further verified that the downregulation of lnc-SNHG16 could overturn the positive action of miR-30c-5p knockdown on ADAM10 expression in ox-LDL-treated hASMCs. That was to say, lnc-SNHG16 preformed as a sponge of miR-30c-5p to upregulate ADAM10 expression, thereby regulating CAS progression.

While the present results highlights the regulation roles of lnc-SNHG16 in CAS, we should note the limitation of this research. One limitation of the present research is, although our initial comparison of the miRNA and circRNA expression level in normal and CAS serum samples was credible, the limited sample size makes further evaluation necessary. Another himation is our research was limited to an in vitro study, therefore, an *in vivo* experiments will be seriously considered in the future. Besides, whether SNHG16 correlates with the progression of atherosclerosis and the effects of targeting SNHG16 on therapy for atherosclerosis may require further investigation.

Together, this study first discovered the regulatory role of the lnc-SNHG16/miR-30c-5p/ADAM10 axis in the ox-LDL induced CAS model. Our findings provided an insight into the molecular basis of carotid atherosclerosis, which will provide us important clues for developing the effective therapeutic strategies.

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Disclosure of interest

The authors declare that they have no financial conflicts of interest

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Figure Legends

Figure 1 Lnc-SNHG16 was upregulated and miR-30c-5p was downregulated in CAS and ox-LDL-treated hASMCs. (A and C) RT-qPCR assay was preformed to detect the expression level of lnc-SNHG16 and miR-30c-5p in 30 pairs of CAS patient serum and healthy volunteers serum (B and D) Expression levels of lnc-SNHG16 **and**

miR-30c-5p in hASMCs treated with ox-LDL at different concentrations (0 ng/mL, 25 ng/mL, 50 ng/mL, 75 ng/mL and 100 ng/mL). (D) The expression association between lnc-SNHG16 and miR-30c-5p in CAS patients was analyzed by Pearson correlation analysis. *P <0.05.

Figure 2 Lnc-SNHG16 knockdown suppressed cell viability, migration, inflammation, promoted differentiation in ox-LDL-treated hASMCs. (A) Lnc-SNHG16 level was measured by RT-qPCR assay in hASMCs treated with Control, ox-LDL, ox-LDL + si-NC and ox-LDL + si-lnc-SNHG16. (B) Cell viability was detected by MTT assay in treated hASMCs. (C) Migration ability was assessed by transwell assay in treated hASMCs. (D) Expression levels of IL-6, IL-β and TNF-α were examined by ELISA in treated hASMCs. (E) The protein levels of SMA and Calponin were tested by western blot assay in treated hASMCs. *P <0.05.

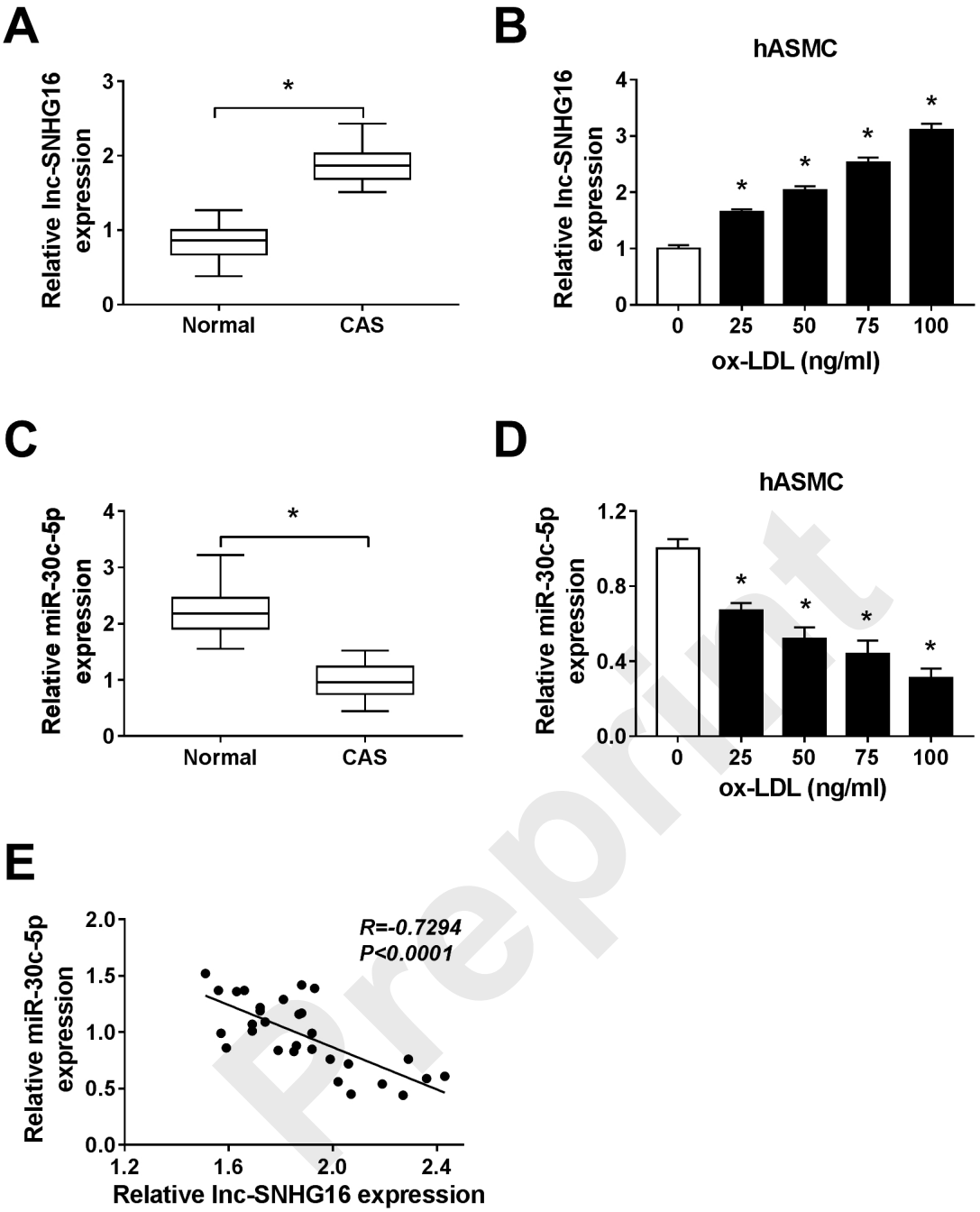
Figure 3 Lnc-SNHG16 directly bound with miR-30c-5p. (A) The binding sites between lnc-SNHG16 and miR-30c-5p were predicted by starBase software. (B) The effects of miR-30c-5p overexpression on luciferase activity of WT-lnc-SNHG16 and MUT-lnc-SNHG16 reporters in hASMCs were measured by dual-luciferase reporter assay. (C-I) HASMCs were stimulated with 50 ng/mL ox-LDL for 24 h. (C) Lnc-SNHG16 level was assessed in hASMCs transfected with Control, pcDNA and lnc-SNHG16. (D) MiR-30c-5p level was measured in hASMCs transfected with Control, si-NC, si-lnc-SNHG16, pcDNA and lnc-SNHG16. (E) MiR-30c-5p level was measured in hASMCs transfected with Control, si-NC, si-lnc-SNHG16, si-lnc-SNHG16 + anti-miR-NC and si-lnc-SNHG16 + anti-miR-30c-5p. (F) Cell viability in treated hASMCs was tested by MTT assay. (G) Migration capacity in treated hASMCs was measured by transwell assay. (H) The levels of IL-6, IL-β and TNF-α in treated hASMCs were by ELISA. (I) SMA and Calponin protein levels in treated hASMCs were western blot assay. *P <0.05.

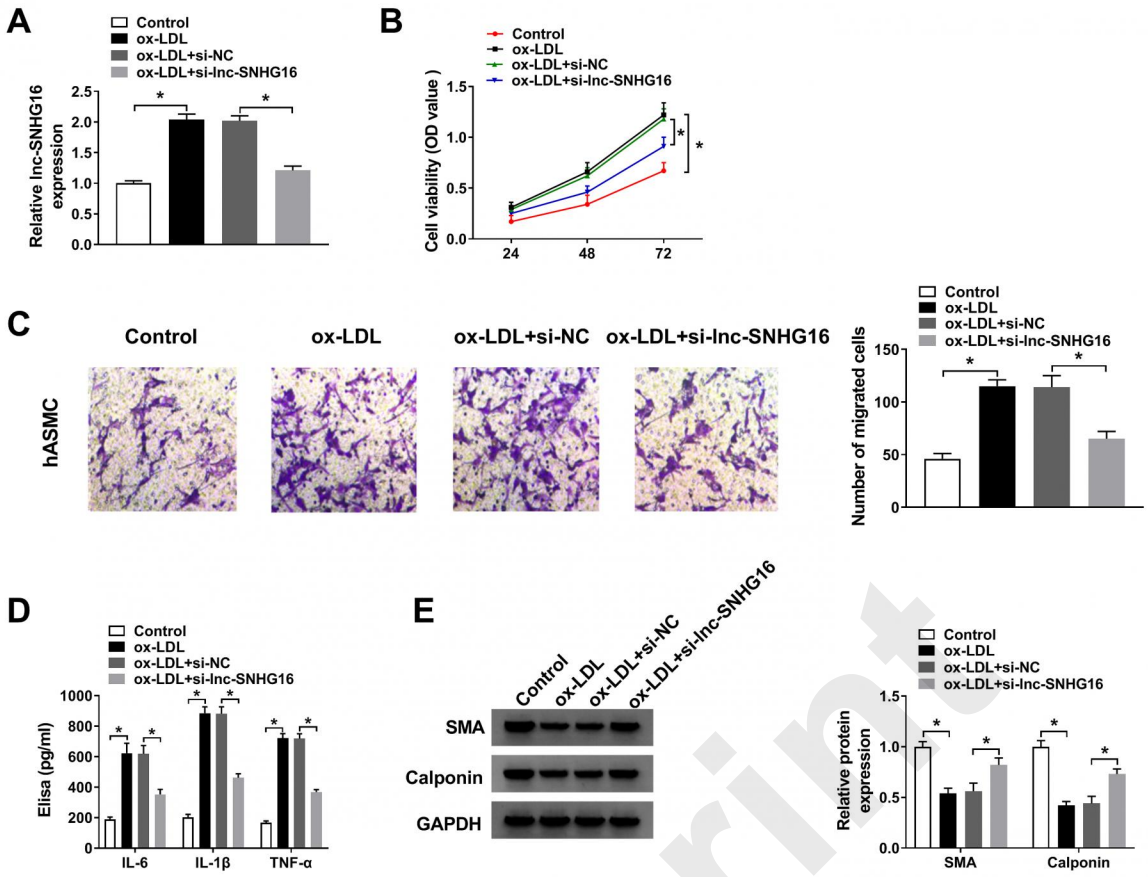
Figure 4 ADAM10 was the target of miR-30c-5p. (A) StarBase software was applied to predict the binding sequence between miR-30c-5p and ADAM10 3'UTR. (B) The dual-luciferase reporter assay was conducted to confirm the binding relationship between miR-30c-5p and ADAM10 3'UTR in hASMCs. (C and D) The mRNA level and protein level of ADAM10 were detected in 30 pairs of CAS patient serum and healthy volunteer serum (E and F) ADAM10 level was measured in hASMCs

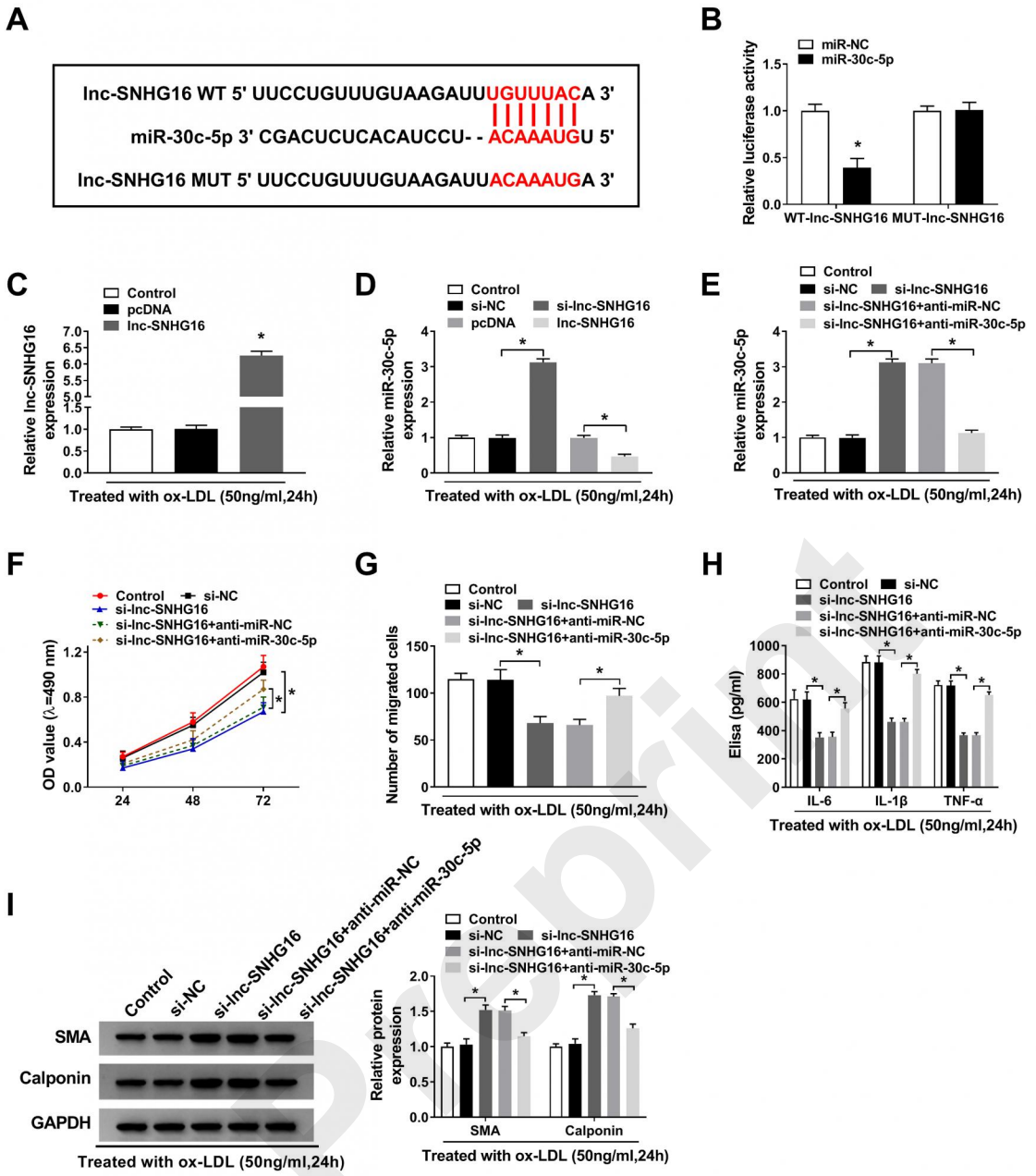
stimulated with ox-LDL at various concentrations (0 ng/mL, 25 ng/mL, 50 ng/mL, 75 ng/mL and 100 ng/mL). (G) Pearson correlation analysis was preformed to appraise the expression association between miR-30c-5p and ADAM10 in CAS patients. (H-J) hASMCs were treated with 50 ng/mL ox-LDL for 24 h. (H) MiR-30c-5p level was examined in hASMCs transfected with Control, anti-miR-NC, anti-miR-30c-5p, miR-NC and miR-30c-5p. (I and J) The mRNA level and protein level of ADAM10 were detected in treated hASMCs. **P* <0.05.

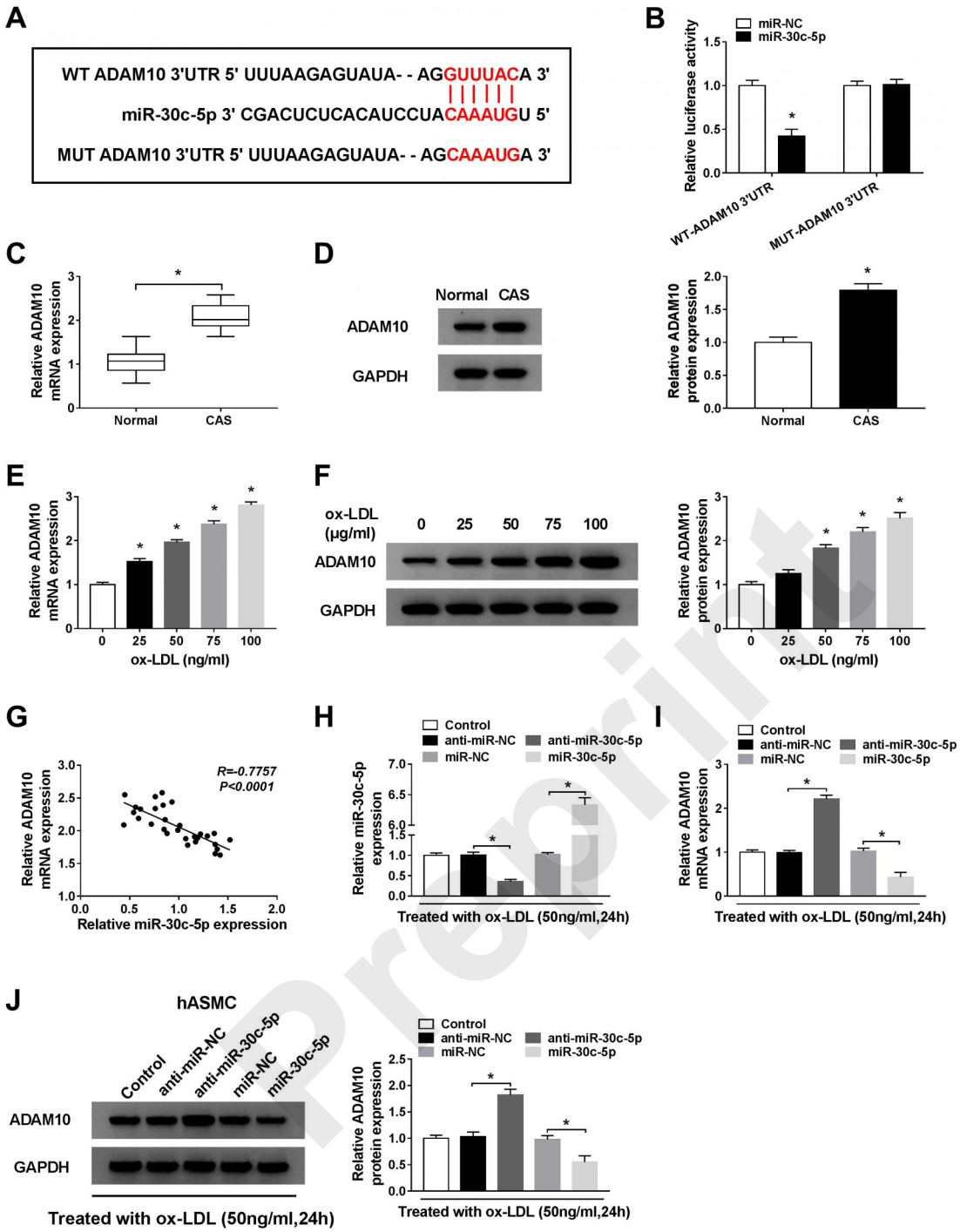
Figure 5 ADAM10 abrogated the effects of miR-30c-5p on cell viability, migration, inflammation and differentiation in ox-LDL-treated hASMCs. HASMCs were treated with 50 ng/mL ox-LDL for 24 h. (A and B) ADAM10 level was tested in hASMCs transfected with Control, pcDNA and ADAM10. (C) MTT assay was used to detect the proliferative ability in hASMCs transfected with Control, miR-NC, miR-30c-5p, miR-30c-5p + pcDNA and miR-30c-5p + ADAM10. (D) Transwell assay was carried out to measure the migration in treated hASMCs. (E) ELISA was preformed to assess the levels of IL-6, IL-β and TNF-α in treated hASMCs. (F) Western blot assay was applied to examine the protein levels of SMA and Calponin in treated HASMCs. **P* <0.05.

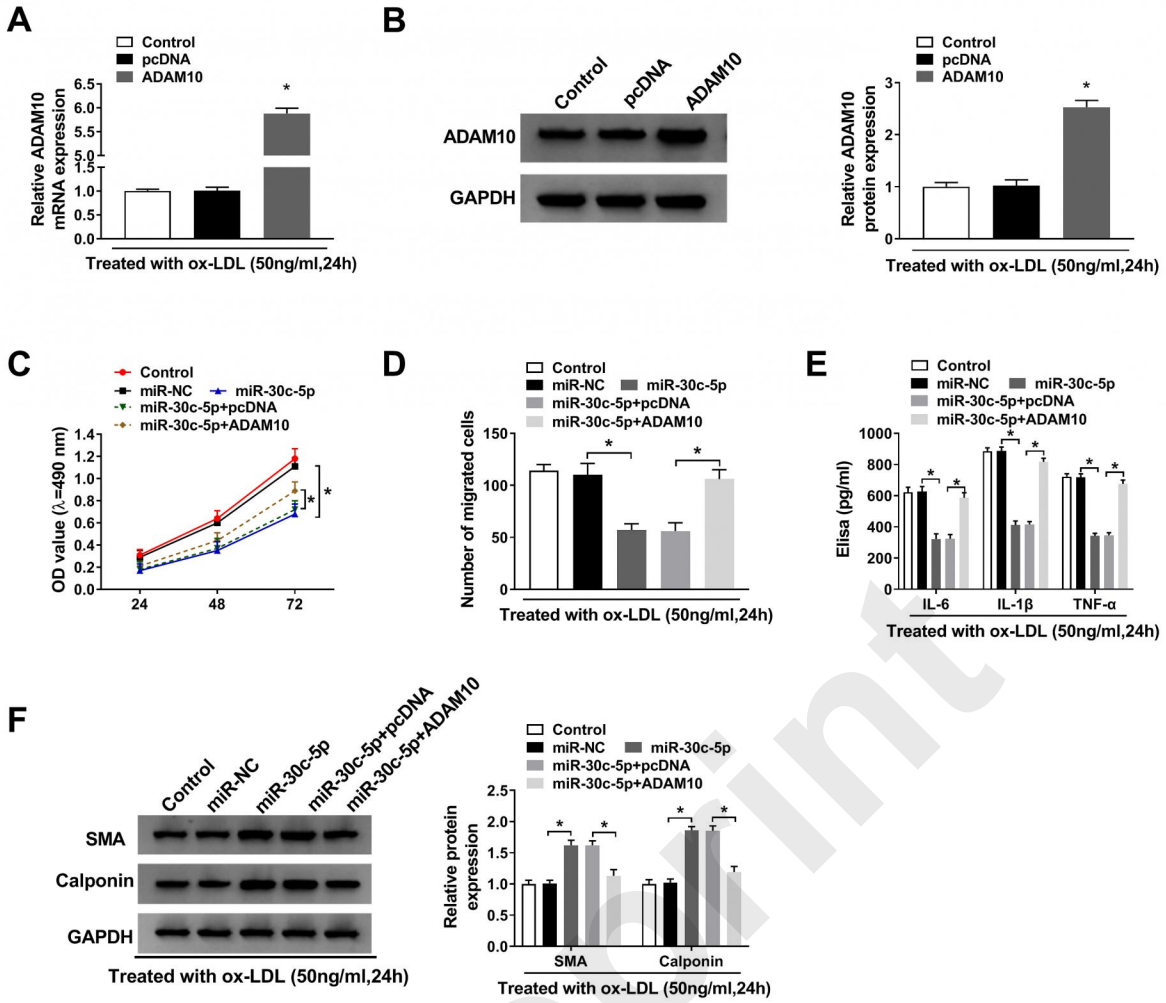
Figure 6 Lnc-SNHG16 regulated ADAM10 expression through sponging miR-30c-5p. HASMCs were treated with 50 ng/mL ox-LDL for 24 h. ADAM10 protein level was detected with Control, anti-miR-NC, anti-miR-30c-5p, anti-miR-30c-5p + si-NC and anti-miR-30c-5p + si-lnc-SNHG16. **P* <0.05.

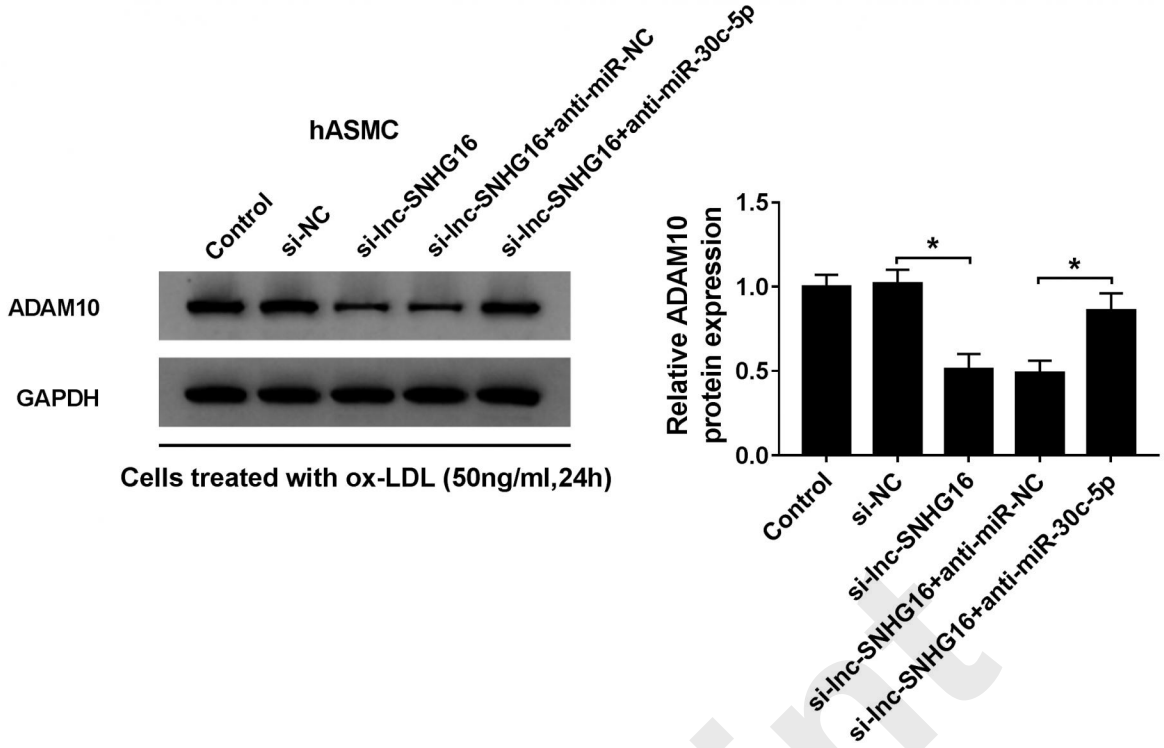

A
Relative lnc-SNHG16 expression
Normal
CAS
B
hASMC
Relative lnc-SNHG16 expression
ox-LDL (ng/ml)
C
Relative miR-30c-5p expression
Normal
CAS
D
hASMC
Relative miR-30c-5p expression
ox-LDL (ng/ml)
E
R=-0.7294
P<0.0001
Relative miR-30c-5p expression
Relative lnc-SNHG16 expression


A
Control
ox-LDL
ox-LDL+si-NC
ox-LDL+si-lnc-SNHG16
Relative lnc-SNHG16 expression
B
Control
ox-LDL
ox-LDL+si-NC
ox-LDL+si-lnc-SNHG16
Cell viability (OD value)
24
48
72
C
Control
ox-LDL
ox-LDL+si-NC
ox-LDL+si-lnc-SNHG16
hASMC
Number of migrated cells
D
Control
ox-LDL
ox-LDL+si-NC
ox-LDL+si-lnc-SNHG16
Elisa (pg/ml)
IL-6
IL-1β
TNF-α
E
Control
ox-LDL
ox-LDL+si-NC
ox-LDL+si-lnc-SNHG16
SMA
Calponin
GAPDH
Relative protein expression


A
lnc-SNHG16 WT 5' UUCCUGUUUGUAAGAUUUGUUUACA 3'
miR-30c-5p 3' CGACUCUCACAUCCU- - ACAAAUGU 5'
lnc-SNHG16 MUT 5' UUCCUGUUUGUAAGAUUACAAAUGA 3'
B
miR-NC
miR-30c-5p
Relative luciferase activity
WT-lnc-SNHG16 MUT-lnc-SNHG16
C
Control
pcDNA
lnc-SNHG16
Relative lnc-SNHG16 expression
Treated with ox-LDL (50ng/ml,24h)
D
Control
si-NC
si-lnc-SNHG16
pcDNA
lnc-SNHG16
Relative miR-30c-5p expression
Treated with ox-LDL (50ng/ml,24h)
E
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p
Relative miR-30c-5p expression
Treated with ox-LDL (50ng/ml,24h)
F
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p
OD value (λ=490 nm)
G
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p
Number of migrated cells
Treated with ox-LDL (50ng/ml,24h)
H
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p
Elisa (pg/ml)
IL-6
IL-1β
TNF-α
Treated with ox-LDL (50ng/ml,24h)
I
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p
SMA
Calponin
GAPDH
Treated with ox-LDL (50ng/ml,24h)
Relative protein expression
SMA
Calponin
Treated with ox-LDL (50ng/ml,24h)


A
WT ADAM10 3'UTR 5' UUUAAGAGUAUA- - AGGUUUACA 3'
miR-30c-5p 3' CGACUCUCACAUCCUACAAAUGU 5'
MUT ADAM10 3'UTR 5' UUUAAGAGUAUA- - AGCAAAUGA 3'
B
miR-NC
miR-30c-5p
Relative luciferase activity
WT-ADAM10 3'UTR
MUT-ADAM10 3'UTR
C
Relative ADAM10 mRNA expression
Normal
CAS
D
Normal CAS
ADAM10
GAPDH
Relative ADAM10 protein expression
Normal
CAS
E
Relative ADAM10 mRNA expression
ox-LDL (ng/ml)
F
ox-LDL (μg/ml)
0 25 50 75 100
ADAM10
GAPDH
Relative ADAM10 protein expression
ox-LDL (ng/ml)
G
R=-0.7757
P<0.0001
Relative ADAM10 mRNA expression
Relative miR-30c-5p expression
H
Control
anti-miR-NC
anti-miR-30c-5p
miR-NC
miR-30c-5p
Relative miR-30c-5p expression
Treated with ox-LDL (50ng/ml,24h)
I
Control
anti-miR-NC
anti-miR-30c-5p
miR-NC
miR-30c-5p
Relative ADAM10 mRNA expression
Treated with ox-LDL (50ng/ml,24h)
J
hASMC
Control
anti-miR-NC
anti-miR-30c-5p
miR-NC
miR-30c-5p
ADAM10
GAPDH
Treated with ox-LDL (50ng/ml,24h)
Control
anti-miR-NC
anti-miR-30c-5p
miR-NC
miR-30c-5p
Relative ADAM10 protein expression
Treated with ox-LDL (50ng/ml,24h)


A
Control
pcDNA
ADAM10
Relative ADAM10 mRNA expression
Treated with ox-LDL (50ng/ml,24h)
B
Control
pcDNA
ADAM10
ADAM10
GAPDH
Relative ADAM10 protein expression
Treated with ox-LDL (50ng/ml,24h)
C
Control
miR-NC
miR-30c-5p
miR-30c-5p+pcDNA
miR-30c-5p+ADAM10
OD value (λ=490 nm)
D
Number of migrated cells
Treated with ox-LDL (50ng/ml,24h)
E
Elisa (pg/ml)
IL-6
IL-1β
TNF-α
Treated with ox-LDL (50ng/ml,24h)
F
SMA
Calponin
GAPDH
Relative protein expression
Treated with ox-LDL (50ng/ml,24h)


hASMC
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p
ADAM10
GAPDH
Cells treated with ox-LDL (50ng/ml,24h)
Relative ADAM10 protein expression
1.5
1.0
0.5
0.0
*
*
Control
si-NC
si-lnc-SNHG16
si-lnc-SNHG16+anti-miR-NC
si-lnc-SNHG16+anti-miR-30c-5p