Silencing of lncRNA *SNHG12* inhibits proliferation and migration of vascular smooth muscle cells via targeting *miR-766-5p/EIF5A* axis

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Abstract

Background. Although long non-coding RNAs (lncRNAs) have been reported to serve as potential biomarkers of atherosclerosis (AS), the role of lncRNA small nucleolar RNA host gene 12 (*SNHG12*) in AS still remains to be elucidated.

Objectives. The present study aimed to investigate the regulatory effects and potential mechanisms of *SNHG12* in human vascular smooth muscle cells (hVSMCs).

Materials and methods. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was employed to determine the expression of *SNHG12*, *miR-766-5p* and eukaryotic translation initiation factor 5A (*EIF5A*) in oxidized low-density lipoprotein (ox-LDL)-induced hVSMCs. After transfection with short hairpin RNA (shRNA)-*SNHG12*, cell viability was estimated using the Cell Counting Kit-8 (CCK-8) assay. Wound healing and transwell assays were used for evaluating migratory capacities of hVSMCs. To further investigate the regulatory mechanisms, binding sites between *SNHG12* and *miR-766-5p*, and *EIF5A* and *miR-766-5p* were predicted using starBase database and validated using luciferase reporter gene assays. Moreover, cell viability and migration were detected following *EIF5A* overexpression and *SNHG12*-knockdown.

Results. *SNHG12* was significantly upregulated in ox-LDL-induced hVSMCs. *SNHG12* silencing inhibited ox-LDL-induced proliferation and migration of hVSMCs. Moreover, *SNHG12* acted as a sponge of *miR-766-5p*, and *miR-766-5p* also interacted with *EIF5A*. *EIF5A* plasmids promoted the capacities of proliferation and migration in ox-LDL-induced hVSMCs. However, shRNA-*SNHG12* counteracted the facilitation of *EIF5A* plasmids on hVSMCs biological behaviors.

Conclusions. Taken together, these findings demonstrated that silencing of *SNHG12* blocks the proliferation and migration of hVSMCs via targeting the *miR-766-5p/EIF5A* axis.

Key words: migration, *SNHG12*, human vascular smooth muscle cells, *miR-766-5p*, eukaryotic translation initiation factor 5A

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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 emerge as crucial regulators of atherosclerosis (AS).[2,3] Atherosclerosis is commonly recognized as a lipid-induced chronic inflammation of the vascular wall associated with activation and dysfunction of resident vascular cells[4] and contributes to stenosis of internal arteries due to plaque accumulation.[5] The number of lncRNAs was reported to be implicated in regulating cholesterol and lipid metabolism, and 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* regulates 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 ameliorate brain microvascular endothelial cell injury.[14] To date, a number of well-studied lncRNAs gave us important clues about their potential for AS treatment.[15] For instance, lincRNA-p21 is downregulated in atherosclerotic plaques of ApoE(–/–) mice, and it can suppress vascular smooth muscle cell (VSMC) 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 AS. However, the status, biological function and regulatory mechanisms of *SNHG12* in AS are still unknown.

Objectives

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 migration. Furthermore, the regulatory mechanisms of *SNHG12* on hVSMCs were explored.

Materials and methods

Cell lines and transfection

The hVSMCs (Cell Bank of the Shanghai Institute of Cell Biology, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; ProCell, Wuhan, China) containing 10% fetal bovine serum (FBS; Gibco, Waltham, USA) under an atmosphere of 95% air and 5% CO_2 at 37°C. The ox-LDL (Solarbio, Beijing, China) was used to stimulate hVSMCs for 48 h. *miR-766-5p* mimic, miR-NC (negative control), and 2 pairs of short hairpin RNA (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). Cells were collected 24 h following transfection, and transfection efficiency was evaluated using reverse-transcription quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR

Total RNA was harvested (TRIzol™ Plus RNA Purification Kit; Invitrogen, Carlsbad, USA) and reverse transcribed into cDNA (M-MLV Reverse Transcriptase; Promega, Madison, USA). TaqMan MicroRNA Assay kit (Applied Biosystems; Thermo Fisher Scientific, Waltham, USA) was 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 the $2^{-\Delta\Delta Ct}$ method.[19] The primers used in this study were as follows: *SNHG12*, forward: 5′-GTGATACTGAGGAGGTGAG-3′ and reverse: 5′-CCTTCTGCTTCCCATAGAG-3′; *EIF5A*, forward: 5′-AGGCCATGGCAAAATAACTG-3′ and reverse: 5′-GGGTGGGGAAAACCAAAATA-3′; *GAPDH*, forward: 5′-AGCCTCCCGCTTCGCTCTCTGC-3′ and reverse: 5′-ACCAGGCGCCCAATACGACCAAA-3′; *miR-766-5p*, forward: 5′-TCGAGTACTTGAGATGGAGTTTT-3′ and reverse: 5′-GGCCGCGTTGCAGTGAGCCGAG-3′; *U6*, forward: 5′-CTCGCTTCGGCAGCACA-3′ and reverse: 5′-AACGCTTCACGAATTTGCGT-3′.

Cell viability assay

The hVSMCs were seeded 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 and 72 h. Absorbance values were recorded on a BioTek microplate reader (BioTek, Winooski, USA) at 450 nm.

Wound healing assay

An amount of 1×10^5 of hVSMCs were plated into each well of a 12-well plate. When 100% confluence was achieved, the culture medium was removed and drew straight from the plate using a 200 µL plastic pipette. The sample was washed gently to remove the floating cells, then serum-free medium was added and maintained in the incubator for 24 h. Samples were photographed at 0 h and 24 h under a microscope (Axioscope 5; Carl Zeiss, Oberkochen, Germany).

Transwell migration assay

For the transwell migration assay, serum-free media containing 5×10^4 of hVSMCs were seeded into the upper chamber of a 24-well transwell filter with 8-µm pore size. The lower chamber was filled with media supplemented

with 10% FBS. Cells were allowed to transgress through the porous filters for 24 h at 37°C. Then, VSMCs were fixed with 4% paraformaldehyde for 20 min. Cells that migrated through the pores of the filter were stained with 1% crystal violet for 30 min. The images were photographed under a fluorescence microscope (BX51; Olympus Corp., Tokyo, Japan), and the number of migrated cells was calculated using ImageJ software (National Institutes of Health, Bethesda, USA).

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). Prior to transfection, cells were seeded into 24-well plates (5×10^3 cells/well) and cultured for 24 h. Afterward, 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 Renilla 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 a radio immunoprecipitation assay lysis buffer containing proteinase inhibitors (Beyotime). After the determination of protein concentrations, equal protein samples (40 μg) were loaded on SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Madison, 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 Cell Signaling Technology, Inc., Danvers, USA) at 4°C overnight. Horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology, Santa Cruz, USA) was used to incubate membranes for 2 h at room temperature. The blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, USA) and subsequently quantified using ImageJ software v. 1.52r (National Institutes of Health).

Statistical analyses

All data were presented as the mean ± standard deviation (SD). The results were analyzed using GraphPad Prism v. 6.0 (GraphPad Software, Inc., San Diego, USA). An unpaired student's t-test was employed to evaluate differences between 2 groups, and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparison of differences between 3 or more groups. A value of $p < 0.05$ was considered statistically significant.

Results

Interference with *SNHG12* inhibits proliferation and migration of ox-LDL-induced hVSMCs

We first investigated the expression of *SNHG12*. The hVSMCs were stimulated with different concentrations of ox-LDL, and, as shown in Fig. 1A, ox-LDL could promote the expression of *SNHG12* in a dose-dependent manner. A volume of 100 mg/L ox-LDL was considered an optimal concentration to induce the transcription of *SNHG12*. To elucidate the function of *SNHG12* in hVSMCs, a 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). Afterward, we estimated the cell viability of hVSMCs in the absence of *SNHG12*, and results showed that sh-SNHG12-1 transfection significantly inhibited the increased cell proliferation caused by ox-LDL stimulation (Fig. 1C). Moreover, the wound healing assay and transwell migration assay indicated that ox-LDL-triggered cell migration was overturned by silencing of *SNHG12* (Fig. 1D–G). These results suggest that disturbing the expression of *SNHG12* could inhibit the viability and migration of hVSMCs induced by ox-LDL.

SNHG12 functions as a sponge of *miR-766-5p*

The LncRNAs are considered competing endogenous RNAs (ceRNAs) to bind with miRNAs and modulate gene expression.[20] Jia et al. demonstrated that *miR-766-5p* participated in cell proliferation, migration and invasion in colorectal cancer.[21] Of note, binding sites between *SNHG12* and *miR-766-5p* were predicted using starBase v. 2.0 (http://starbase.sysu.edu.cn) (Fig. 2A), and *miR-766-5p* mimic was validated to be effective to elevate the expression of *miR-766-5p* (Fig. 2B). The luciferase reporter gene assay demonstrated that *miR-766-5p* mimic inhibited luciferase activity in hVSMCs transfected with *SNHG12*-WT (Fig. 2C). Additionally, it was found that sh-SNHG12-1 elevated the expression of *miR-766-5p* (Fig. 2D). Moreover, the level of *miR-766-5p* in hVSMCs treated with ox-LDL was notably decreased (Fig. 2E). Collectively, this data reveal that *miR-766-5p* is remarkably downregulated in ox-LDL-treated hVSMCs, and *SNHG12* directly targeted *miR-766-5p*.

EIF5A is a direct target gene of *miR-766-5p*

As mentioned above, *SNHG12* directly targeted *miR-766-5p* and served as a ceRNA to bind with *miR-766-5p*. The ceRNA activity forms a large-scale cross-talk network among the transcriptome. The miRNAs are generally regarded as active regulatory elements which reduce

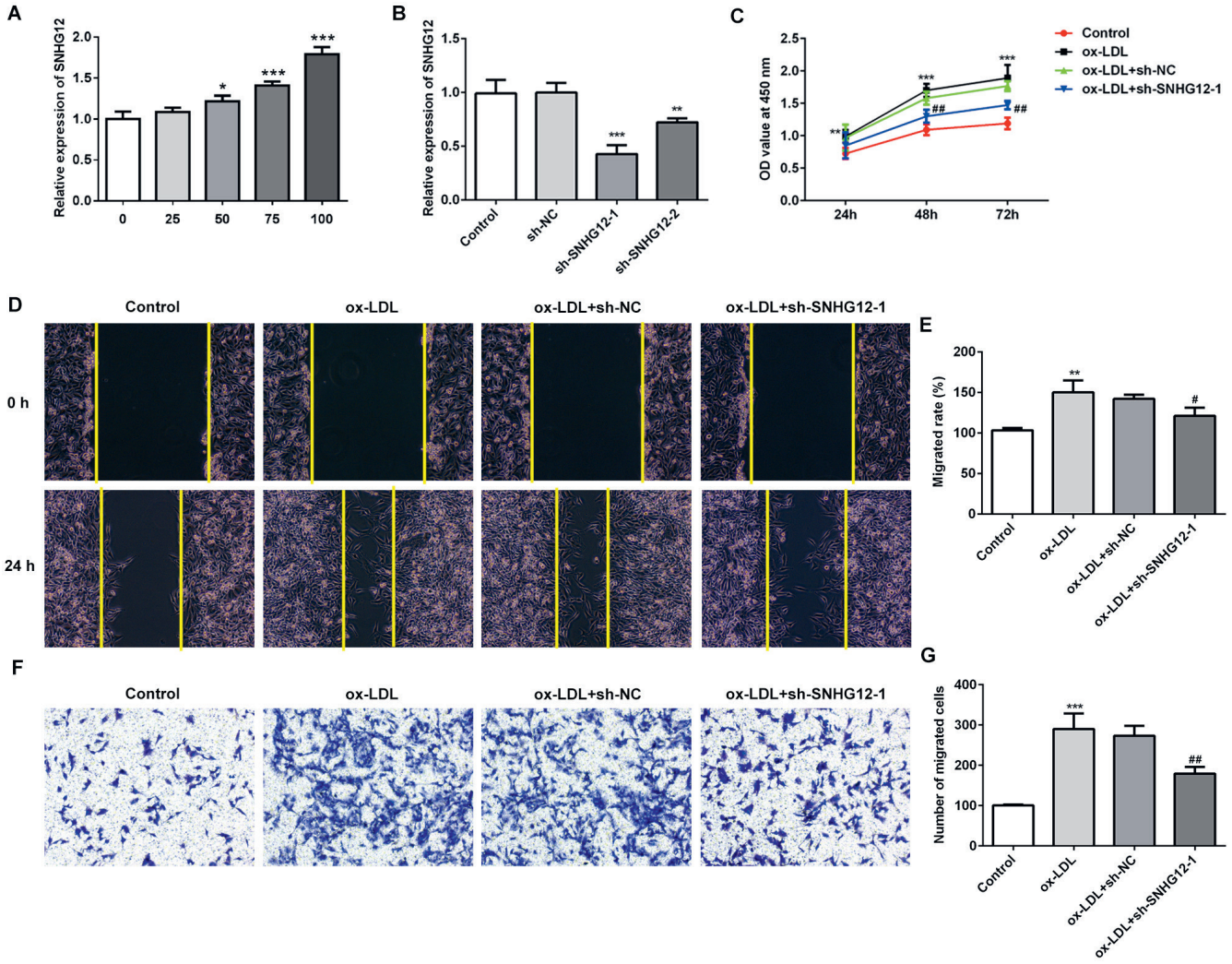

Fig. 1. Interference with *SNHG12* inhibits proliferation and migration of hVSMCs

A. hVSMCs were stimulated with different concentrations of ox-LDL (0 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L), and the expression of *SNHG12* was explored using RT-qPCR; *p < 0.05, ***p < 0.001 compared to 0 mg/L ox-LDL; B. The expression of *SNHG12* in hVSMCs transfected with sh-SNHG12-1 or sh-SNHG12-2 was estimated using RT-qPCR; **p < 0.01, ***p < 0.001 compared to the sh-NC group; C. hVSMCs were stimulated with 100 mg/L ox-LDL for 24 h, 48 h and 72 h, and the cell viability was explored using CCK-8 assay; **p < 0.01, ***p < 0.001 compared to the control group; ##p < 0.01 compared to the ox-LDL+sh-NC group. The capability of cell migration was assessed using wound healing assay (D and E) and transwell migration assay (F and G); **p < 0.01, ***p < 0.001 compared to the control group; #p < 0.05, ##p < 0.01 compared to the ox-LDL+sh-NC group; ×100 magnification.

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, *EIF5A* was predicted as a potential target of *miR-766-5p* (Fig. 3A). Luciferase reporter gene analysis was employed to test the potential interaction between them. It was observed that *miR-766-5p* mimic apparently decreased the luciferase activity of *EIF5A*-WT in hVSMCs, and mutation of *EIF5A* abrogated the function of *miR-766-5p* mimic (Fig. 3B). Subsequently, overexpression of *miR-766-5p* reduced the transcription and translation of *EIF5A* (Fig. 3C,D). All of these data indicate that *EIF5A* may be a target mRNA of *miR-766-5p*.

SNHG12 regulates the proliferation and migration of ox-LDL-induced hVSMCs via regulating *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, and 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,B). 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, while

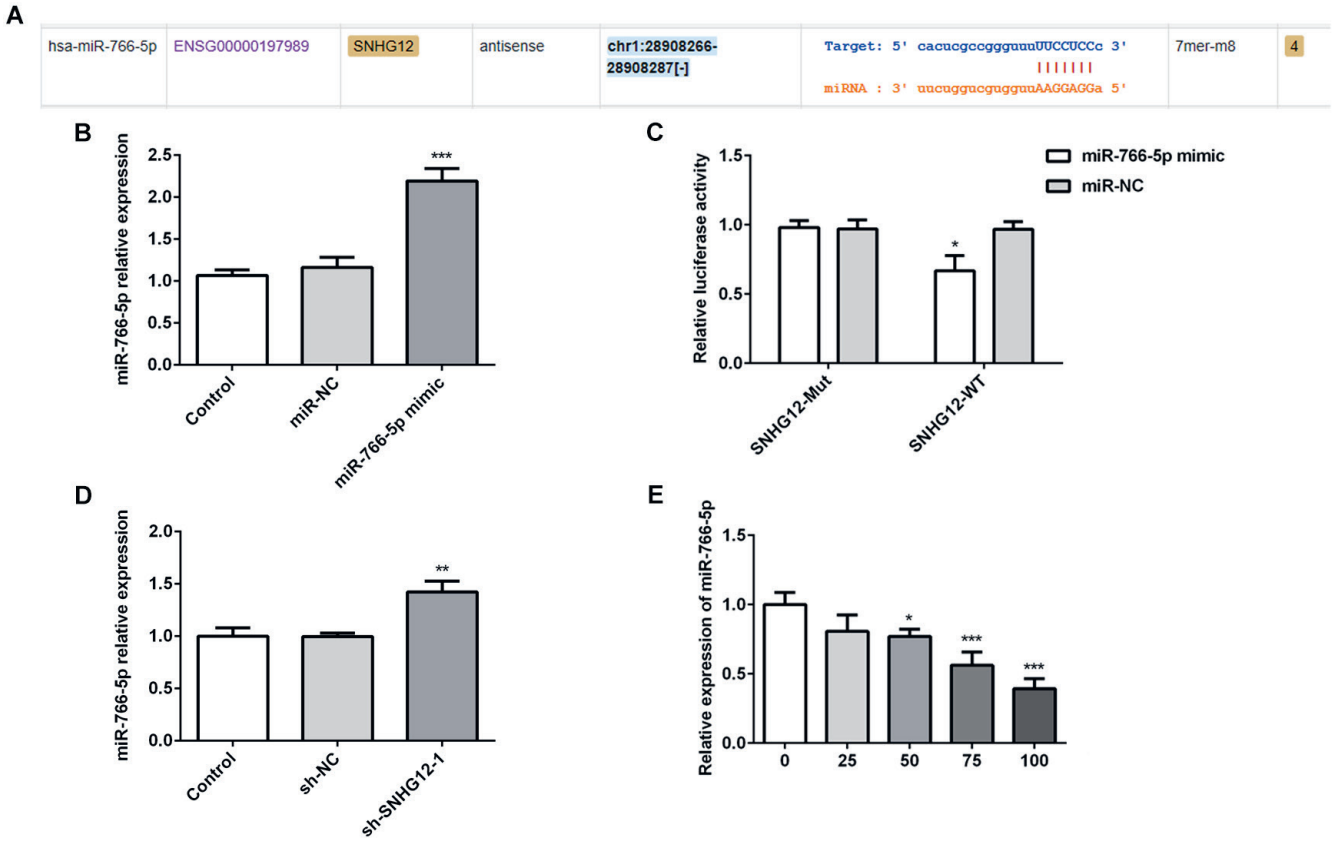

Fig. 2. *SNHG12* functions as a sponge of *miR-766-5p*

A. The potential binding sites were predicted using starBase v. 2.0; B. RT-qPCR was used to determine the expression of *miR-766-5p* in hVSMCs transfected with *miR-766-5p* mimic or miR-NC; ***p < 0.001 compared to miR-NC group; C. The interaction between *SNHG12* and *miR-766-5p* was validated using luciferase reporter gene assay; *p < 0.05 compared to miR-NC group; D. The expression of *miR-766-5p* in hVSMCs transfected with sh-SNHG12-1 or sh-NC; **p < 0.01 compared to the sh-NC group; E. RT-qPCR was used to determine the expression of *miR-766-5p* in hVSMCs exposed to ox-LDL (0 mg/L, 25 mg/L, 50 mg/L, 75 mg/L, 100 mg/L); *p < 0.05, ***p < 0.001 compared to 0 mg/L ox-LDL.

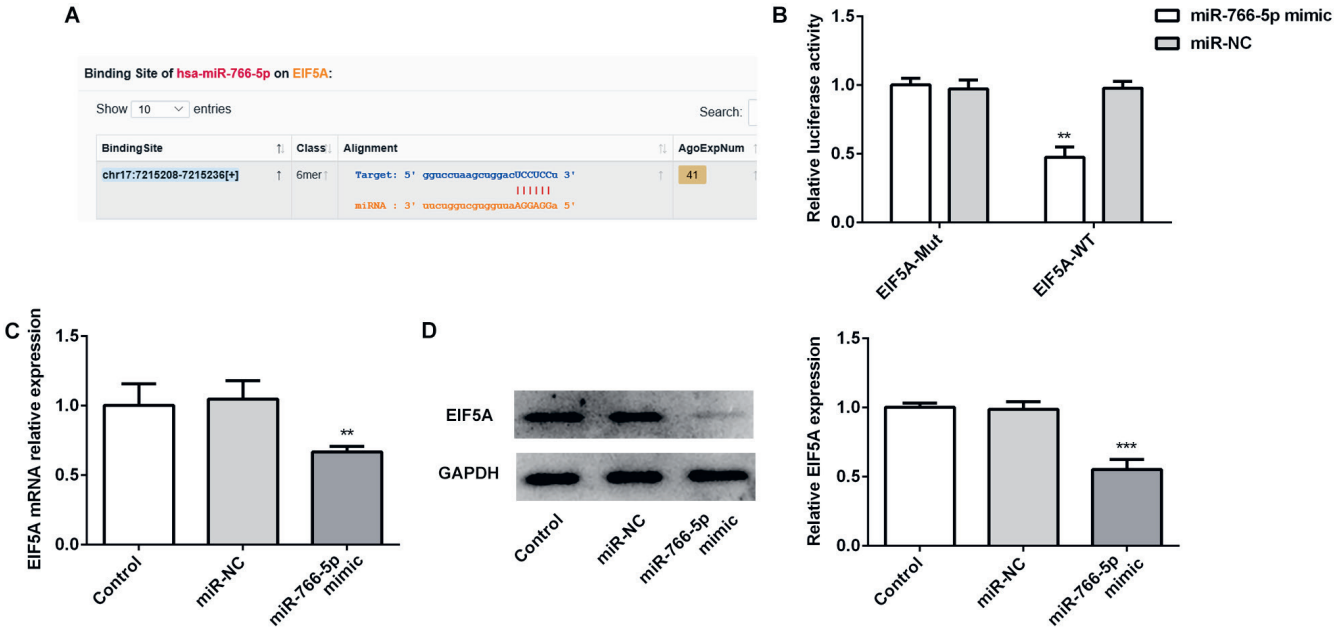

Fig. 3. *EIF5A* is target gene of *miR-766-5p*

A. The potential binding sites were predicted using starBase v. 2.0; B. The interaction between *miR-766-5p* and *EIF5A* was validated using luciferase reporter gene assay; **p < 0.01 compared to miR-NC group; C. RT-qPCR was used to determine the expression of *EIF5A* in hVSMCs transfected with *miR-766-5p* mimic or miR-NC; **p < 0.01 compared to the miR-NC group; D. western blotting was used to determine the protein levels of *EIF5A* in hVSMCs; ***p < 0.001 compared to the miR-NC group.

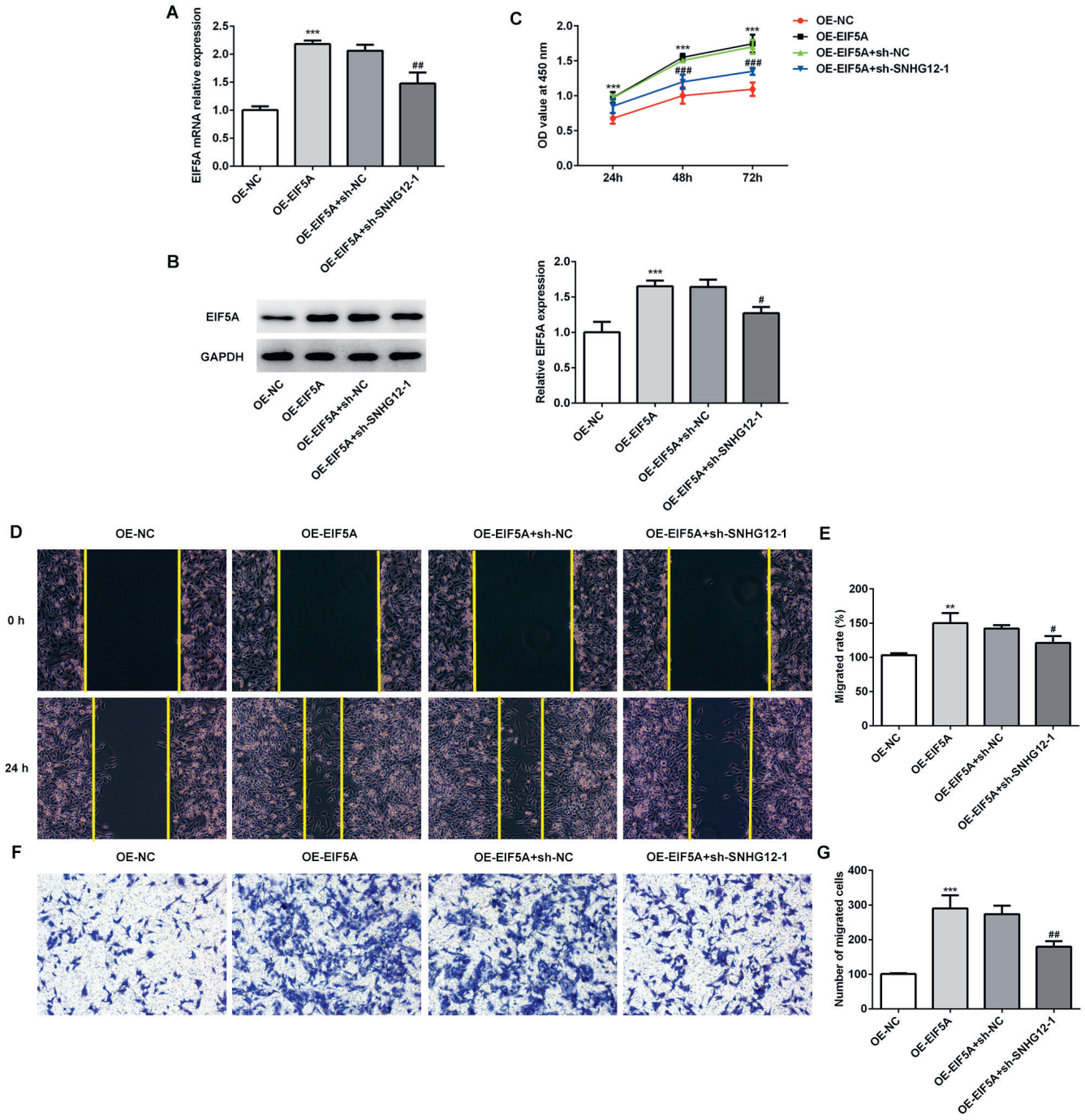

Fig. 4. *SNHG12* regulates the proliferation and migration of hVSMCs via mediation of *EIF5A*

A. The expression of *EIF5A* was examined using RT-qPCR; ***p < 0.001 compared to the OE-NC group; ##p < 0.01 compared to the OE-EIF5A+sh-NC group; B. western blot analysis was used to determine the protein levels of *EIF5A* in hVSMCs; ***p < 0.001 compared to the OE-NC group; #p < 0.05 compared to the OE-EIF5A+sh-NC group; C. The cell viability was estimated using CCK-8 assay; ***p < 0.001 compared to the OE-NC group; ###p < 0.001 compared to the OE-EIF5A+sh-NC group. The capability of cell migration was assessed using wound healing assay (D and E) and transwell migration assay (F and G); **p < 0.01, ***p < 0.01 compared to the control group; #p < 0.05, ##p < 0.01 compared to the ox-LDL+sh-NC group; ×100 magnification.

SNHG12 knockdown exhibited an inhibition of cell migration (Fig. 4D,E). Consistently, transwell migration assays showed a similar result with that of the wound healing assay (Fig. 4F,G). Taken together, these results indicate that *SNHG12* mediated the migratory capacities of hVSMCs through regulating *EIF5A*.

Discussion

In recent years, numerous studies have demonstrated that lncRNAs regulate various cellular process including cell proliferation, migration, invasion, and apoptosis.[24–26] It was reported that lncRNA MIAT activates the PI3K/Akt

signaling pathway, thereby exacerbating atherosclerotic damage in AS mice.[3] LncRNA activated by transforming growth factor (TGF) expression is significantly higher in AS patients compared with healthy patients, and it could enhance the expression of caspase-3 in human vascular endothelial cells (HUVECs).[27] Furthermore, the proliferation and migration of VSMCs were promoted by lncRNA 430945.[28]

The hVSMCs are the major cell type observed in blood vessel walls, and play a considerable role in the regulation of multiple physiological and pathological situations.[29] Aberrant proliferation and migration of VSMCs are key events in the progression of AS and restenosis after percutaneous coronary intervention.[30] A large amount of studies have suggested that ox-LDL exert a promotion effect in the development of AS by stimulating the proliferation of hVSMCs within the vessel wall; therefore, ox-LDL was widely used to stimulate hVSMCs for investigating the related mechanisms of AS.[31,32] Studies have implicated *SNHG12* in various cancers, and it functions as a potential candidate for cancer-directed interventions.[33,34] The altered expression of *SNHG12* is associated with cell viability, proliferation, metastasis, and invasion, thereby affecting the progression and diagnosis of cancer.[13] However, the function of *SNHG12* in AS has 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 cell migration induced by ox-LDL.

Previous reports have described that lncRNAs interact with miRNA as ceRNAs and protect miRNAs from binding to and repressing target RNAs,[22,35] suggesting a complicated crosstalk among diverse RNA species. Accumulating reports have been made to understand the effect of miRNAs in VSMC biology, especially in cellular proliferation and migration.[36–38] In our study, luciferase reporter gene assays 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 between *miR-766-5p* and *EIF5A* sequence were predicted using starBase v. 2.0. Subsequently, the interaction between *miR-766-5p* and *EIF5A* was further validated using luciferase reporter gene assay and RT-qPCR. Finally, we found that overexpression of *EIF5A* expedited the proliferation and migration of hVSMCs, whereas the effect was reversed by *SNHG12* silencing.

Conclusions

The present study illustrates that *SNHG12* was highly expressed 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*-knockdown inhibited the proliferation and migration of hVSMCs through targeting the *miR-766-5p*/*EIF5A* axis. Further research is necessary for investigating the impact of *SNHG12*/*miR-766-5p*/*EIF5A* signaling pathway on other pathological alterations in AS progression.

The data supporting our findings are available from the corresponding author upon reasonable request.

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