Pathology - Research and Practice 215 (2019) 152575

Contents lists available at ScienceDirect

Pathology - Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original article

Silencing lncRNA SNHG6 suppresses proliferation and invasion of breast cancer cells through miR-26a/VASP axis

Kai Li[a,1], Yan-bin Ma[a,1], Yi-hao Tian[b,1], Xiao-long Xu[a], Yang Gao[a], Yan-qi He[a], Wen-ting Pan[a], Jing-wei Zhang[c], Chun-jiang He[d], Lei Wei[a,*]

[a] Department of Pathology and Pathophysiology, Hubei Provincial Key Laboratory of Developmentally Originated Disease, School of Basic Medical Sciences, Wuhan University, Wuhan 430071, Hubei, China
[b] Department of Anatomy, School of Basic Medical Sciences, Wuhan University, Wuhan 430071, Hubei, China
[c] Department of Breast and Thyroid Surgery, Zhongnan Hospital, Hubei Key Laboratory of Tumor Biological Behaviors, Hubei Cancer Clinical Study Center, Wuhan University, Wuhan 430071, Hubei, China
[d] Hubei Provincial Key Laboratory of Developmentally Originated Disease, Wuhan University, Wuhan 430071, Hubei, China

ARTICLE INFO

Keywords:
LncRNA
SNHG6
miR-26a
VASP
Breast cancer

ABSTRACT

The important role of LncRNA in the development of breast cancer is attracting more and more attention. In the previous study, we found that the expression level of LncRNA SNHG6 in breast cancer tissues and cells was significantly increased, but its mechanism in the development of breast cancer was still unclear. Our study found that knockdown of SNHG6 significantly inhibited the proliferation, migration and invasion of breast cancer cells MCF-7 and MDA-MB-231 cells. Further study showed that knockdown of SNHG6 significantly inhibited the expression level of VASP. More importantly, SNHG6 and VASP both can bind directly to miR-26a, suggesting that SNHG6 could act as a ceRNA to sponge miR-26a, thereby promoting the expression of VASP, which leading to activated proliferation, migration and invasion of breast cancer cells. Taken together, this study revealed the important role of the SNHG6/miR-26a/VASP regulatory network in the development of breast cancer, and provided a reference for exploring new pathogenesis and biomarkers of breast cancer.

1. Introduction

As one of the most common female malignancies in the world, the high occurrence of breast cancer is still a global challenge [1]. Although it has different treatment options such as surgery, chemotherapy, radiotherapy, ablation, hormone therapy and combination therapy, it still remains high mortality and recurrence rate [2]. Thereby, there is an urgent need to identify new therapy targets and biomarkers for breast cancer.

The vasodilator-stimulated phosphoprotein (VASP) gene is located in the region of human chromosome 19q13.2-13.3. As one of the Ena/VASP protein family members, it is an actin-related skeleton protein widely present in different tissues and cells. It plays an important role in various life activities that depend on cytoskeletal rearrangement [3]. Spatial and differential expression of VASP in normal lung tissues and lung adenocarcinomas suggests that it may be involved in the differentiation of normal lung cells into adenocarcinomas. A positive correlation between VASP expression and pathological stage indicates that it may regulate the invasion behavior of lung adenocarcinoma [4]. Our previous series of work showed that VASP was a key target protein for regulating the migration of various tumor cells: high expression of VASP was positively correlated with poor differentiation of gastric adenocarcinoma [5]; In cervical, gastric and breast cancer cells, knockdown of VASP expression can significantly inhibit tumor cell migration [6]; In addition, we found that the inflammatory factor TNF-α can inhibit breast cancer cell adhesion and promote proliferation via the HIF-1α/VASP pathway [7,8]; hsa-miR-610 can inhibit the migration and invasion of gastric cancer cells by targeting the 3′UTR region of VASP [9]; Berberine can inhibit the migration of breast cancer cells by targeting VASP and changing its spatial structure [10]. These studies indicated that VASP was involved in the assembly and aggregation of actin filaments, which in turn regulated cell migration and promoted tumor invasion and metastasis.

In this study, through bioinformatics analysis, we found that miR-

* Corresponding author at: Department of Pathology and Pathophysiology, Hubei Provincial Key Laboratory of Developmentally Originated Disease, School of Basic Medical Sciences, Wuhan University, 185 Donghu Road, Wuchang District, Wuhan 430071, Hubei, China.
E-mail address: leiwei@whu.edu.cn (L. Wei).
[1] Contributed equally.

https://doi.org/10.1016/j.prp.2019.152575
Received 12 May 2019; Received in revised form 16 July 2019; Accepted 31 July 2019
0344-0338/

26a can not only bind to SNHG6, but also bind to the 3′UTR region of VASP protein. Therefore, it is hypothesized that SNHG6 and VASP act synergistically to promote the development of breast cancer through the bridge action of miR-26a. This study will help clarify the mechanism of SNHG6 and VASP in breast cancer, and further explore the molecular mechanisms of SNHG6/miR-26a/VASP regulatory network in breast cancer progression, providing a theoretical basis for exploring new mechanism and biomarkers of breast cancer.

2. Materials and methods

2.1. Human breast cancer samples

Twenty cases of breast cancer tissues and cancer adjacent normal tissues were collected from Affiliated Zhongnan Hospital of Wuhan University and diagnosed by the Department of Pathology. All patients were informed and agreed. Our research was approved by the Ethics Board of School of Basic Medical Sciences, Wuhan University and was based on all relevant principles of the Declaration of Helsinki. The breast cancer data used to evaluate the expression of SNHG6, miR-26a and VASP was from TCGA (https://cancergenome.nih.gov/) and analyzed by R language. The relationship of SNHG6, miR-26a and VASP expression with the survival percent of breast cancer patients was analyzed by the Kaplan Meier plotter database [11].

2.2. Cell culture

The cell lines used for this study were purchased from the China Center for Type Culture Collection (CCTCC, Chinese Academy of Sciences, Shanghai, China). EFM192A, AU565, UACC893, MDA-MB-415, HS742 T, MDA-MB-231, MCF-7 and HEK293 T cells were cultured in medium (DMEM, HyClone, USA, SH30022.01B) supplemented with 10% fetal bovine serum (FBS) (Gibco, Milano, Italy, 10099-141). MCF-10A cells were cultured in Dulbecco's Modifies Eagle Medium (DMEM/F-12; GIBCO, Milano, Italy, 12,400,024) with F-12 supplemented with 5% horse serum, 20 ng/mL EGF, 100 ng/mL cholera toxin, 0.5 mg/mL hydrocortisone and 10 mg/mL insulin.

2.3. Plasmids, siRNA and cell transfection

To meet the experimental needs, the full-length VASP (Accession: NM_003370.3) 3′UTR region and the mutants of miR-26a binding site were constructed into pMIR-report-luci vector, and the restriction sites were SpeI and HindIII. pMIR-report-con (pMIR-REPORT System miRNA Expression Reporter Vector, AM5795, ABI), pMIR-report-VASP 3′UTR (foward: 5′-GGACTAGTCCACAGGGACCCAGAAGACCC-3′; reverse: 5′−CCAAGCTTCTGCAGGGGCCCATTTTCAAG-3′), pMIR-report-VASP 3′UTR mut (foward: 5′-CAAGGTGCTCGGGCGGTCCAACGGCAGGGTGACAGGCCGGGT-3′; reverse: 5′−CCTGCCGTTGGACCGCCCGAGCACCTTGGAATTGGCTGAAGA-3′), pMIR-report-SNHG6 (Accession: NR_002599.2) 3′UTR (foward: 5′-GGACTAGTCTTTCCCGCGCGACCGGC-3′; reverse: 5′−CCAAGCTTGTAAGGGGGAATTTGATTGCTA-3′), pMIR-report-SNHG6 3′UTR mut (foward: 5′-GAGCATTTTGTCCAGTGTTGATAACATCACAAT-3′; reverse: 5′-TTATCAACACTGGACAAAATGCTCATTTTCATC-3′).

The siRNAs (small interfering RNAs) of SNHG6 were purchased from GenePharma Company in Suzhou (A10001). si-SNHG6#1 was at nucleotides 104 bp (foward: 5′-GAGGUGCAAGAAAGCCUUUTT-3′; reverse: 5′-AAAGGCUUUCUUGCACCUCTT-3′), si-SNHG6#2 at nucleotides 204 bp (foward: 5′-GCGGCAUGUAUUGAGCAUATT-3′; reverse: 5′-UAUGCUCAAUACAUGCCGCTT-3′), and si-SNHG6#3 at nucleotides 273 bp (foward: 5′-GCUUCGUUACCUCAAGUGUTT-3′; reverse: 5′-ACACUUGAGGUAACGAAGCTT-3′), and the negative si-RNA control (foward: 5′-UUCUCCGAACGUGUCACGUTT-3′; reverse: 5′-ACGUGACACGUUCGGAGAATT-3′).

All of these plasmids and siRNAs were sequenced and confirmed. HEK 293 T cells with a density of 1×10^5 were seeded into 24-well plates to achieve a healing rate of 80% on the next day. Then, siRNAs and plasmids were transfected with Lipofectamine 2000 reagent (Invitrogen Co, Ltd. 11668-027). The transfection procedure is referred to the instruction manual. After transfection for 48 h, cells were harvested to detect the biological effect or target gene expression level.

2.4. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Trizol reagent (Invitrogen, Carlsbad, CA, 15596-026) was applied for extracting total RNA of cells and tissues. 2 μg RNA was added for first-strand cDNA synthesis with GeneAmp™ RNA PCR Core Kit (Thermo Scientific, USA, K1622). Then the expression level of target genes was analyzed by 2 μL cDNA. The mRNA expression level was detected and analyzed by qPCR (Applied Biosystem Inc.) [12]. For qPCR, the primers of mRNA are SNHG6 (foward: 5'-CCTACTGACAACATCGACGTTGAAG-3′; reverse: 5′-GGAGAAAACGCTTAGCCATACAG-3′), VASP (foward: 5′-ATGGCAACAAGCGATGGCT-3′; reverse: 5′-CGATGGCACAGTTGATGACCA-3′), and GAPDH (foward: 5′-AATGGACAACTGGTCGTGGAC-3′; reverse: 5−CCCTCCAGGGGATCTGTTTG-3′). As for miR-26a, foward: 5′-TTCAAGTAATCCAGGATAGGCT-3′; reverse was Universal Primer (Qiagen,Germany). When the reaction system is well prepared, put it on the Fluorescence quantitative PCR (BIO-RAD, CFX96, 15000193, Singapore) and run it.

2.5. Western blotting

The protein was extracted with RIPA lysis buffer (biosharp, BL504A), and quantified with BCA Protein Assay Kit (Beyotime Biotechnology Co, Jiangsu, China, P0010S). Preparing polyacrylamide gel with 12% separation gel and adding the protein sample to the sample hole with 20 μg. The protein was bind to the PVDF membrane (Millipore, Billerica, MA, IPVH00010) by electrophoretic and wet transmembrane with running buffer and transfer buffer. After blocking the nonspecific sites on the membrane with 5% skim milk powder at room temperature about 2 h, a primary antibody (Proteintech, USA) (Including GAPDH, ag0766, dilution of 1:1000; VASP, Ag17679, dilution of 1:1000) should be prepared. And then incubate the PVDF membrane overnight at 4 ℃. The membrane was incubated with the corresponding secondary antibody (dilution of 1:1000, 10285-1-AP) for 1 h at room temperature, and finally detected by ECL reagents (Tanon, Shanghai, China, 180-5001) and digital imager (Tanon, 5200). The optical density of bands was measured by a computer-assisted imaging analysis system (Tanon, Shanghai, China) and the relative protein expression levels were normalized to GAPDH.

2.6. RNA isolation of nuclear and cytoplasmic fractions

RNA isolation of nuclear and cytoplasmic fractions was performed using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, 78,833), according to the manufacturer's protocol. The RNA expression level of SNHG6, miR-26a and VASP were analyzed by qRT-PCR. U6 was used as nuclear control transcript, and GAPDH as cytoplasmic control transcript.

2.7. RNA binding protein immunoprecipitation (RIP) assay and RNA pull down assay

The Ago2-RIP assay was performed using RNA Immunoprecipitation Kit (Millipore, Germany, 17-701) according to the manufacturer's protocol. The magnetic beads were incubated with AGO2 antibody or IgG. The immunoprecipitated RNAs were further analyzed by qRT-PCR. Total RNAs served as input control.

The RNA pull down assay was conducted using Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Scientific, 20164). Biotin labeled

SNHG6 and NC were transfected into cells for 48 h. The cell extract was incubated with streptavidin labeled magnetic beads at 4 °C for 4 h. Then, the coprecipitated RNAs were isolated and detected by qRT-PCR, according to the manufacturer's protocol.

2.8. Cell proliferation assay

CCK-8, colon formation and EdU assays were used to detect the proliferative capacity of cells. For the CCK-8 assay (Cell Counting Kit-8, Zomanbio, Beijing, ZP328-2), cells (1×10^3 cells/well) were seeded in 96-well plates. When cells were cultured for 0, 12, 24, and 48 h, the CCK-8 solution was added respectively. After continued to culture 2 h, the value of OD450 was measured by automated microplated reader (Bio-Tek, USA, FLx800).

For the colony formation assay, cells (2×10^2 cells/well) were seeded in 6-well plates. About one week later, the cells were fixed 20 min with 4% paraformaldehyde and stained 30 min with 5% crystal violet, then counted the colony number under a camera. As for the Edu assay (Cell-Light™ EdU ApoIIo567 In Vitro Kit, Ribobio, China, C10310-1), cells (4×10^3~1×10^5 cells/well) seeded in 24-well plates for 24 h, then cultured for 2 h in the medium with EdU in appropriate concentration. Based on the specific reaction of EdU and Apollo594 fluorescent dyes, it can rapidly detect the cell proliferation.

2.9. Wound healing assay

Cells (4×10^5 cells/2 mL) were seeded in 6-well plates and cultured for 24 h. After the cells were scratched, followed by washing with PBS, and treated with serum-free medium. After 0, 24 and 48 h of incubation, cells were fixed and photographed under a light microscope (100×). The number of cells migrated into the scratched area was calculated.

2.10. Transwell assay

The experiment used some 24-well plates and a polyvinyl-pyrrolidone-free polycarbonate filter (8 μm pore size). The lower chamber was filled with 500 μl medium containing 10% FBS, the upper chamber filled serum-free medium containing 2×10^4 cells. After incubation 24 h, cells were fixed and stained with 4% paraformaldehyde and 5% crystal violet, and counted the cell number in the surface of the lower chamber under light microscope (100×). The assay could be used to detect the ability cell invasion and migrate. Differently, when it was used to detect the ability of cell invasion, the 8 microspore was coated with Matrigel (BD Biosciences, USA).

2.11. Cell cycle analysis

Cell cycle was quantified by flow cytometry. Briefly, cells were trypsinized and collected, then fixed with pre-cooled anhydrous ethanol. Removing ethanol, per tube was added with 200 μL PBS and 2 μL RNase (0.25 mg/mL) (incubate at 37 °C for 30 min). Then the cell pellet was mixed with 0.5 mL of 50 μg/ml PI solution, staining for 30 min at room temperature in dark. The fluorescence intensity was analyzed by flow cytometry (BD Biosciences, USA), and the model of the cytometer have to be indicated was a flow cytometer software (BD FACSDiva 7.0).

2.12. Immunofluorescence

The sterilized circular glass slides were placed in a 24-well plate and cultured at 37 °C with the cells of logarithmic growth period seeded on the glass plates. When the cells successfully seeded, the cells were transfected into groups and cultured for 48 h. After being fixed 30 min with 4% paraformaldehyde, transparent 30 min with 0.5% triton, and blocked 1 h with goat serum, the antibody (VASP, Ag17679, dilution of 1:500) was added and incubated overnight at 4 °C. The next day, after washing the glass, the second antibody with fluorescent labeling (Alexa Fluor 488-conjuncted Affinipure Goat Anti-Mouse IgG, A23310-1, proteintech, China) was added, treating for 2 h, then washing and sealing the slide, and observing the staining results under fluorescence microscope (Olympus, Janpan) [13].

2.13. Luciferase assay

pMIR-report-control, pMIR-report-VASP, pMIR-report-VASP mut, pMIR-report-SNHG6 3′UTR, or pMIR-report-SNHG6 3′UTR mut were co-transfected with si-NC, si-SNHG6, control, miR-26a mimics, or miR-26a inhibit into HEK293 T cells by using Lipofectamine 2000, using PLR-TK (Promega, USA) as reference, and the transfection ratio was 10:30:1. At 48 h after transfection, the relative luciferase activities were measured by a Dual-Luciferase reporter Assay Kit (Promega, Madison, WI, USA, E1910) according to the manufacturer's guide and the GloMax 20/20 Luminometer (Promega, E5311).

2.14. Statistical analysis

Statistical analysis was performed using software SPSS 13.0. Each experiment was repeated for 3 times. The data was expressed as the mean ± standard deviation (SD). The variance analysis between groups was performed using a one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Upregulation of lncRNA SNHG6 and VASP and downregulation of miR-26a were presented in breast cancer tissues and cells, which were related to prognosis of breast cancer patients

The expression of SNHG6, VASP and miR-26a were analyzed using RNA-sequencing date from TCGA database. Compared with adjacent normal tissues, breast cancer tissues showed higher expression level of VASP and lower miR-26a, while there was no statistical difference with SNHG6 (Fig. 1A-C). Furthermore, Kaplan-Meier survival analysis showed that expression of VASP was negatively correlated, while miR-26a was positively, to the prognosis of breast cancer patients. However, there was no statistical difference with SNHG6 (Fig. 1D-F).

As shown in Fig. 1G, the expression of SNHG6 and VASP was significantly increased in breast cancer tissues, while miR-26a decreased, compared to adjacent normal tissues. In addition, we also revealed that SNHG6 and VASP were significantly increased in breast cancer cell lines EFM192A, AU565, UACC893, MDA-MB-415, HS742 T, MDA-MB-231 and MCF-7, while miR-26a decreased, compared to normal breast cell lines MCF-10A (Fig. 1H). Furthermore, we collected MDA-MB-231 and MCF-7 cells to extract the cytoplasm and nuclear, the results showed that SNHG6, miR-26a and VASP mRNA were mainly located in the cytoplasm (Fig. 1I).

3.2. Knockdown of SNHG6 inhibited breast cancer cell proliferation

Three siRNAs was constructed to knock down SNHG6 in breast cancer cells, and the efficiency of knockdown was detected by qPCR, which showed that all of the three siRNAs of SNHG6 was effective (Fig. 2A). Then we used si-SNHG6#2 to knock down SNHG6 and evaluated the effect of SNHG6 knockdown on the cell proliferation, which was detected by CCK-8 assay and colony formation assay. The results showed that SNHG6 knockdown inhibited the proliferation of MCF-7 and MDA-MB-231 cells (Fig. 2B and C). Furthermore, EdU staining assay also showed that knocking down SNHG6 significantly decreased the number of EdU-labeled (red fluorescence) MCF-7 and MDA-MB-231 cells (Fig. 2D). In addition, we also found that after knocking down SNHG6, the proportion of MCF-7 and MDA-MB-231

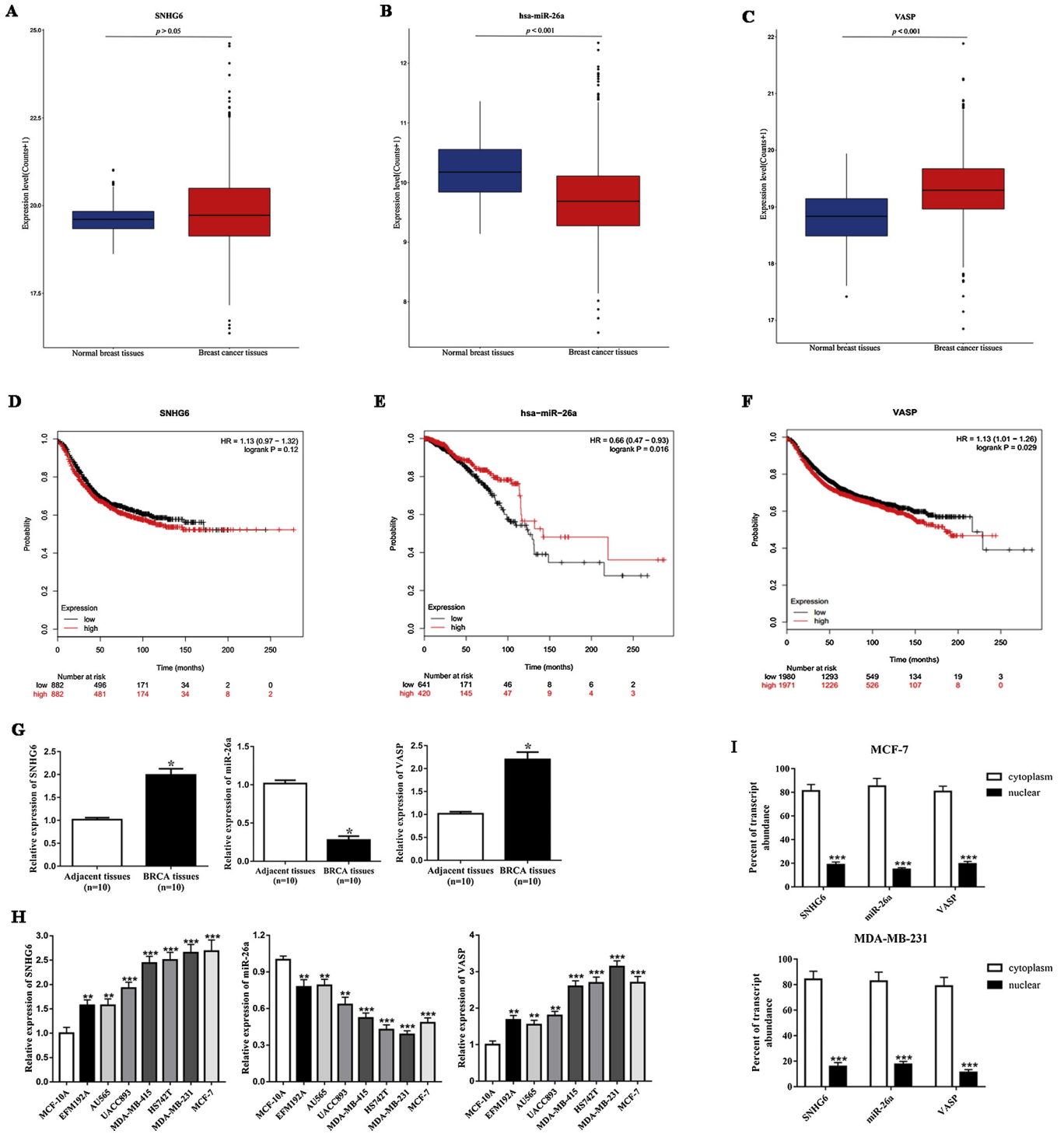


Fig. 1. Upregulation of lncRNA SNHG6 and VASP and downregulation of miR-26a were presented in breast cancer tissues and cells, which were related to prognosis of breast cancer patients. The differential expression of SNHG6 *(A)*, miR-26a *(B)* and VASP *(C)* in breast cancer samples and adjacent normal breast tissues was shown, according to TCGA database. The relationship of SNHG6 *(D)*, miR-26a *(E)* and VASP *(F)* with survival percents of breast cancer patients was analyzed by Kaplan-Meier survival analysis. *(G)* The relative expression level of SNHG6, miR-26a and VASP in breast cancer tissues (n = 10) and corresponding normal tissues (n = 10) were detected by qRT-PCR. *(H)* The relative expression level of SNHG6, miR-26a and VASP in breast cancer cell lines EFM192A, AU565, UACC893, MDA-MB-415, HS742 T, MDA-MB-231, MCF-7 and normal breast cell MCF-10A were detected by qRT-PCR. *(I)* The expression of SNHG6, miR-26a and VASP in cytoplasm and nuclear which were detected by qRT-PCR. *P < 0.05, **P < 0.01, ***P < 0.001.

cells at G2/M phase were both decreased, while the proportion of cells at G1 phase were increased (Fig. 2E).These results indicated that the knockdown of SNHG6 induced to G1 arrest in MCF-7 and MDA-MB-231 cells, exerted suppressive effects of cell proliferation.

3.3. Knockdown of SNHG6 inhibited migration and invasion of breast cancer cells

To further explore the effects of SNHG6 knockdown on migration and invasion abilities of breast cancer cells, wound healing and

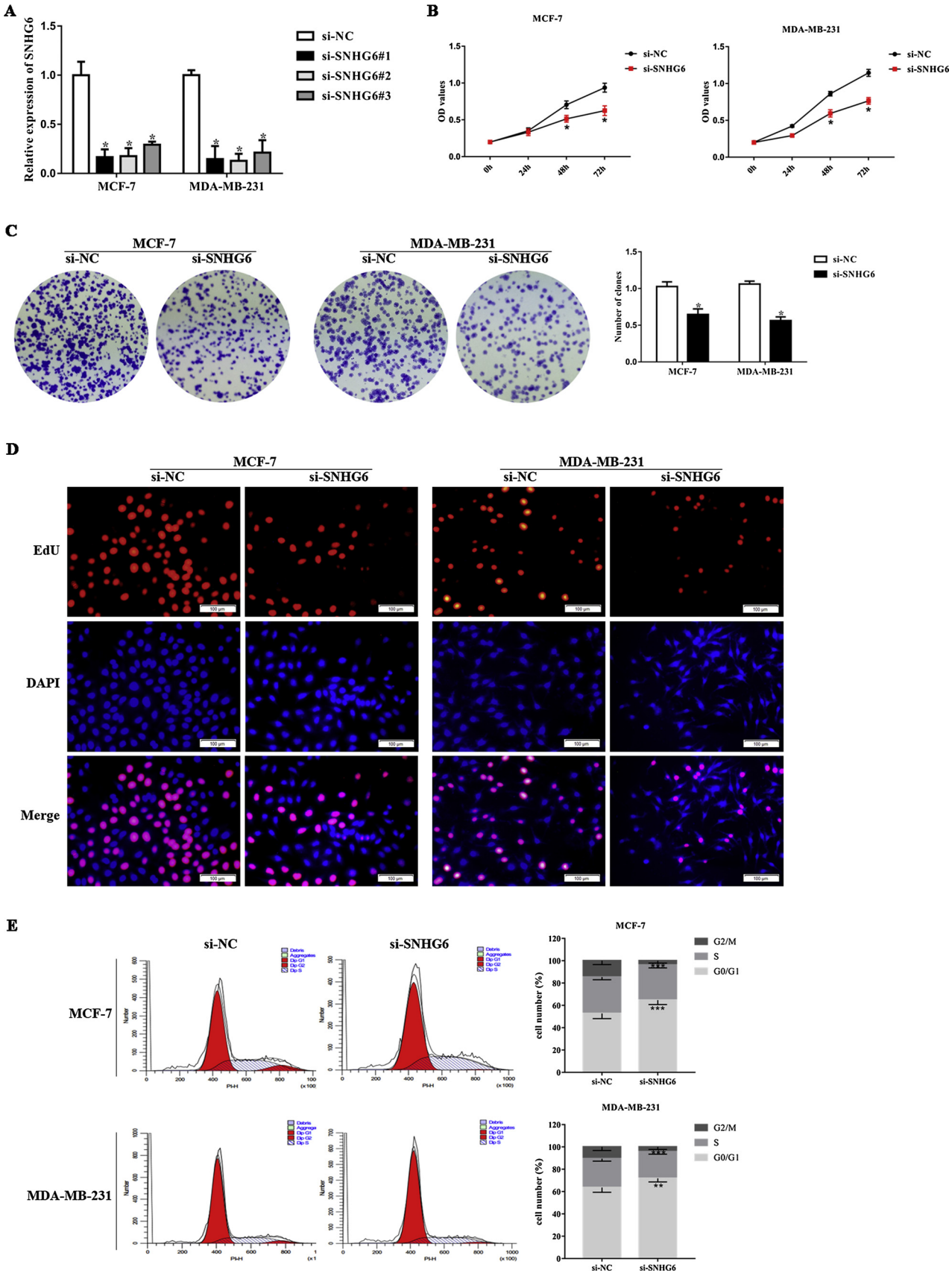


Fig. 2. Knockdown of SNHG6 inhibited breast cancer cell proliferation. *(A)* The expression of SNHG6 was significantly decreased by transfection of SNHG6 siRNA in MCF-7 and MDA-MB-231 cells, which were detected by qRT-PCR. *(B)* Growth curves of MCF-7 and MDA-MB-231 cells after transfection with si-SNHG6 or si-NC were determined via CCK-8 assays. *(C)* Colony formation assays showed that knockdown of SNHG6 inhibited breast cancer cell proliferation. *(D)* Suppression of SNHG6 expression attenuated the proliferation of breast cancer cells by EdU assay. *(E)* Flow cytometry assay showed that si-SNHG7 resulted in G1 arrest in breast cancer cells. The cell cycle distribution was exhibited. *P < 0.05, **P < 0.01, ***P < 0.001.

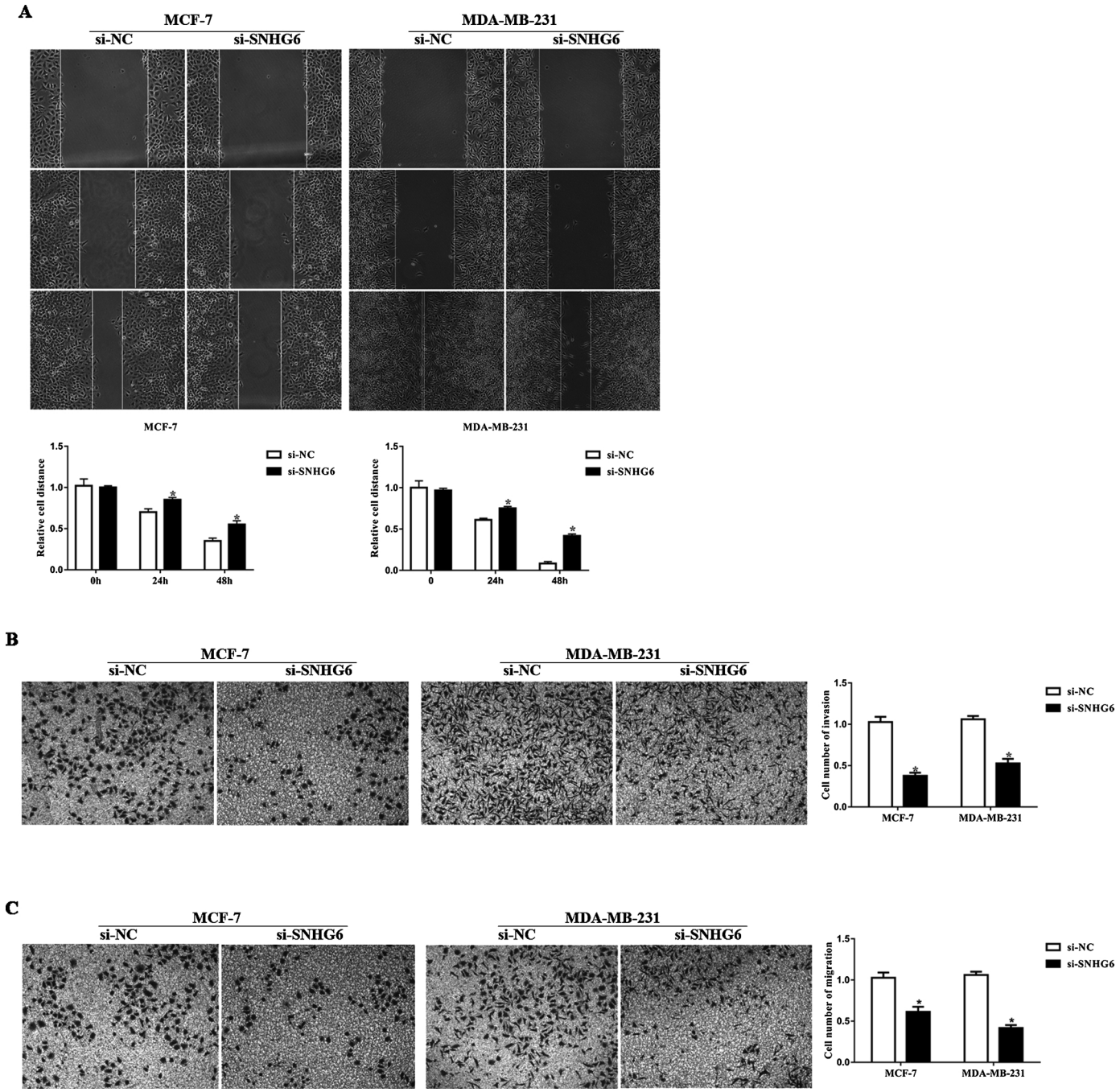
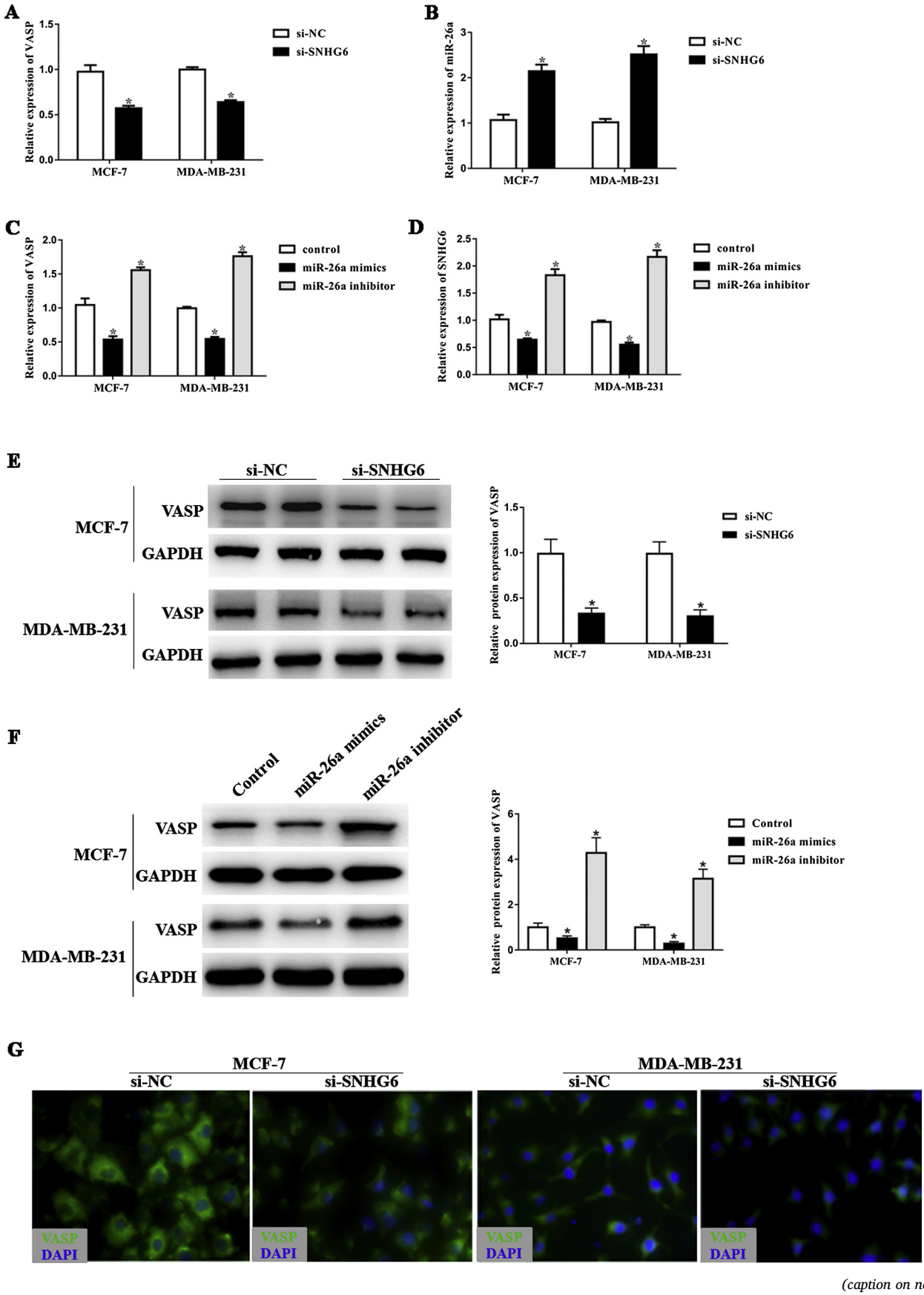


Fig. 3. Knockdown of SNHG6 inhibited migration and invasion of breast cancer cells. *(A)* si-SNHG6 resulted in a slower closing of scratch wound in MCF-7 and MDA-MB-231 cells by wound healing assay. *(B)* Transwell invasion assay was measured and the results were expressed as the number of invaded cells per field. *(C)* Transwell migration assay was measured and the results were expressed as the number of invaded cells per field. *P < 0.05.

Transwell assays were performed. The results indicated that compared to the si-NC groups, the numbers of migratory cells were obviously attenuated in MCF-7 and MDA-MB-231 cells which were transfected with si-SNHG6 (Fig. 3A-B). Moreover, the invasive abilities of MCF-7 and MDA-MB-231 cells were also inhibited by knocking down SNHG6 (Fig. 3C). Therefore, these results demonstrated that knock down of SNHG6 inhibited cell migration and invasion in breast cancer.

3.4. SNHG6 and miR-26a could regulate VASP expression in breast cancer cells

In addition, we found that knockdown of SNHG6 could significantly inhibit the expression of VASP, while promoted the expression of miR-26a in MCF-7 and MDA-MB-231 cells (Fig. 4A-B). Moreover, miR-26a mimics could significantly inhibit the mRNA expression of VASP and SNHG6, while miR-26a inhibitor promoted the expression of VASP and miR-26a in MCF-7 and MDA-MB-231 cells (Fig. 4C-D). Furthermore, we also detected the effect of SNHG6 and miR-26a on the protein expression of VASP. The western blotting results showed that SNHG6 knockdown and miR-26a mimics both significantly inhibited the protein expression level of VASP, while miR-26a inhibitor significantly promoted the expression of VASP (Fig. 4E-F). The results of cell immunofluorescence were consistent with western blotting, which showed that the intensity of fluorescence was significantly weakened after knocking down SNHG6 (Fig. 4G). These above results showed that SNHG6 and miR-26a both can regulate VASP expression, while miR-26a



(caption on next page)

Fig. 4. SNHG6 and miR-26a could regulate VASP expression in breast cancer cells. *(A)* The relative mRNA expression level of VASP was detected by qRT-PCR in MCF-7 and MDA-MB-231 cells transfected with si-SNHG6 and si-NC. *(B)* The relative mRNA expression level of miR-26a was detected by qRT-PCR in MCF-7 and MDA-MB-231 cells transfected with si-SNHG6 and si-NC. *(C)* The relative mRNA expression level of VASP was detected by qRT-PCR in MCF-7 and MDA-MB-231 cells transfected with miR-26a mimics, miR-26a inhibitor and negative control (control). *(D)* The relative mRNA expression level of SNHG6 was detected by qRT-PCR in MCF-7 and MDA-MB-231 cells transfected with miR-26a mimics, miR-26a inhibitor and negative control (control). *(E)* The relative protein expression level of VASP was detected by western blotting in MCF-7 and MDA-MB-231 cells transfected with si-SNHG6 and si-NC. *(F)* The relative protein expression level of VASP was detected by western blotting in MCF-7 and MDA-MB-231 cells transfected with miR-26a mimics, miR-26a inhibitor and negative control (control). *(G)* The protein expression of VASP was detected by cell immunofluorescence in MCF-7 and MDA-MB-231 cells transfected with si-SNHG6 and si-NC. *P < 0.05.

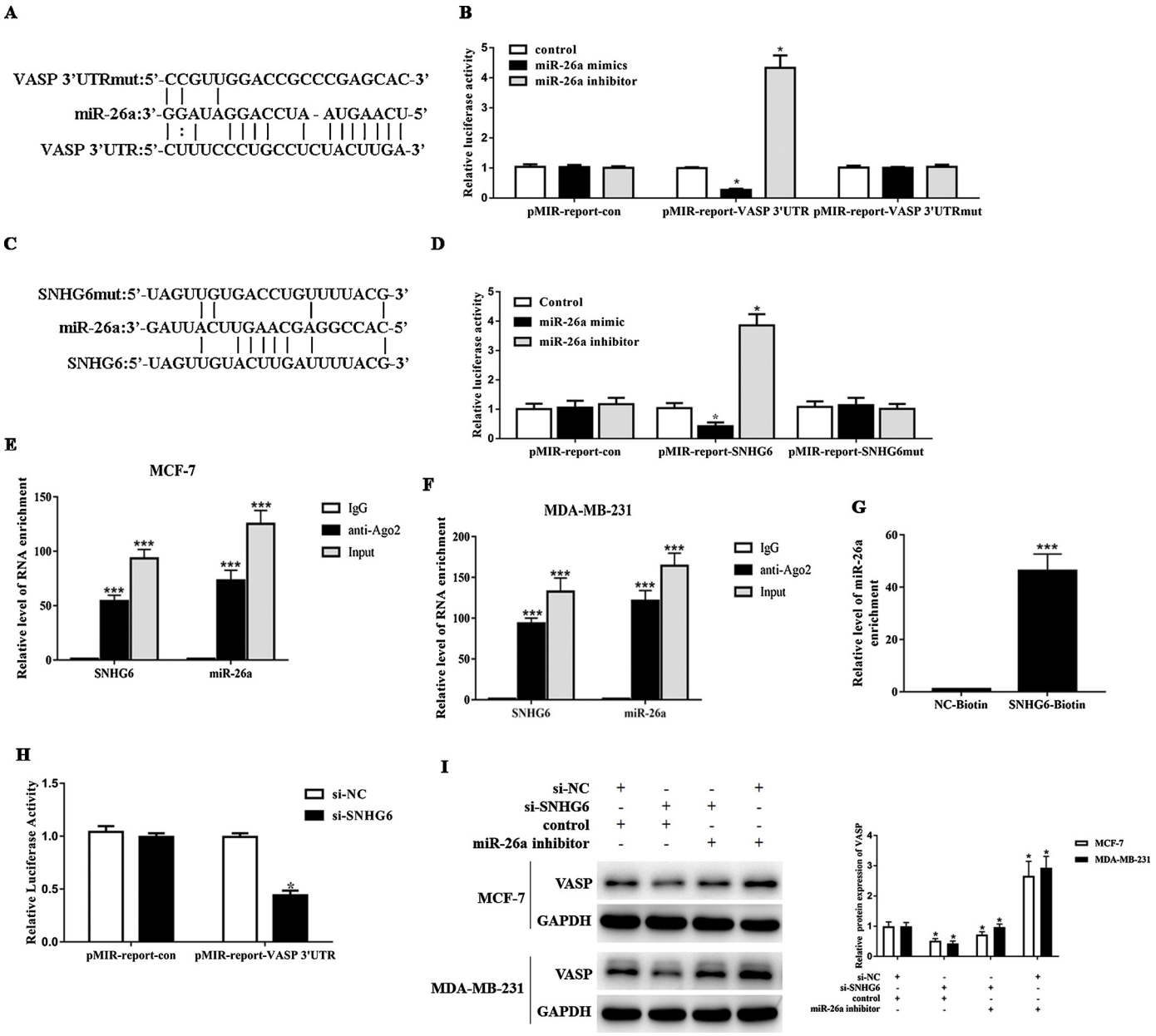


Fig. 5. SNHG6 and miR-26a regulated the expression of VASP via a ceRNA mechanism. *(A)* The predicted binding sites and mutated sites of miR-26a to the VASP sequence were shown. *(B)* Luciferase activity of HEK293 T cells cotransfected with miR-26a mimics, miR-26a inhibitor and luciferase reporters containing VASP 3′UTR or VASP 3′UTRmut transcript were analyzed. *(C)* The predicted binding sites and mutated sites of miR-26a to the SNHG6 sequence were shown. *(D)* Luciferase activity of HEK293 T cells cotransfected with miR-26a mimics, miR-26a inhibitor and luciferase reporters containing SNHG6 or SNHG6mut transcript were analyzed. *(E, F)* The Ago2-RIP assay showed that SNHG6 and miR-26a interacted with Ago2 protein in MCF-7 and MDA-MB-231. ***(G)*** SNHG6 was labeled with Biotin, and then the interaction of SNHG6 with miR-26a was detected by RNA pull down assay in MCF-7 cells. *(H)* Luciferase activity of HEK293 T cells cotransfected with si-SNHG6, si-NC and luciferase reporters containing VASP 3′UTR were analyzed. *(I)* The levels of VASP transfected with miR-26a inhibitor or si-SNHG6 in MCF-7 and MDA-MB-231 cell were analyzed by western blotting. *P < 0.05, ***P < 0.001.

can also inhibit the expression of SNHG6 and VASP.

3.5. SNHG6 and miR-26a regulated the expression of VASP via a ceRNA mechanism

Based on the results and bioinformatics analysis, we speculated that SNHG6 and miR-26a regulated the expression of VASP via ceRNA mechanism. The results of bioinformatics analysis showed that there were possible binding sites between miR-26a and VASP, SNHG6 (Fig. 5A, C). Then we constructed reporter gene plasmids of VASP and SNHG6, and their mutated plasmids which included mutated binding sites. The reporter gene assays showed that miR-26a mimics could significantly inhibit the activities of VASP and SNHG6 reporter gene plasmids, while had no effect on their mutated plasmids (Fig. 5B, D). Furthermore, the interaction of SNHG6 with miR-26a was detected by Ago2-RIP assay (Fig. 5E, F) and RNA pull down assay (Fig. 5G). The results showed that SNHG6 could directly bind to miR-26a in breast cancer cells. In addition, knockdown of SNHG6 could significantly inhibit the activity of VASP reporter gene plasmid (Fig. 5H). Furthermore, we found that miR-26a inhibitor could significantly reverse the inhibitory effect of SNHG6 knockdown on the expression of VASP (Fig. 5I). These results showed that SNHG6 and miR-26a regulated the expression of VASP via ceRNA mechanism.

3.6. MiR-26a reversed the effect of SNHG6 and VASP on the proliferation and migration of breast cancer cells

To determine whether SNHG6 exerted its biology function in breast cancer cells through miR-26a, we performed the rescue experiments. The results of colony formation assay and Transwell invasion assay showed that VASP knockdown and SNHG6 knockdown both can significantly inhibit proliferation and migration of MDA-MB-231 cells, while miR-26a inhibitor can significantly promote proliferation and migration of MDA-MB-231 cells. Furthermore, miR-26a inhibitor could significantly reverse the inhibitory effect of SNHG6 and VASP knockdown on the proliferation and migration of MDA-MB-231 cells (Fig. 6A-B). These results indicated that SNHG6, miR-26a and VASP formed an interactive regulatory network to exert an influence on the proliferation and migration of breast cancer cells.

4. Discussion

With the development of society, the incidence of breast cancer has been increasing year by year [1]. Currently, the prevention and treatment measures for breast cancer are still not perfect, so there are still serious threats to human health. Therefore, exploring new pathogenesis and effective molecular markers is a top priority for all cancer researchers. In this study, we observed a close relationship among SNHG6, miR-26a with VASP in human breast cancer tissues and cells, suggesting that SNHG6/miR-26a/VASP network may be involved in the progression of breast cancer. However, there is no research to explore the mechanism of SNHG6/miR-26a/VASP network in the development of breast cancer.

More and more evidence shows that non-coding RNA (ncRNA) is an important regulatory molecule involved in various physiological and pathological cellular processes [14]. In addition, previous studies have revealed that it can be involved in many diseases including cancer [15], diabetes [16], and neurological diseases [17]. SNHG6 (U87HG) is a housekeeping gene of the 5′TOP family that encodes two non-coding RNAs (ncRNAs): U87 C/D box snoRNA (SNORD87) [18]. According to previous studies, SNHG6 acts as a carcinogenic lncRNA involved in the development of various cancers through different mechanisms [19]. For example, acts as an oncogene in colorectal cancer (CRC), knockdown of SNHG6 inhibits colorectal cancer cell proliferation, cell cycle progression and induces apoptosis [20]. The expression of SNHG6 is increased in osteosarcoma tissues and cells, and its expression level is negatively correlated with the prognosis of patients with osteosarcoma [21]. Studies have also shown that SNHG6 promotes tumor growth and metastasis by inducing epithelial-mesenchymal transition, and affects the development of hepatocellular carcinoma (HCC) by interacting with ZEB1 and regulating Smad7 expression [22]. Abnormally high expression of SNHG6 in HCC cells promotes cell proliferation and induces drug resistance [23]. Recent studies showed that SNHG6 was also upregulated in breast cancer, which promoted cell proliferation, migration and invasion by regulating miR-26a-5p/MAPK6 [24]. But another mechanism in which SNHG6 involved in breast cancer was unclear. In this study, we found that SNHG6 shows a high expression trend in breast cancer tissues, and its expression level is negatively correlated with the survival percent of patients in most breast cancer patients, but they are not statistically significant, which may be related to the background noise of the online database. To further validate our hypothesis, we examined the expression of SNHG6 in breast cancer tissues and cells, and found that it was overexpressed in breast cancer tissues and cells. Then we observed that knockdown of SNHG6 significantly inhibited breast cancer cell proliferation, migration and invasion in vitro, suggesting that SNHG6 functions as an oncogene in breast cancer.

Furthermore, Bioinformatics analysis revealed a possible binding site among miR-26a, VASP with SNHG6. Recent study showed that miR-26a could target SNHG6 [24], which was consistent with our findings. However, we revealed the novel interaction sites of miR-26a with SNHG6. It was found that miR-26a acted as a tumor suppressor gene with a significantly reduced expression level in the development of various tumors [25–27], including breast cancer [28]. In this study, we found that there may be a negative regulatory relationship between miR-26a and VASP, SNHG6 in breast cancer through bioinformatics analysis, as well as tissue and cell level study.

The intracellular competitive endogenous RNA (ceRNA), including mRNA, lncRNA, pseudogene, can compete for the same miRNA through miRNA response element (MRE), leading to upregulation of the other one [29], which was the ceRNA mechanism. In the present study, we found that knockdown of SNHG6 significantly inhibited mRNA and protein expression of VASP, while promoted miR-26a expression. Overexpression of miR-26a significantly inhibited the expression levels of SNHG6 and VASP. Conversely, knockdown of miR-26a significantly promoted the expression levels of SNHG6 and VASP. Based on the binding sites predicted by bioinformatics, we found that the binding sites of miR-26a are indeed present in the SNHG6 and VASP nucleotide sequences by reporter gene experiments. Moreover, SNHG6 can reverse the upregulation of VASP protein by miR-26a knockdown. Therefore, our results suggest that SNHG6 may act as a ceRNA to upregulate VASP expression by sponging miR-26a. Furthermore, knockdown of miR-26a could significantly reverse the inhibitory effect of SNHG6 and VASP knockdown on the proliferation and migration of MDA-MB-231 cells. Our study indicated that SNHG6, miR-26a and VASP formed an interactive regulatory network to exert an influence on the proliferation and migration of breast cancer cells.

In summary, our study revealed that the expression of SNHG6 is significantly increased in breast cancer tissues and cells, knockdown of SNHG6 can significantly inhibit the proliferation, migration and invasion of breast cancer cells, suggesting that SNHG6 may be an oncogene in the development of breast cancer. SNHG6 can act as a ceRNA to promote VASP expression by sponging miR-26a, thereby promoting the proliferation and migration of breast cancer cells (Fig. 6C). These findings provided useful information to find new biomarkers for early diagnosis and therapeutic application in breast cancer.

Funding

This work was supported by the National Natural Science Foundation of China (81572943) and the Key Project of Science and Technology Department of Hubei Province (2015CFA070).

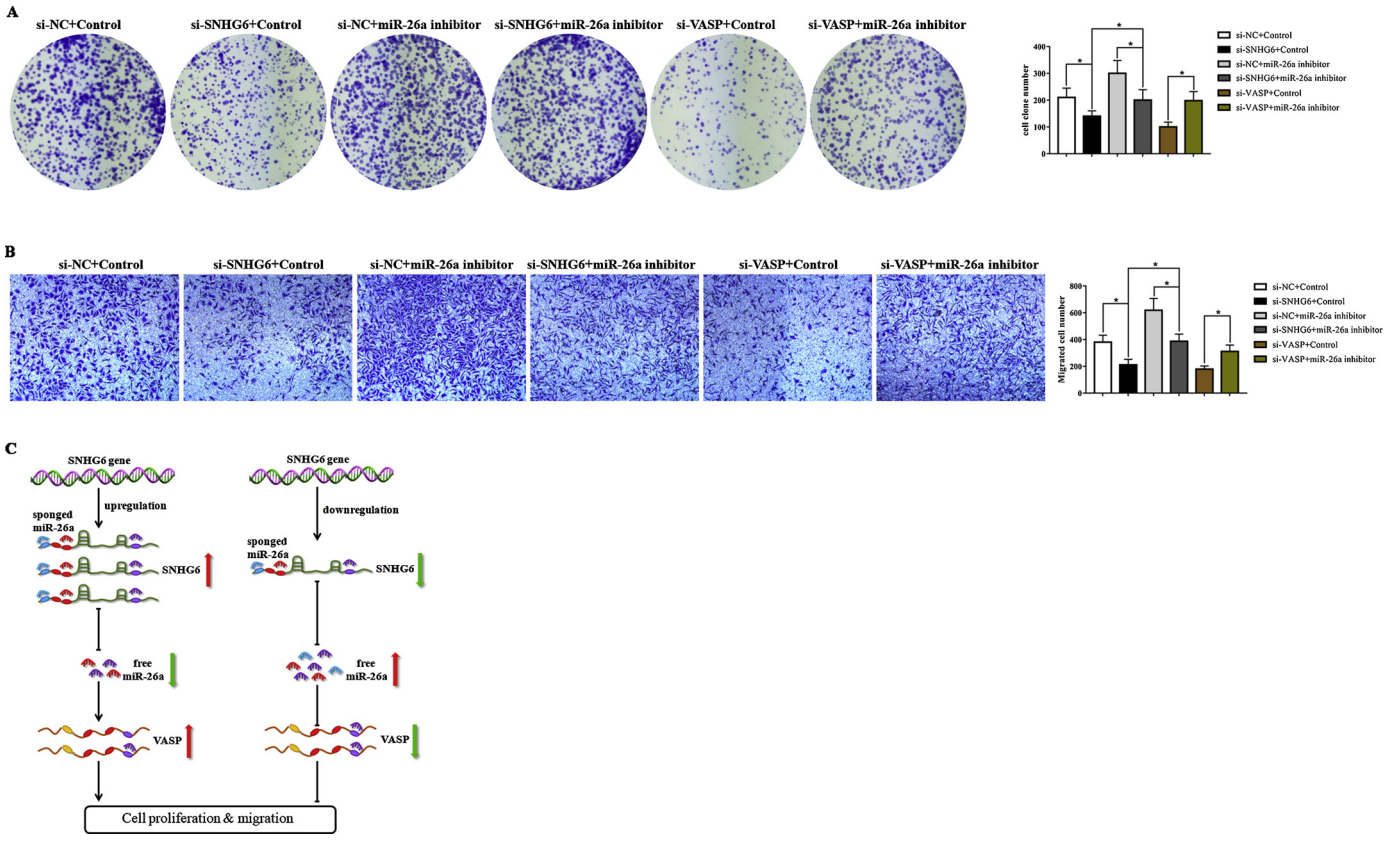


Fig. 6. MiR-26a reversed the effect of SNHG6 and VASP on the proliferation and migration of breast cancer cells. *(A and B)* Functional assays identified the phenomenon of SNHG6 and VASP regulated each other to compete for the binding of miR-26a by colony formation and transwell invasion assay in MDA-MB-231 cell lines. *(C)* Working model for the regulation of proliferation and migration of breast cancer cells by SNHG6/miR-26a/VASP network via ceRNA mechanism. In breast cancer, upregulated SNHG6 will sponge more miR-26a, leading to less free miR-26a to inhibit the expression of VASP, which returns to promote proliferation and migration of breast cancer cells.

Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Declaration of Competing Interest

No conflict of interest.

Acknowledgements

Not applicable.

References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer Statistics, 2017, CA Cancer J. Clin. 67 (2017) 7–30.
[2] R. Benedetti, C. Dell'Aversana, C. Giorgio, R. Astorri, L. Altucci, Breast Cancer vaccines: new insights, Front. Endocrinol. (Lausanne) 8 (2017) 270.
[3] M. Zimmer, T. Fink, L. Fischer, W. Hauser, K. Scherer, P. Lichter, U. Walter, Cloning of the VASP (vasodilator-stimulated phosphoprotein) genes in human and mouse: structure, sequence, and chromosomal localization, Genomics 36 (1996) 227–233.
[4] L. Dertsiz, G. Ozbilim, Y. Kayisli, G.A. Gokhan, A. Demircan, U.A. Kayisli, Differential expression of VASP in normal lung tissue and lung adenocarcinomas, Thorax 60 (2005) 576–581.
[5] G. Han, B. Fan, Y. Zhang, X. Zhou, Y. Wang, H. Dong, Y. Wei, S. Sun, M. Hu, J. Zhang, L. Wei, Positive regulation of migration and invasion by vasodilator-stimulated phosphoprotein via Rac1 pathway in human breast cancer cells, Oncol. Rep. 20 (2008) 929–939.
[6] Y. Zhang, G. Han, B. Fan, Y. Zhou, X. Zhou, L. Wei, J. Zhang, Green tea (-)-epigallocatechin-3-gallate down-regulates VASP expression and inhibits breast cancer cell migration and invasion by attenuating Rac1 activity, Eur. J. Pharmacol. 606 (2009) 172–179.
[7] M. Tang, Y. Tian, D. Li, J. Lv, Q. Li, C. Kuang, P. Hu, Y. Wang, J. Wang, K. Su, L. Wei, TNF-alpha mediated increase of HIF-1alpha inhibits VASP expression, which reduces alveolar-capillary barrier function during acute lung injury (ALI), PLoS One 9 (2014) e102967.
[8] K. Su, Y. Tian, J. Wang, W. Shi, D. Luo, J. Liu, Z. Tong, J. Wu, J. Zhang, L. Wei, HIF-1alpha acts downstream of TNF-alpha to inhibit vasodilator-stimulated phosphoprotein expression and modulates the adhesion and proliferation of breast cancer cells, DNA Cell Biol. 31 (2012) 1078–1087.
[9] J. Wang, J. Zhang, J. Wu, D. Luo, K. Su, W. Shi, J. Liu, Y. Tian, L. Wei, MicroRNA-610 inhibits the migration and invasion of gastric cancer cells by suppressing the expression of vasodilator-stimulated phosphoprotein, Eur. J. Cancer 48 (2012) 1904–1913.
[10] K. Su, P. Hu, X. Wang, C. Kuang, Q. Xiang, F. Yang, J. Xiang, S. Zhu, L. Wei, J. Zhang, Tumor suppressor berberine binds VASP to inhibit cell migration in basal-like breast cancer, Oncotarget 7 (2016) 45849–45862.
[11] B. Gyorffy, A. Lanczky, A.C. Eklund, C. Denkert, J. Budczies, Q. Li, Z. Szallasi, An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients, Breast Cancer Res. Treat. 123 (2010) 725–731.
[12] K. Li, Y.B. Ma, Z. Zhang, Y.H. Tian, X.L. Xu, Y.Q. He, L. Xu, Y. Gao, W.T. Pan, W.J. Song, X. He, L. Wei, Upregulated IQUB promotes cell proliferation and migration via activating Akt/GSK3beta/beta-catenin signaling pathway in breast cancer, Cancer Med. 7 (2018) 3875–3888.
[13] K. Li, X. Xu, Y. He, Y. Tian, W. Pan, L. Xu, Y. Ma, Y. Gao, J. Gao, Y. Qi, L. Wei, J. Zhang, P21-activated kinase 7 (PAK7) interacts with and activates Wnt/beta-catenin signaling pathway in breast cancer, J. Cancer 9 (2018) 1821–1835.
[14] S. Memczak, M. Jens, A. Elefsinioti, F. Torti, J. Krueger, A. Rybak, L. Maier, S.D. Mackowiak, L.H. Gregersen, M. Munschauer, A. Loewer, U. Ziebold, M. Landthaler, C. Kocks, F. le Noble, N. Rajewsky, Circular RNAs are a large class of animal RNAs with regulatory potency, Nature 495 (2013) 333–338.
[15] A. Bhan, M. Soleimani, S.S. Mandal, Long noncoding RNA and Cancer: a new paradigm, Cancer Res. 77 (2017) 3965–3981.
[16] A.H. Mirza, S. Kaur, F. Pociot, Long non-coding RNAs as novel players in beta cell function and type 1 diabetes, Hum. Genomics 11 (2017) 17.
[17] X. Huang, Y.L. Luo, Y.S. Mao, J.L. Ji, The link between long noncoding RNAs and depression, Prog. Neuropsychopharmacol. Biol. Psychiatry 73 (2017) 73–78.
[18] I.K. Gogolevskaya, J.A. Makarova, L.N. Gause, V.A. Kulichkova, I.M. Konstantinova, D.A. Kramerov, U87 RNA, a novel C/D box small nucleolar RNA from mammalian cells, Gene 292 (2002) 199–204.

[19] T. Guo, H. Wang, P. Liu, Y. Xiao, P. Wu, Y. Wang, B. Chen, Q. Zhao, Z. Liu, Q. Liu, SNHG6 acts as a genome-wide hypomethylation trigger via coupling of miR-1297-mediated S-adenosylmethionine-dependent positive feedback loops, Cancer Res. 78 (2018) 3849–3864.
[20] M. Li, Z. Bian, S. Yao, J. Zhang, G. Jin, X. Wang, Y. Yin, Z. Huang, Up-regulated Expression of SNHG6 Predicts Poor Prognosis in Colorectal Cancer, Pathology, Research and Practice, (2018).
[21] J. Ruan, L. Zheng, N. Hu, G. Guan, J. Chen, X. Zhou, M. Li, Long noncoding RNA SNHG6 promotes osteosarcoma cell proliferation through regulating p21 and KLF2, Arch. Biochem. Biophys. 646 (2018) 128–136.
[22] L. Chang, Y. Yuan, C. Li, T. Guo, H. Qi, Y. Xiao, X. Dong, Z. Liu, Q. Liu, Upregulation of SNHG6 regulates ZEB1 expression by competitively binding miR-101-3p and interacting with UPF1 in hepatocellular carcinoma, Cancer Lett. 383 (2016) 183–194.
[23] C. Cao, T. Zhang, D. Zhang, L. Xie, X. Zou, L. Lei, D. Wu, L. Liu, The long non-coding RNA, SNHG6-003, functions as a competing endogenous RNA to promote the progression of hepatocellular carcinoma, Oncogene 36 (2017) 1112–1122.
[24] A. Jafari-Oliayi, M.H. Asadi, SNHG6 is upregulated in primary breast cancers and promotes cell cycle progression in breast cancer-derived cell lines, Cell. Oncol. (Dordr.) 42 (2019) 211–221.
[25] X. Zhang, X. Zhang, T. Wang, L. Wang, Z. Tan, W. Wei, B. Yan, J. Zhao, K. Wu, A. Yang, R. Zhang, L. Jia, MicroRNA-26a is a key regulon that inhibits progression and metastasis of c-Myc/EZH2 double high advanced hepatocellular carcinoma, Cancer Lett. 426 (2018) 98–108.
[26] Y.Y. Lu, Y. Lin, D.X. Ding, S. Su, Q.Q. Chi, Y.C. Zhang, J. Sun, X. Zhang, H.M. Zhu, Q.S. Huang, Y.L. Chi, G.Z. Ye, S. Tao, S.J. Dong, MiR-26a functions as a tumor suppressor in ambient particulate matter-bound metal-triggered lung cancer cell metastasis by targeting LIN28B-IL6-STAT3 axis, Arch. Toxicol. 92 (2018) 1023–1035.
[27] M. Rizzo, G. Berti, F. Russo, S. Fazio, M. Evangelista, R. D'Aurizio, M. Pellegrini, G. Rainaldi, Discovering the miR-26a-5p targetome in prostate Cancer cells, J. Cancer 8 (2017) 2729–2739.
[28] S. Yndestad, E. Austreid, K.O. Skaftnesmo, P.E. Lonning, H.P. Eikesdal, Divergent activity of the pseudogene PTENP1 in ER-positive and negative breast cancer, Mol. Cancer Res. 16 (2018) 78–89.
[29] S. Yamamura, M. Imai-Sumida, Y. Tanaka, R. Dahiya, Interaction and cross-talk between non-coding RNAs, Cell. Mol. Life Sci. 75 (2018) 467–484.