LncRNA SNHG12 promotes proliferation and migration of vascular smooth muscle cells via targeting miR-766-5p/ EIF5A

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Research

Keywords: SNHG12, hVSMCs, migration, miR-766-5p, EIF5A

Posted Date: May 12th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-27364/v1

Abstract

Background

Although lncRNAs have reported to serve as potential biomarkers of atherosclerosis (AS), the role of lncRNA SNHG12 in AS are still unknown.

Methods

In present study, we investigated the regulatory effects of SNHG12 on human vascular smooth muscle cells (hVSMCs). RT-qPCR were employed to determine the expressions of SNHG12, miR-766-5p and eukaryotic translation initiation factor 5A (EIF5A). Cell viability was estimated via the Cell Counting Kit-8 assay. Wound healing and Transwell invasion assays were used for evaluation of hVSMCs migratory capacity. To further investigate the regulatory mechanisms, binding sites between SNHG12 and miR-766-5p, EIF5A and miR-766-5p were speculated via starBase V2.0, and validated using luciferase reporter gene assay.

Results

It was identified that SNHG12 was up-regulated in oxidized low-density lipoprotein (ox-LDL)-insulted hVSMCs. Silencing SNHG12 inhibited ox-LDL-induced proliferation and migration of hVSMCs. Moreover, we found that SNHG12 acted as a sponge of miR-766-5p, and miR-766-5p also interacted with EIF5A. EIF5A plasmids promoted the proliferation and migratory capacities of hVSMCs, however, shRNA-SNHG12 counteracted the facilitation of EIF5A plasmids on biological behaviors of hVSMCs.

Conclusions

These findings of this study demonstrated that SNHG12 facilitated the migration and invasion of hVSMCs via targeting miR-766-5p/EIF5A axis.

Background

Long non-coding RNAs (lncRNAs) are a heterogeneous class of non-coding RNAs greater than 200 nucleotides in length without protein-coding capacity [1]. Recently, studies have found that lncRNAs emerges as crucial regulators of atherosclerosis (AS) [2, 3]. AS is commonly recognized as a lipid-induced chronic inflammation of the vascular wall associated with activation and dysfunction of resident vascular cells [4], it contributes to stenosis of internal artery due to plaque accumulation [5]. Number of lncRNAs was reported to implicate in regulating cholesterol and lipid metabolism, they also play diverse roles in a variety of atherosclerotic processes including cell proliferation, migration, inflammation differentiation, and apoptosis [6].

Small nucleolar RNA host gene 12 (SNHG12) is one of the classes of SNHGs [7]. Studies revealed that SNHG12 regulate cell proliferation, migration, invasion and metastasis in several cancers [8–12],

indicating a potential target for cancer-directed interventions [13]. Except for its role in cancers, SNHG12 could also ameliorates brain microvascular endothelial cell injury [14]. To date, a number of well-studied lncRNAs gave us important clues about the potential of them for AS treatment [15]. For instance, lincRNA-p21 is downregulated in atherosclerotic plaques of ApoE(-/-) mice, it can suppress vascular smooth muscle cells (VSMCs) proliferation and induce apoptosis [16]. HIF1α-AS1 regulates the proliferation and apoptosis of VSMCs [17]. The expression of H19 is higher in serum of AS patients [18], serving as a potential biomarker for diagnosing atherosclerosis. However, the status, biological function and the regulatory mechanisms of SNHG12 in AS are still unknown.

Here, we examined the expression of SNHG12 in human VSMCs (hVSMCs) exposed to oxidized low density lipoprotein (ox-LDL), and evaluated the influence of SNHG12 on cell biological activities. Furthermore, the regulatory mechanisms of SNHG12 on biological activities of hVSMCs were explored.

Materials And Methods

Cell lines and transfection

hVSMCs were cultured in DMEM (Procell, Wuhan, China) containing 10% FBS (Gibco, NY, USA) under an atmosphere of 95% air and 5% CO_2 at 37 °C. Ox-LDL (Solarbio, Beijing, China) was utilized to stimulate hVSMCs for 48 h. MiR-766-5p mimic, miR-NC (negative control) and shRNA-SNHG12 (sh-SNHG12-1, sh-SNHG12-2) were obtained from GenePharma Co., Ltd (Shanghai, China). Overexpression plasmids of EIF5A and the negative control were generated with the help of Sangon Biotech (Shanghai, China).

RT-qPCR

Total RNA was harvested (TRIzol™ Plus RNA Purification Kit, Invitrogen) and reversely transcribed into cDNA (M-MLV Reverse Transcriptase, Promega). TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) were employed to quantify miR-766-5p, the relative expression of miR-766-5p was normalized to U6, and others were normalized to GAPDH based on $2^{-\triangle\triangle}$Ct method [19]. The primers were listed in Table 1:

Table 1: the sequences of primers.

Gene	Primers
SNHG12	forward: 5′-GTGATACTGAGGAGGTGAG-3′ reverse: 5′-CCTTCTGCTTCCCATAGAG-3′
EIF5A	forward: 5′-AGGCCATGGCAAAATAACTG-3′ reverse: 5′-GGGTGGGGAAAACCAAAATA-3′
GAPDH	forward: 5′-AGCCTCCCGCTTCGCTCTCTGC-3′ reverse: 5′-ACCAGGCGCCCAATACGACCAAA-3′
miR-766-5p	forward: 5′-TCGAGTACTTGAGATGGAGTTTT-3′ reverse: 5′-GGCCGCGTTGCAGTGAGCCGAG-3′
U6	forward: 5′-CTCGCTTCGGCAGCACA-3′ reverse: 5′-AACGCTTCACGAATTTGCGT-3′

Cell Viability Assay

hVSMCs were seed into a 96-well plate, then cells were incubated with 10 μL Cell Counting Kit-8 (CCK-8) solution (Beyotime, Jiangsu, China) at 24 h, 48 h, 72 h. Absorbance value was recorded on a BioTek microplate reader (BioTek, VA, USA) at 450 nm. The results were represented as the relative percentage of the control group.

Wound Healing Assay

An amount of 1×10^5 hVSMCs were planted into each well of a 12-well plate, when 100% confluence was achieved, removed culture medium and drew straightly via a 200 μL plastic pipette. Washed gently to remove the floating cells, then added serum-free medium and maintained in the incubator for 12 h. Photographed at 0, 12 h under microscope (Zeiss, Oberkochen, Germany).

Transwell Invasion Assay

The bottom Transwell chamber (Costar, USA) was supplemented with 600 μl DMEM medium, containing 20% FBS, hVSMCs were resuspended in 200 μL serum-free DMEM medium and subsequently added into the upper chamber and cultured for 24 h in the incubator. 24 h later, the invasive cells were fixed with methanol and stained with 0.1% crystal viole for 30 min.

Luciferase Reporter Gene Assay

SNHG12 or EIF5A sequences containing the wild-type (WT) binding site or mutated-type (Mut) binding site for miR-766-5p were synthesized by Vigorous Biotechnology Beijing Co., Ltd (Beijing, China) and

cloned into the pmiR-GLO vector (Promega Corporation, WI, USA). Prior to transfection, cells were seeded into 24-well plates (5×10^3 cells/well) and cultured for 24 h. Afterwards, the WT or Mut of SNHG12 was transiently co-transfected with miR-766-5p mimics or miR-NC using lipofectamine 3000 reagent for another 48 h, the firefly luciferase activity normalized to that of Renilla was represented the value of relative luciferase activity. Likewise, EIF5A WT or Mut co-transfected with miR-766-5p mimic or miR-NC was similar to the above method.

Western Blotting

Total protein from treated cells was extracted using radio immunoprecipitation assay lysis buffer containing proteinase inhibitors (Beyotime, Jiangsu, China). After determination of protein concentrantions, the equal protein samples were loaded on SDS-PAGE gels and transferred onto PVDF membranes (Millipore, Madison, WI, USA). Then, the membranes were blocked with 5% non-fat milk for 2 h, and incubated with primary antibodies against EIF5A and GAPDH (both obtained from Invitrogen) at 4 °C overnight. HRP-conjugated antibody (Santa Cruz Biotechnology) was used to incubate membranes for 2 h at room temperature.

Statistical analysis

All data were presented as the mean ± standard deviation. The results were analyzed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student's t-test was employed to evaluate differences between two groups and one-way analysis of variance was used for comparison of differences between three or more groups. $P < 0.05$ was considered statistically significant.

Results

Interference with SNHG12 inhibits migration and invasion of hVSMCs

We first investigated the expression of SNHG12. HVSMCs were stimulated with different concentration of ox-LDL, as shown in Fig. 1A, ox-LDL could promote the expression of SNHG12 in a dose-dependent manner. 100 mg/L ox-LDL was considered an optimal concentration to induce the transcription of SNHG12. To elucidate the function of SNHG12 in hVSMCs, loss-of-function study was performed via transfecting sh-SNHG12 into cells. It was identified that sh-SNHG12-1 presented a better outcome for silencing SNHG12 (Fig. 1B). Afterwards, we estimated the cell viability of hVSMCs in absence of SNHG12, results showed that sh-SNHG12-1 significantly inhibited the increased cell viability caused by ox-LDL (Fig. 1C). Moreover, wound healing assay indicated that ox-LDL-triggered cell migration was overturned by silencing SNHG12 (Fig. 1D, 1E). Likewise, sh-SNHG12-1 reduced invasive cells induced by ox-LDL (Fig. 1F, 1G). These result suggested that disturbing the expression of SNHG12 could inhibit migration and invasion of hVSMCs.

SNHG12 Functions As A Sponge Of miR-766-5p

LncRNAs are considered as competing endogenous RNAs (ceRNAs) to bind with miRNAs and modulate gene expression [20]. Jia *et al* have demonstrated that miR-766-5p participated the cell proliferation, migration and invasion in colorectal cancer [21]. Of note, binding sites between SNHG12 and miR-766-5p were speculated via starBase V2.0 (Fig. 2A), and miR-766-5p mimic was validated to be effective to elevated the expression of miR-766-5p (Fig. 2B). Luciferase reporter gene assay demonstrated that miR-766-5p mimic inhibited the luciferase activity in hVSMCs transfected with SNHG12-WT, while distinction was failed to observe in hVSMCs transfected with SNHG12-Mut (Fig. 2C). We found sh-SNHG12-1 elevated the expression of miR-766-5p (Fig. 2D). In addition, the level of miR-766-5p in hVSMCs treated with ox-LDL was decreased (Fig. 2E). Collectively, we found that miR-766-5p was down-regulated in ox-LDL-treated hVSMCs and SNHG12 directly targeted miR-766-5p.

EIF5A Is Target Gene Of miR-766-5p

As mentioned above, SNHG12 directly targeted miR-766-5p and served as a ceRNAs to bind with miR-766-5p. CeRNAs activity forms a large-scale cross-talk network among the transcriptome, miRNAs are generally regarded as active regulatory elements, which reduce the stability of target RNAs or inhibit their translation [22]. Therefore, target mRNAs are considered as silencing objects of miRNAs. EIF5A is a small molecule protein in eukaryotic cells, which plays an important role in cell growth, survival and senescence. It is especially essential for cell proliferation [23]. Of note, binding site of miR-766-5p on EIF5A were speculated (Fig. 3A). Luciferase reporter gene analysis was employed to testify the potential interaction of them. It was manifested that miR-766-5p mimic apparently decreased the luciferase activity of EIF5A-WT in hVSMCs, mutation of EIF5A abrogated the function of miR-766-5p mimic (Fig. 3B). Overexpression of miR-766-5p reduced the transcription and translation of EIF5A (Fig. 3C, 3D). All of these data indicating that EIF5A may be target mRNA of miR-766-5p.

SNHG12 regulates the migration and invasion of hVSMCs via targeting EIF5A

In order to further explore the interaction between EIF5A and SNHG12, gain-of-function and loss-of-function studies were applied in subsequent experiments. Overexpression plasmids were constructed and transfected into hVSMCs with or without sh-SNHG12-1, high-expression of EIF5A validated the plasmids could overexpress EIF5A successfully. However, sh-SNHG12-1 drastically impeded the mRNA and protein levels of EIF5A (Fig. 4A, 4B). Cell viability was elevated in EIF5A overexpression group, while the effect was abolished by knockdown of SNHG12 (Fig. 4C). Migratory capacity represented by wound width illustrated that EIF5A promoted hVSMCs migration, SNHG12 knockdown exhibited an inhibition of cell migration (Fig. 4D, 4E). In addition, Transwell invasion assay manifested sh-SNHG12-1 suppressed invasive capacity of hVSMCs enhanced by EIF5A overexpression plasmids (Fig. 4F, 4G). Taken together, these results revealed that SNHG12 mediated the migratory and invasive capacities of hVSMCs may through targeting EIF5A.

Discussion

In recent years, numerous studies have demonstrated that lncRNAs regulates various cellular process including cell proliferation, migration, invasion and apoptosis [24–26]. It was reported that lncRNA MIAT activated the PI3K/Akt signaling pathway, thereby exacerbated atherosclerotic damage in AS mice [3]. LncRNA ATB expression was significantly higher in atherosclerosis patients compared with the healthy, and it could enhance the expression of caspase-3 in HUVECs [27]. Furthermore, the proliferation and migration of VSMCs were promoted by lncRNA 430945 [28].

Studies have implicated SNHG12 in various cancers, it functions as a potential candidate for cancer-directed interventions [29, 30]. The altered expression of SNHG12 is associated the cell viability, proliferation, metastasis, and invasion, affecting the progression and diagnosis of cancer [13]. However, the function of SNHG12 in AS have not yet been clearly elucidated. In this study, it was found that ox-LDL facilitated the expression of SNHG12 in hVSMCs. Deletion of SNHG12 impeded the cell migration and invasion induced by ox-LDL.

Previous reports have described lncRNAs interact with miRNA as ceRNAs and protect miRNAs from binding to and repressing target RNAs [22, 31], suggesting a complicated crosstalk among diverse RNA species. In our study, luciferase reporter gene assay revealed an interplay between SNHG12 and miR-766-5p, and SNHG12 knockdown enhanced the expression of miR-766-5p. To further examine the target RNA regulated by lncRNA-miRNA, binding sites of miR-766-5p on EIF5A sequence were predicted using starBase V2.0. Subsequently, the interaction between miR-766-5p and EIF5A was further validated via luciferase reporter gene assay and RT-qPCR. Finally, we found that overexpression of EIF5A expedited the migration and invasion of hVSMCs, whereas the effect was reversed by silencing SNHG12.

Conclusions

In summary, the present study illustrated that SNHG12 was upregulated in ox-LDL-challenged hVSMCs. An intricate interplay among SNHG12, miR-766-5p and EIF5A was discovered and all of these results indicated that SNHG12 promoted the migration and invasion of hVSMCs may through targeting miR-766-5p/EIF5A axis. Further research is necessary for investigating the impact of SNHG12/miR-766-5p/EIF5A signal pathway on other pathological alterations in AS progression.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

LW performed the experiments and draft the manuscript. CJH and GY analyzed the data. SL and JYY helped to perform the experiments. YS designed the experiment in this study and draft the manuscript.

Funding

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interests.

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Figures

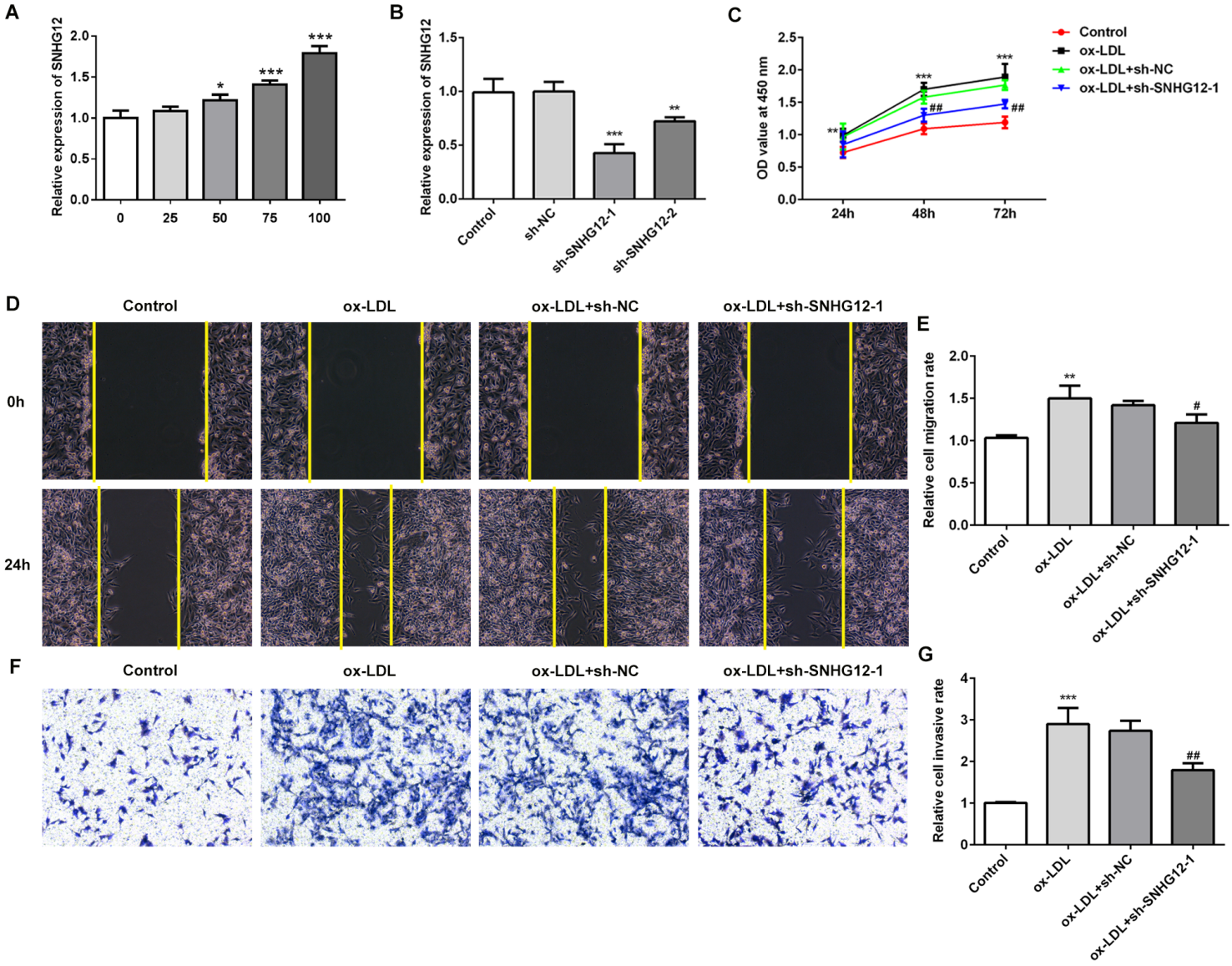

Figure 1

Interference with SNHG12 inhibits migration and invasion of hVSMCs. (A) hVSMCs were stimulated with different concentrations of ox-LDL, the expression of SNHG12 was explored by RT-qPCR. *p<0.05, ***p<0.001 versus 0 mg/L ox-LDL. (B) The expression of SNHG12 in hVSMCs transfected with sh-SNHG12-1 or sh-SNHG12-2 was estimated by RT-qPCR. *p<0.05, **p<0.01 versus sh-NC group. (C) hVSMCs were stimulated with 100 mg/L ox-LDL for 12 h, 24 h and 48 h, the expression of SNHG12 was explored by RT-qPCR. **p<0.01, ***p<0.001 versus control group; ##p<0.01 versus ox-LDL+sh-NC group. (D, E) The capability of cell migration was assessed by wound healing assay. **p<0.01 versus control group; #p<0.05 versus ox-LDL+sh-NC group. (F, G) The capability of cell invasion was assessed by Transwell invasion assay. ***p<0.001 versus control group; ##p<0.01 versus ox-LDL+sh-NC group.

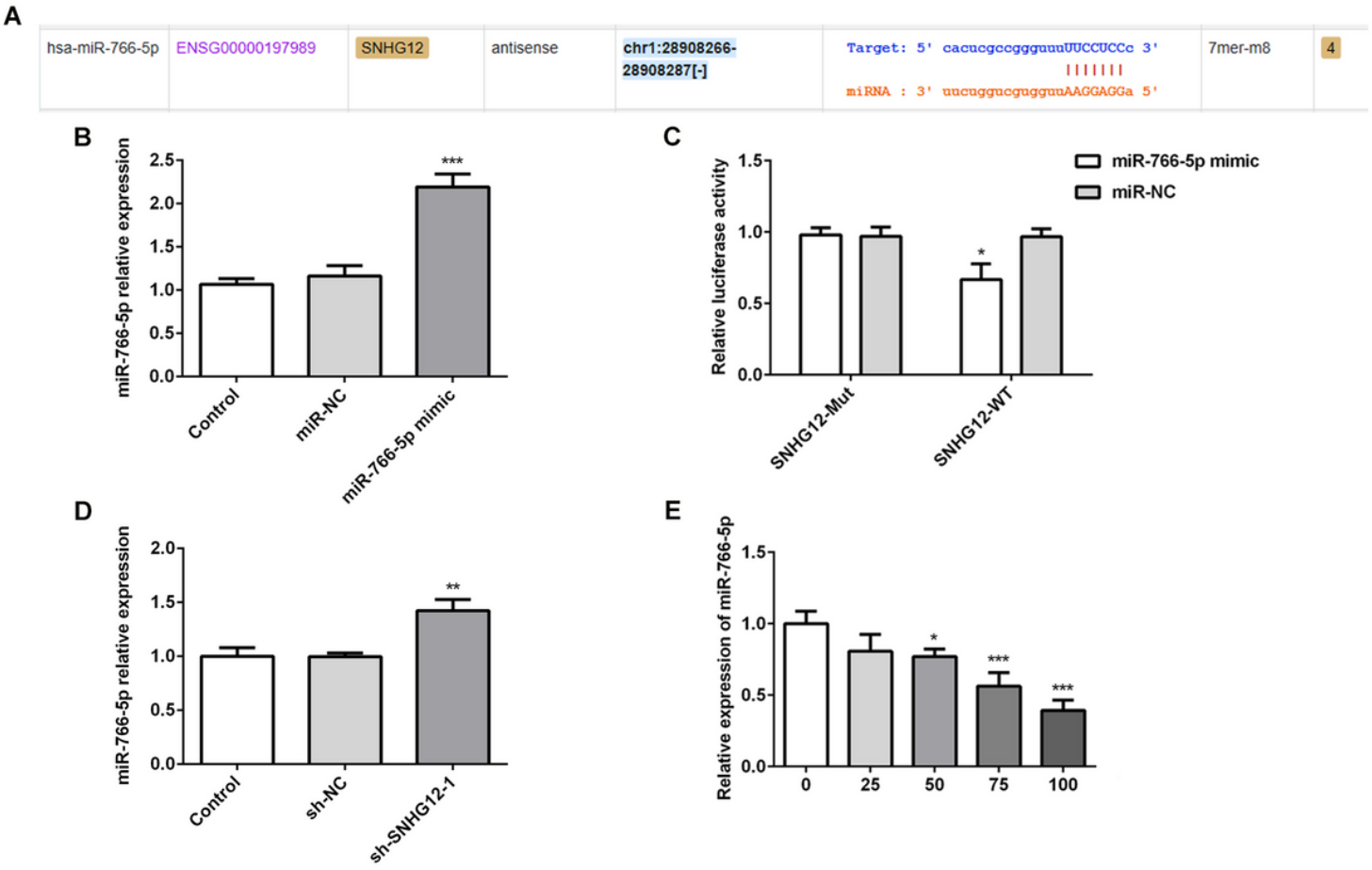

Figure 2

SNHG12 functions as a sponge of miR-766-5p. (A) The potential binding sites were speculated via starBase V2.0. (B) RT-qPCR was utilized to determine the expression of miR-766-5p in hVSMCs transfected with miR-766-5p mimic or miR-NC. ***p<0.001 versus miR-NC group. (C) The interaction between SNHG12 and miR-766-5p was validated by luciferase reporter gene assay. *p<0.05 versus miR-NC group. (D) The expression of miR-766-5p in hVSMCs transfected with sh-SNHG12-1 or sh-NC. **p<0.01 versus sh-NC group. (E) RT-qPCR was utilized to determine the expression of miR-766-5p in hVSMCs exposed to ox-LDL (0, 25, 50, 75, 100 mg/L). *p<0.05, ***p<0.001 versus 0 mg/L ox-LDL.

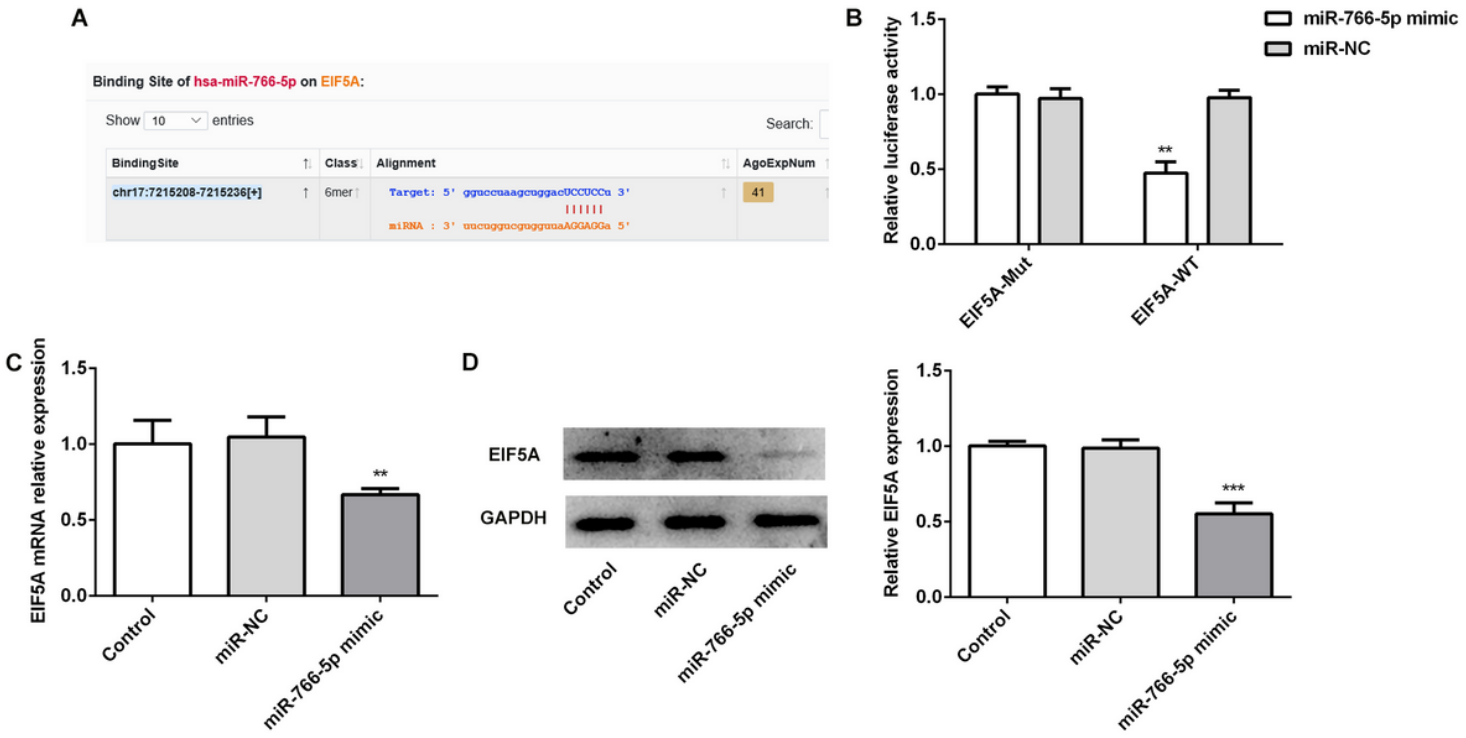

Figure 3

EIF5A is target gene of miR-766-5p. (A) The potential binding sites were speculated via starBase V2.0. (B) The interaction between miR-766-5p and EIF5A was validated by luciferase reporter gene assay. **p<0.01 versus miR-NC group. (C) RT-qPCR was utilized to determine the expression of EIF5A in hVSMCs transfected with miR-766-5p mimic or miR-NC. **p<0.01 versus miR-NC group. (D) Western blottig was utilized to determine the protein levels of EIF5A in hVSMCs. ***p<0.001 versus miR-NC group.

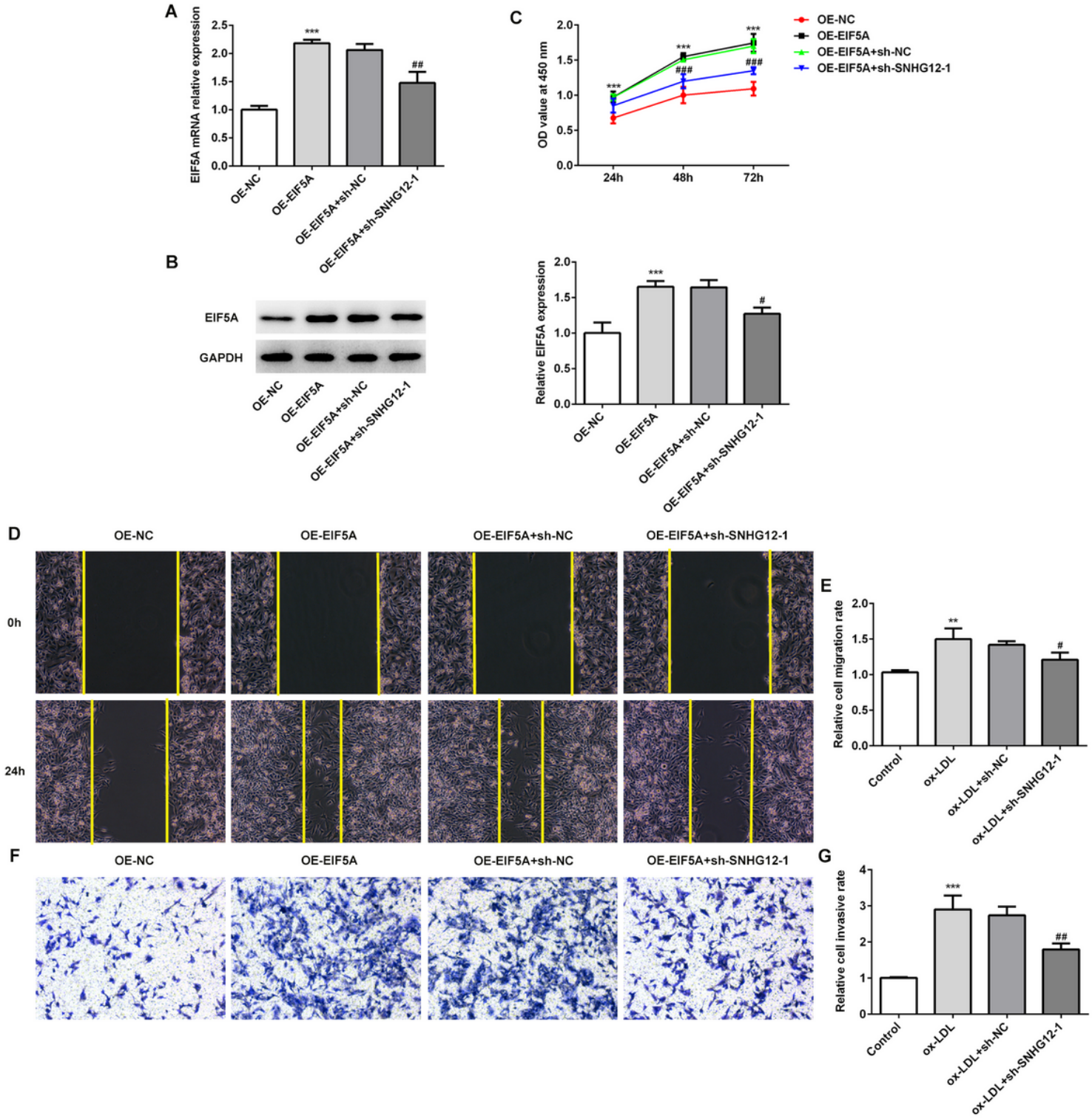

Figure 4

SNHG12 regulates the migration and invasion of hVSMCs via targeting EIF5A. (A) The expression of EIF5A was examined via RT-qPCR. ***p<0.001 versus OE-NC group. ##p<0.01 versus OE-EIF5A+sh-NC group. (B) Western blottig was utilized to determine the protein levels of EIF5A in hVSMCs. ***p<0.001 versus OE-NC group. #p<0.05 versus OE-EIF5A+sh-NC group. (C) The cell viability was estimated by CCK-8 assay. ***p<0.001 versus OE-NC group. ###p<0.001 versus OE-EIF5A+sh-NC group. (D, E) The

capability of cell migration was assessed by wound healing assay. **p<0.01 versus control group. #p<0.05 versus ox-LDL+sh-NC group. (F, G) The capability of cell invasion was assessed by Transwell invasion assay. ***p<0.01 versus control group. ##p<0.01 versus ox-LDL+sh-NC group.