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Original Article

SNHG3 promotes migration, invasion, and epithelial-mesenchymal transition of breast cancer cells through the miR-186-5p/ZEB1 axis

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Abstract: Increasing evidence suggests that the long non-coding RNAs (lncRNAs) participate in the development and progression of breast cancer. The lncRNA small nucleolar RNA host gene 3 (SNHG3) reportedly acts as an oncogene in hepatocellular carcinoma and colorectal cancer; however, little is known about the biological function and oncogenic mechanisms of SNHG3 in breast cancer. We demonstrated that the expression of SNHG3 was abnormally high in breast cancer tissues and cells, and transgenic expression of SNHG3 promoted the proliferation, migration, and invasion of breast cancer cell lines (MCF-7 and MDA-MB-231). The mean volume of the xenografts from the SNHG3-knockdown MCF-7 cells was lower than that of the control tumor cells. Moreover, the expression of zinc finger E-box binding homeobox 1 (ZEB1) increased after SNHG3 overexpression and *vice versa*. Overexpression of ZEB1 triggered cellular migration and invasion behaviors. Analysis of the mechanism underlying these effects suggested that SNHG3 is an effective sink for miR-186-5p and modulates ZEB1 repression, conferring an additional level to its post-transcriptional regulation. In conclusion, SNHG3 promotes the migration and invasion of breast cancer cells through miR-186-5p/ZEB1 regulation and the induction of the epithelial to mesenchymal transition, indicating that SNHG3 is a potential treatment target for breast cancer.

Keywords: Long non-coding RNA, SNHG3, ZEB1, epithelial to mesenchymal transition, breast cancer

Introduction

According to the latest global cancer statistics, breast cancer (BC), accounting for 30% of all new cancer diagnoses and the most commonly diagnosed cancer in women, is the leading cause of cancer-related deaths among females worldwide [1, 2]. Moreover, different subtypes of BC, including HER2-enriched, basal-like, luminal A, luminal B, and normal-like, can be distinguished based on their various clinical behaviors and outcomes [3]. Although immunotherapy and personalized medicine have improved the survival rate and recovery of patients to some extent, these approaches cannot limit or prevent recurrence and metastasis of the different subtypes of the BC. Distinct subtypes of BC are characterized by high heterogeneity and complex entities in morphology and molecular phenotype [4]. Thus, studies of the key signaling molecules and their respective pathways that have been implicated in BC development and progression are clinically important.

Long non-coding RNAs (lncRNAs) are non-coding RNA molecules of more than 200 nucleotides in length [5]. According to previous reports, although lncRNAs do not encode proteins, they are widely involved in abnormal cellular biological processes [6], including the pathogenesis and development of cancer [7], energy metabolism of tumors [8], osteogenic differentiation of stem cells [9], immune system-related diseases [10], and other pathophysiological processes. For example, lncRNA H19 reduces the degradation of the miR-152 target gene DNMT1 (DNA methyltransferase), thereby enhancing the proliferation and invasion ability of BC cells [11]. ZFAS1 promotes the

Table 1. siRNA sequence

siRNA	Sequence (5'→3')
si-NC	Sense: UUCUCCGAACGUGUCACGUTT
	Anti-sense: ACGUGACACGUUCGGAGAATT
si-*SNHG3*-1	Sense: GCAUUUAGCUAGGAAUGCATT
	Anti-sense: UGCAUUCCUAGCUAAAUGCTT
si-*SNHG3*-2	Sense: CUAGCAUGAUAGCUUCAGUTT
	Anti-sense: ACUGAAGCUAUCAUGCUAGTT
si-*SNHG3*-3	Sense: GGGAUCAUCUAGAAGGUAATT
	Anti-sense: UUACCUUCUAGAUGAUCCCTT

metastasis of liver cancer, GAS5 regulates the apoptosis of prostate cancer cells, and SNHG6 promotes the progress of liver cancer as a competing endogenous RNA, suggesting that lncRNAs play a crucial role in regulating cell homeostasis and cancer biology [12-14]. Epithelial-mesenchymal transition (EMT), characterized by epithelial tumor cells losing their intercellular adhesion and polarity, recombination with the actin cytoskeleton, and alterations in cell morphology, lead to cancer cells gaining migration and invasion abilities [15, 16]. The EMT is modulated by some signaling pathways, such as WNT, NOTCH, transforming growth factor-β, and mitogen-activated protein kinase, as well as key transcription factors (ZEB1, Twist1, Snail1) [17-20].

In a preliminary study, we employed lncRNA expression profiling microarrays to detect gene expression differences in MDA-MB-231 cells transfected with green fluorescent protein- and bone morphogenetic protein 9 (BMP9)-containing adenoviruses to illustrate the inhibitory mechanism of BMP9 on BC progression. BMP9 has been shown by microarray screening to significantly reduce the mRNA level of SNHG3. Additionally, based on our previous research, BMP9 inhibited the proliferation and migration of MDA-MB-231 and SK-BR-3 BC cells [12, 21]. SNHG3, located on 1q35.3, is a newly identified lncRNA. Zhang et al. found that SNHG3 overexpression significantly led to a malignant status and poor prognosis of patients with hepatocellular carcinoma [22]. Furthermore, the lncRNA SNHG3 promotes the malignant development of colorectal cancer [23]. However, little is known about the exact function and potential mechanisms of SNHG3 in BC. Herein, we studied the expression and biological role of SNHG3 in BC tumorigenesis and how SNHG3 leads BC cell lines to EMT. The study provides a foundation for determining the molecular mechanism of SNHG3 in BC progression and identifying a prognostic marker and therapeutic target for breast cancer.

Materials and methods

Cell lines

MCF-7, the MDA-MB-231 human breast cell line, and HEK-293T cells were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Plasmid construction, siRNA and miRNA synthesis, and cell transfections

The SNHG3 cDNA sequence was synthetically cloned into the pUC57 vector backbone (Genescript Technology, Nanjing, China) and subcloned into pcDNA 3.1+ (Invitrogen, Carlsbad, CA, USA). A pcDNA3.1(+)-SNHG3-MUT vector was produced by site-directed mutagenesis, which contained mutations at the assumed miR-186-5p binding site of SNHG3. For luciferase reporter assays, the wild-type (WT) and variant of SNHG3 cDNA sequences (containing a putative miR-186-5p binding site) were synthesized by Genscript Co., Ltd. (Nanjing, China) and inserted into the pGL6-miR vector (Beyotime Biotechnology, Shanghai, China) to obtain plasmids RLuc-SNHG3-WT and RLuc-SNHG3-MUT. The RLuc-ZEB1-3'-untranslated region (UTR)-WT (wild-type 3'-UTR cDNA of ZEB1) and RLuc-ZEB1-3'-UTR-MUT vector containing mutations at the assumed miR-186-5p binding site were inserted into a pGL6-miR vector (Beyotime Biotechnology). The plasmid of EX-Z5750-M35 and ZEB1 were purchased from Genecopoeia (Rockville, MD, USA). Three different short interfering RNAs (siRNAs) targeting SNHG3, miRNA mimics, and inhibitors (for miR-186-5p) were synthesized by Shanghai GenePharma, Co., Ltd. (Shanghai, China). The sequences are shown in **Table 1**. After the MDA-MB-231 and MCF-7 cells were seeded into 6-well plates overnight, the plasmid and miRNA were transfected using Lipofectamine 2000 (Invitrogen)

Table 2. Primer sequences

Primers	Sequences (5'→3')
SNHG3	Forward: *CAGTGGTCGCTTCTTCTCCTT*
	Reverse: *GGCATGAAATGCACCTCAAT*
SNHG3-1	Forward: TTCAAGCGATTCTCGTGCC
	Reverse: AAGATTGTCAAACCCTCCCTGT
SNHG3-2	Forward: TCGCTTCTTCTCCTTGGATTTG
	Reverse: AGGCATGAAATGCACCTCAA
β-Actin	Forward: CCACGAAACTACCTTCAACTCC
	Reverse: GTGATCTCCTTCTGCATCCTGT
E-cadherin	Forward: CGAGAGCTACACGTTCACGG
	Reverse: GGGTGTCGAGGGAAAAATAGG
N-cadherin	Forward: CCATCAAGCCTGTGGGAATC
	Reverse: GCCGCTTTAAGGCCCTCAT
Vimentin	Forward: TCTGGATTCACTCCCTCTGGTT
	Reverse: ATCGTGATGCTGAGAAGTTTCGT
MMP9	Forward: CCCTTGTGCTCTTCCCTGGA
	Reverse: TCTGCCACCCGAGTGTAACC
MMP2	Forward: ACATCAAGGGCATTCAGGAGC
	Reverse: CACAGTCCGCCAAATGAACC
Snail	Forward: TCCAGCAGCCCTACGACCAG
	Reverse: AGGCCGAGGTGGACGAGAA
ZEB1	Forward: CCAAGCTTATGAAAGTTACAAATTATAA
	Reverse: CGGGATCCCTTCAAAGGACTTTGTAGAT
Claudin 1	Forward: CCTCCTGGGAGTGATAGCAA
	Reverse: GGCAACTAAAATAGCCAGACCT
Fibronection	Forward: CGGTGGCTGTACGTCAAAG
	Reverse: AAACCTCGGCTTCCTCCATAA
GAPDH	Forward: ACAACTTTGGTATCGTGGAAGG
	Reverse: GCCATCACGCCACAGTTTC
U6	Forward: CGCTTCGGCAGCACATATAC
	Reverse: AAATATGGAACGCTTCACGA
miR-NC	Sense: UUCUCCGAACGUGUCACGUTT
	Anti-sense: ACGUGACACGUUCGGAGAATT
miR-186-5p mimics	Sense: CAAAGAAUUCUCCUUUUGGGCU
	Anti-sense: CCCAAAAGGAGAAUUCUUUGUU
SNHG3-sh	Forward: CCGGGCACTGGCTGCCAACATAAATCT CGAGATTTATGTTGGCAGCCAGTGCTTTTTG
	Reverse: AATTCAAAAAGCACTGGCTGCCA ACATAAATCTCGAGATTTATGTTGGCA GCCAGTGC

and siRNA duplexes were transfected with R4000 reagent according to the manufacturer's protocols.

qRT-PCR

Total RNA, harvested and extracted from the cells transfected with corresponding plasmid or siRNA and incubated for 36 h with TRIzol reagent (Invitrogen), was used for cDNA synthesis by reverse transcription PCR (TaKaRa, Shiga, Japan), followed by fluorogenic quantitative PCR with SYBR Green PCR Master Mix (TaKaRa). β-Actin was used as the endogenous control gene, and $2^{-\Delta\Delta CT}$ values were normalized to β-actin levels. The primers are listed in **Table 2**.

Antibodies and western blot analysis

Primary antibodies against the following were used: N-cadherin, E-cadherin, and MMP-2

(Abcam, Cambridge, UK); MMP-9, vimentin, Snail, and β-actin (Cell Signaling Technology, Danvers, MA, USA); and ZEB1 (ImmunoWay, Plano, TX, USA). The cells were lysed in RIPA lysis buffer (Beyotime), and total protein (35 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The PVDF membranes were incubated with the corresponding primary antibodies overnight at 4°C. Next, the PVDF membranes were washed three times with Tris-buffered saline containing Tween 20 and incubated with an appropriate secondary antibody conjugated to horseradish peroxidase (Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h at 37°C, followed by washing three times with Tris-buffered saline containing Tween 20. Finally, the membranes were visualized by immunoblotting using an ECL immunoblotting kit (Beyotime).

Scratch wound healing assay

BC cells (1×10^6 cells/well) were treated with the specified reagents, and wounds were made with 200-μL plastic pipette tip. The wound size was measured after 0, 24, and 48 h of wound formation, and the wound was imaged. The cell migration area was tested between dashed regions using ImageJ software (NIH, Bethesda, MD, USA) and normalized to control cells. The wound healing rate was calculated as follows: (0, 24, or 48 h width)/0 h width × 100%.

Transwell assay

Transwell chambers (24-well Transwell chambers; Corning, Inc., Corning, NY, USA) were used for migration and invasion assays. The membrane was coated with 1:4 diluted Matrigel (BD Matrigel™, BD Biocoat, BD Biosciences, Franklin Lakes, NJ, USA) in invasion assays in advance. After transfection for 48 h, 3×10^4 cells per well were harvested for seeding into the Transwell upper chamber with 300 μL of 0% FBS culture medium, whereas the lower chamber contained 700 μL of 10% FBS culture medium. The cells were cultured for 24 h in migration assays and 48 h in invasion assays and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and stained with 0.1% crystal violet for 20 min, after which the cells were counted in five random fields under bright field microscopy.

RNA-fluorescence in situ hybridization (FISH)

Human BC cells were seeded into a 24-well plate on chamber coverslips. After 12 h, the degree of cell confluence reached 60-70%, and the cells were fixed with 4% PFA for 10 min at RT. Permeabilization was carried out with precooled 0.5% Triton-X-100 for 5 min at 4°C, followed by washing with PBS three times for 5 min each time. The cover slides were prehybridized for 30 min at 37°C with 200 μL prehybridization buffer (1% blocking solution, 99% hybridization buffer). LncRNA FISH Probe Mix Storage solution (2.5 μL 20 μM; Rio™ LncRNA FISH Probe Mix) was added to 100 μL hybridization buffer and incubated at 37°C overnight in a humidified chamber in the dark. The cover slides were washed three times for 5 min each with 4 × SSC, 2 × SSC for 5 min, and 1 × SSC for 5 min at 42°C, followed by a 5-min wash in 1 × PBS at RT in the dark. We mounted cover slides with DAPI nuclear stain.

Dual-luciferase reporter assay

Plasmids pGL6-SNHG3 and pGL6-ZEB1 were constructed using the pGL6-miR firefly luciferase vector and 3'UTRs of SNHG3 and ZEB1, respectively. HEK293 cells were transfected with 400 ng pGL6-SNHG3 or pGL6-ZEB1 plasmids, 10 ng pRL-Tk Renilla reporter for transfection control, and normalization, and miR-186-5p mimic and seeded (8×10^4 cells per well) into 24-well plates. Luciferase activity was measured by the Dual Glo Luciferase Assay System (Promega, Madison, WI, USA) in duplicate on different days.

Animal experiments

Female nude mice (4-6 weeks old) were purchased from and fed in the Animal Experimental Center of Chongqing Medical University. All procedures for the *in vivo* nude mouse study were approved by the Animal Care Committee of Chongqing Medical College. We randomly allocated eight mice into two groups: control group (control) and SNHG3-sh group (SNHG3-sh). A total of 4.5×10^6 MCF-7 cells with stable knockdown of SNHG3 and their control cells were inoculated subcutaneously into control and SNHG3-sh nude mice groups, respectively.

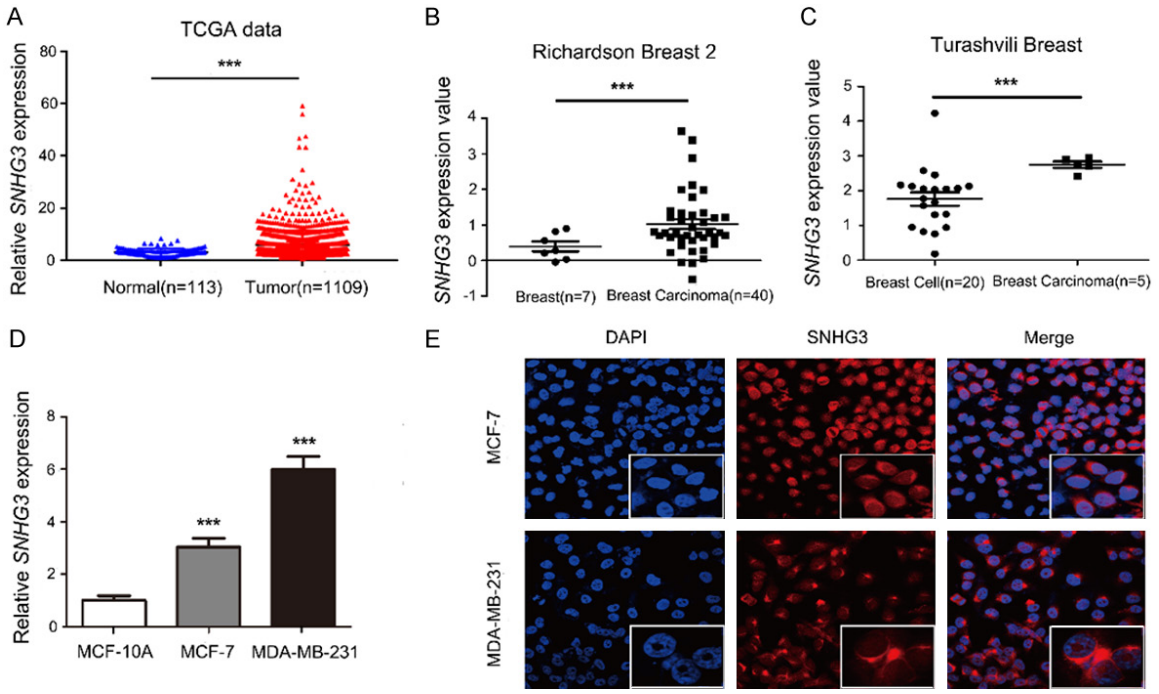

Figure 1. SNHG3 was strongly upregulated in BC. A. Expression of SNHG3 in normal breast (n = 113) and BC (n = 1109) tissue samples from TCGA. B, C. mRNA expression levels of SNHG3 in samples from the Oncomine database. D. SNHG3 expression levels in normal breast cell line (MCF-10A) and BC cell lines (MCF-7, MDA-MB-231) determined by RT-qPCR analysis. E. Representative FISH images showing the expression of SNHG3 in BC cells (400 ×).

After five weeks, tumor tissues were isolated and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry (IHC) analysis. All procedures, including primary care, handling, and treatment of animals, were carried out in strict accordance with the recommendations of the Regulations on the Control of Experimental Animals.

Immunohistochemical staining

The tissues from nude mice were fixed with 10% PFA and embedded in paraffin wax. IHC was performed on sections using a primary antibody against E-cadherin (Inmmunoway), vimentin (Cell Signaling Technology), proliferating cell nuclear antigen (PCNA; Cell Signaling Technology), and an horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology).

Online databases and statistical analysis

Gene expression analyses were performed in The Cancer Genome Atlas (TCGA). A set of microarray data from Richardson Breast 2 and Turashvili Breast were downloaded from the Oncomine Compendium of Expression Array data (www.oncomine.org). Research data are presented as the mean ± standard error of the mean of at least three independent experiments. All statistical analyses were performed using GraphPad Prism 5 software (GraphPad, Inc., La Jolla, CA, USA). A value of $P < 0.05$ was regarded as statistically significant ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Results

SNHG3 was strongly upregulated in BC

First, differences in SNHG3 gene expression in BC clinical samples were analyzed using online databases to evaluate the relationship between SNHG3 expression levels and BC features. TCGA data indicated that the expression of SNHG3 was aberrantly higher in 1109 BC clinical tissues compared to in 113 normal tissues (**Figure 1A**), and microarray analysis confirmed a similar result from Richardson Breast 2 and Turashvili Breast using the Oncomine database (**Figure 1B**, **1C**), which showed significantly higher SNHG3 expression in BC tissues com-

pared to in non-tumor tissues. Additionally, the mRNA expression of SNHG3 in BC cells was measured. Consistently, the expression of SNHG3 was remarkably higher in BC cell lines than in the immortalized, normal breast epithelial cell line MCF-10A (**Figure 1D**). Furthermore, FISH confirmed that SNHG3 was mainly localized in the cytoplasm of MCF-7 and MDA-MB-231 cells (**Figure 1E**). These results demonstrate that SNHG3 upregulation was closely associated with BC development.

SNHG3 overexpression increased the proliferation, migration, and invasion of BC cells

Next, the pcDNA3.1+/SNHG3 expression plasmid was employed to drive SNHG3 overexpression in MCF-7 and MDA-MB-231 cells, and an empty pcDNA3.1+ vector was used as a control to investigate the effect of SNHG3 overexpression on BC cells (**Figure 2A**). Interestingly, overexpression of SNHG3 visibly increased the proliferation abilities of MCF-7 cells based on the colony formation assay. Microscopic observation revealed a single colony of MCF-7 cells in the overexpression SNHG3 group, which was also larger than that in the control group, confirming that SNHG3 promoted the proliferation ability of MCF-7 BC cells (**Figure 2B**). Moreover, the migration rate of cells overexpressing SNHG3, as assessed by wound healing experiments, was higher than that in the control group (**Figure 2C**). Furthermore, upregulation of SNHG3 greatly enhanced the migration and invasion of BC cells compared with control cells (**Figure 2D**, **2E**). In summary, these data show that SNHG3 overexpression promoted the proliferation, migration, and invasion of BC cells.

Downregulation of SNHG3 expression repressed the migration and invasion of BC cells

To clarify the biological function of SNHG3 in BC, three different siRNAs against SNHG3 (designated as siRNA 1-3) were used to knock down SNHG3 in MCF-7 and MDA-MB-231 cells. siRNA-3 exhibited a significant decrease in endogenous SNHG3 expression (**Figure 3A**); therefore, we selected siRNA-3 for subsequent experiments. The migration rate of the downregulation group was lower than that of the control group as measured by wound healing and Transwell migration assays (**Figure 3B**, **3C**). We observed that the downregulation group contained fewer invasive cells than the control group (**Figure 3D**). The results revealed that lowering the expression of SNHG3 reduced cell migration and invasion of BC cells.

SNHG3 depletion reduced tumor size and metastasis

To further examine the function of SNHG3 in tumorigenesis *in vivo*, MCF-7 cells stably transfected with control vector or SNHG3-shRNA were inoculated into 5 different female nude mice, separately. The SNHG3 expression levels on BC were validated by RT-qPCR, which demonstrated that the mRNA expression of SNHG3 was reduced efficiently in SNHG3 shRNA tumor tissues (**Figure 4B**). SNHG3 shRNA decreased the tumor size compared to in the control group (**Figure 4A**). SNHG3 knockdown dramatically reduced the expression of ZEB1, MMP2, and Snail compared to in the control group, as observed in western blotting assays in BC tissue (**Figure 4D**). Additionally, the IHC results showed that the protein levels of E-cadherin were increased, whereas the protein levels of vimentin were attenuated in SNHG3 shRNA-treated MCF-7 tumor tissues. Moreover, the expression of proliferation-related proteins PCNA markedly decreased in SNHG3 shRNA-treated MCF-7 tumor tissues (**Figure 4C**). According to these results, SNHG3 promoted tumor growth and the expression of EMT markers *in vivo*.

SNHG3 regulated ZEB1 expression by binding miR-186-5p

To illustrate the potential mechanism of SNHG3 in BC tumorigenesis, we predicted whether miRNAs participate in BC progression. A recent study suggested that cytoplasmic lncRNAs can directly bind to miRNA and act as sponges or competing endogenous RNAs, which could control the availability of miRNA for binding to target mRNAs. Analysis using DIANA-LncBase software and StarBase [24, 25] showed that SNHG3 contained a binding site for miR-186-5p. BC cells transfected miR-186-5p mimics were verified to overexpress miR-186-5p by RT-qPCR (**Figure 5A**). The luciferase reporter activity of MCF-7 cells co-transfected with miR-186-5p mimics and the pGL6-SNHG3-WT vector was approximately decreased compared with that in cells transfected with miR-NC and pGL6-SNHG3-MUT (**Figure 5B**). The potential target genes of miR-186-5p were characterized

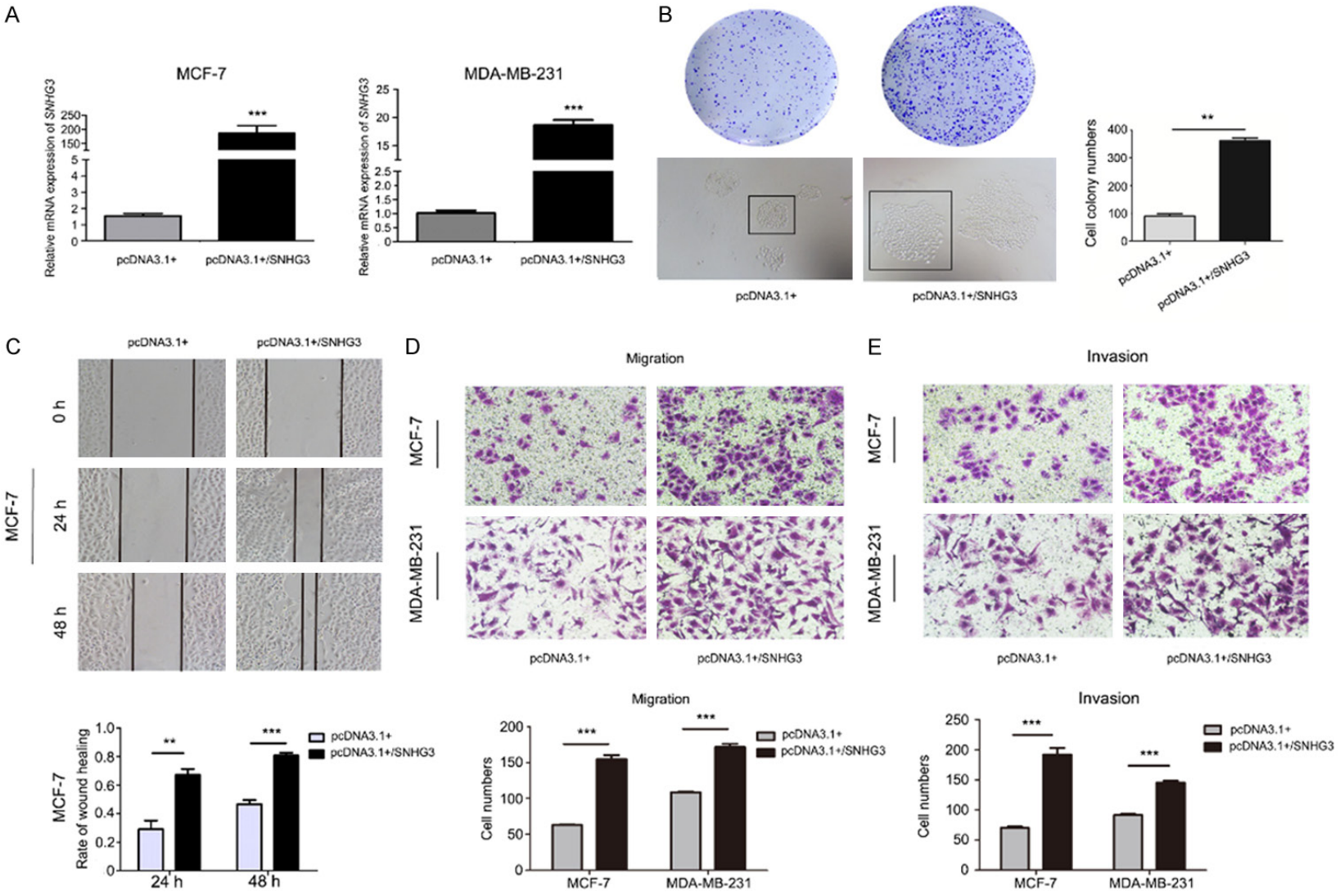

Figure 2. SNHG3 overexpression increased the proliferation, migration, and invasion of BC cells. A. RT-qPCR confirmed the overexpression efficiency of SNHG3 in BC cell lines (MCF-7 and MDA-MB-231). B. Colony formation assays showed that SNHG3 overexpression promoted the proliferation of MCF-7 cells. C, Healing abilities of MCF-7 cells were determined in a wound healing assay after 24 and 48 h. D, E. Transwell experiments showing the effects of SNHG3 upregulation on migration and invasion of BC cells. Cells were stained with 0.1% crystal violet (400 ×). Data are presented as the mean ± standard deviation of triplicate independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

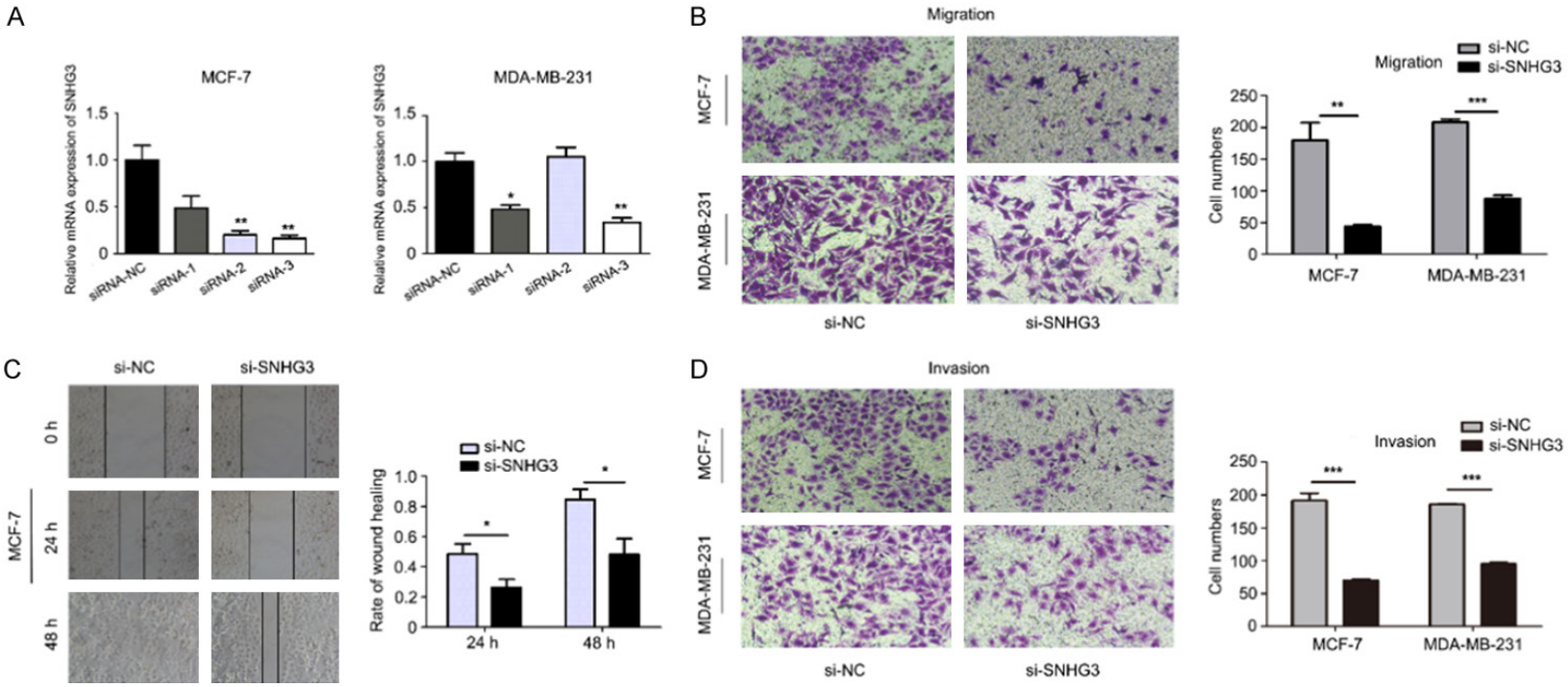

Figure 3. Downregulation of SNHG3 expression repressed the migration and invasion of BC cells. A. RT-qPCR analysis of SNHG3 expression following SNHG3 knockdown in BC cells; B, C. Cell migration was evaluated by wound healing and Transwell migration assays; D. Cell invasion was assessed using Transwell invasion assay, after the cells were stained with 0.1% crystal violet (400 ×). Data are presented as the mean ± standard deviation of triplicate independent experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

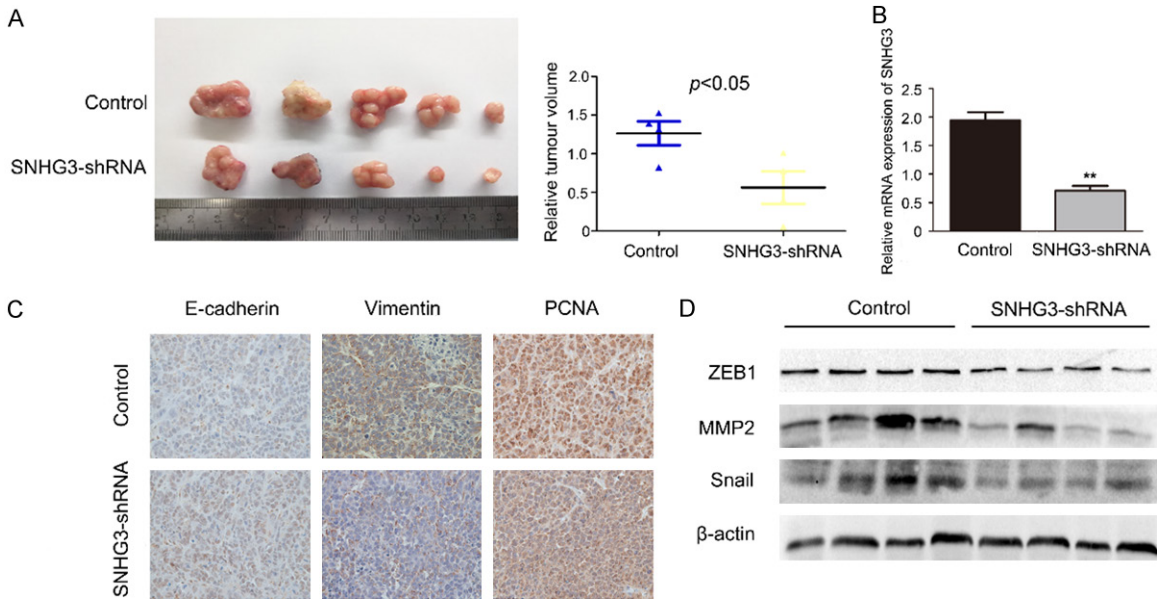

Figure 4. SNHG3 depletion reduced tumor size and metastasis. A. Each group contained five nude mice. Tumor volumes during the xenograft experiment were measured ($P < 0.05$). B. Expression of SNHG3 was verified by RT-qPCR. C. Immunohistochemical staining of E-cadherin, vimentin, and PCNA in tumor tissues (400 ×). D. Western blotting of the expression of ZEB1, MMP2, and Snail in tumor tissues.

using TargetScan Human 7.0 [26]. miR-186-5p contained a potential binding site for the ZEB1 3'-UTR according to bioinformatics-based analysis. ZEB1 is a target of miR-185-5p and an important EMT-inducing transcription factor. We performed luciferase reporter assays with vectors, which contained the 3'-UTR of ZEB1, which revealed that miR-186-5p mimics attenuated the luciferase activity of pGL6-ZEB1-3'-UTR WT, whereas ZEB1-3'-UTR MUT abrogated the suppressive effect. This indicates that ZEB1 was a direct target of miR-186-5p (**Figure 5C**). Moreover, upregulation of miR-186-5p significantly inhibited ZEB1 protein levels in BC cells. (**Figure 5D**). To assess whether SNHG3 regulated ZEB1 expression by targeting miR-186-5p binding sites in the SNHG3 sequence, SNHG3-siRNA was employed to silence endogenous SNHG3 and transfected cells with either pcDNA3.1-SNHG3-MUT, or pcDNA3.1-SNHG3-WT. As shown in **Figure 5D**, SNHG3-siRNA transfection reduced the expression of ZEB1. In addition, in rescue experiments, transfection with pcDNA3.1-SNHG3-WT reversed the trend of decreased ZEB1 expression, whereas pcDNA3.1-SNHG3-MUT transfection did not. SNHG3 regulated ZEB1 expression by blocking endogenous miR-186-5p. These experiments suggest that SNHG3 eliminates miRNA-induced inhibition of ZEB1 by binding miR-186-5p as an endogenous "sponge".

SNHG3 induced EMT pathway by increasing ZEB1 expression to facilitate the migration and invasion of BC cells

To further investigate the relationship between SNHG3 and ZEB1 expression in BC, the expression of ZEB1 was measured in BC cells and MCF-10A cells by RT-qPCR and western blotting (**Figure 6A**). The results showed that ZEB1 was highly expressed in BC cells. Moreover, the expression of ZEB1 increased after overexpression of SNHG3 and *vice versa* in BC cells, indicating that the expression of ZEB1 was significantly positively correlated with SNHG3 expression (**Figure 6B**). As a key transcription factor of EMT associated with the enhancement of cancer cell invasion and metastasis, ZEB1 regulates the expression of EMT markers [27]. We predicted that SNHG3 induced EMT by increasing ZEB1 expression. Western blotting or RT-qPCR analyses suggested increased expression of epithelial markers (E-cadherin), whereas mesenchymal markers (N-cadherin, vimentin, and fibronectin) and the transcription factors of EMT (Snail) showed decreased expression in SNHG3-knockdown BC cells (**Figure 6C**, **6D**). In addition, depletion of SNHG3 significantly

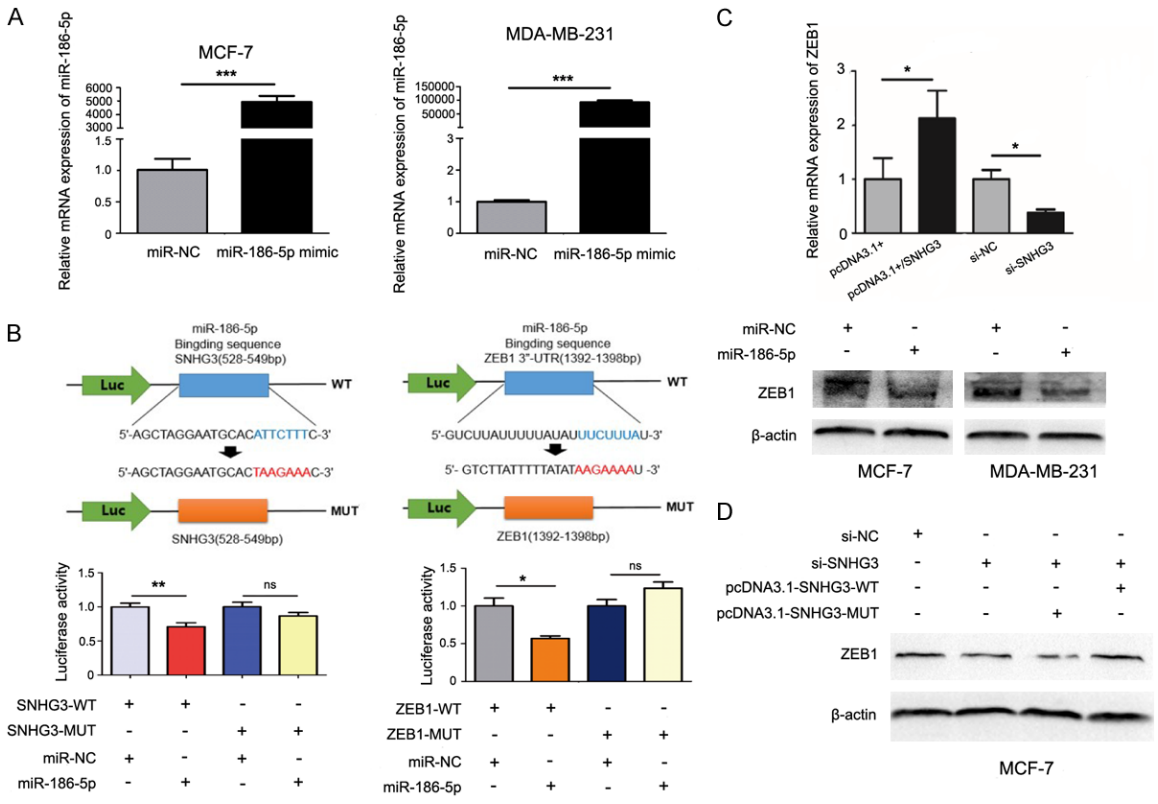

Figure 5. SNHG3 regulates ZEB1 expression by binding miR-186-5p. A. RT-qPCR confirmed the overexpression efficiency of SNHG3 in MCF-7 and MDA-MB-231 cells. B. WT SNHG3, or MUT sequences containing putative miR-186-5p recognition sites were cloned downstream of the luciferase gene in the pGL6 vector. The luciferase reporter plasmid containing WT or mutant SNHG3 was then co-transfected into MCF-7 cells with miR-186-5p mimics. Luciferase activity was determined by a dual-luciferase assay and normalized to Renilla activity by the relative luciferase activity. WT ZEB1 or MUT sequences containing putative miR-186-5p recognition sites were cloned downstream of the luciferase gene in the pGL6 vector. The luciferase reporter plasmid containing WT or mutant ZEB1 3'-UTR was then co-transfected into 293T cells with miR-186-5p mimics. Luciferase activity was determined in a dual-luciferase assay and normalized to Renilla activity by relative luciferase activity. C. RT-qPCR and western blotting analysis showing the expression of ZEB1 in BC cells transfected with miR-186-5p mimics. D. Western blotting analysis of ZEB1 expression after transfection of MCF-7 cells with si-SNHG3 or co-transfection with si-SNHG3 and pcDNA3.1-SNHG3-MUT or pcDNA3.1-SNHG3-WT.

decreased the expression of matrix metalloproteinases MMP-2 and MMP-9, which are closely associated with metastasis (**Figure 6C**, **6D**). Similarly, overexpression of SNHG3 in BC cells led to opposite results, inducing EMT in BC cells (**Figure 6E**, **6F**).

ZEB1 overexpression promoted BC cell migration and invasion

The pEZ-ZEB1 plasmid was transfected into MCF-7 and MDA-MB-231 cells to evaluate the effect of ZEB1 on BC cell behavior. As shown in **Figure 7A**, **7B**, the mRNA and protein of expression levels were increased after transfection. The results showed that overexpression of ZEB1 promoted the migration and invasion of BC cells as evaluated in Transwell chamber migration and invasion assays (**Figure 7C**, **7D**). In summary, the results suggest that SNHG3 promoted the migration and invasion of BC cells to activate the EMT pathway by upregulating ZEB1.

Discussion

The development of BC involves a cascade of complex and diverse biological processes that are driven by dysregulated molecular interactions among different gene products. Despite long-term progress in understanding the tumor-related molecular mechanisms underlying BC,

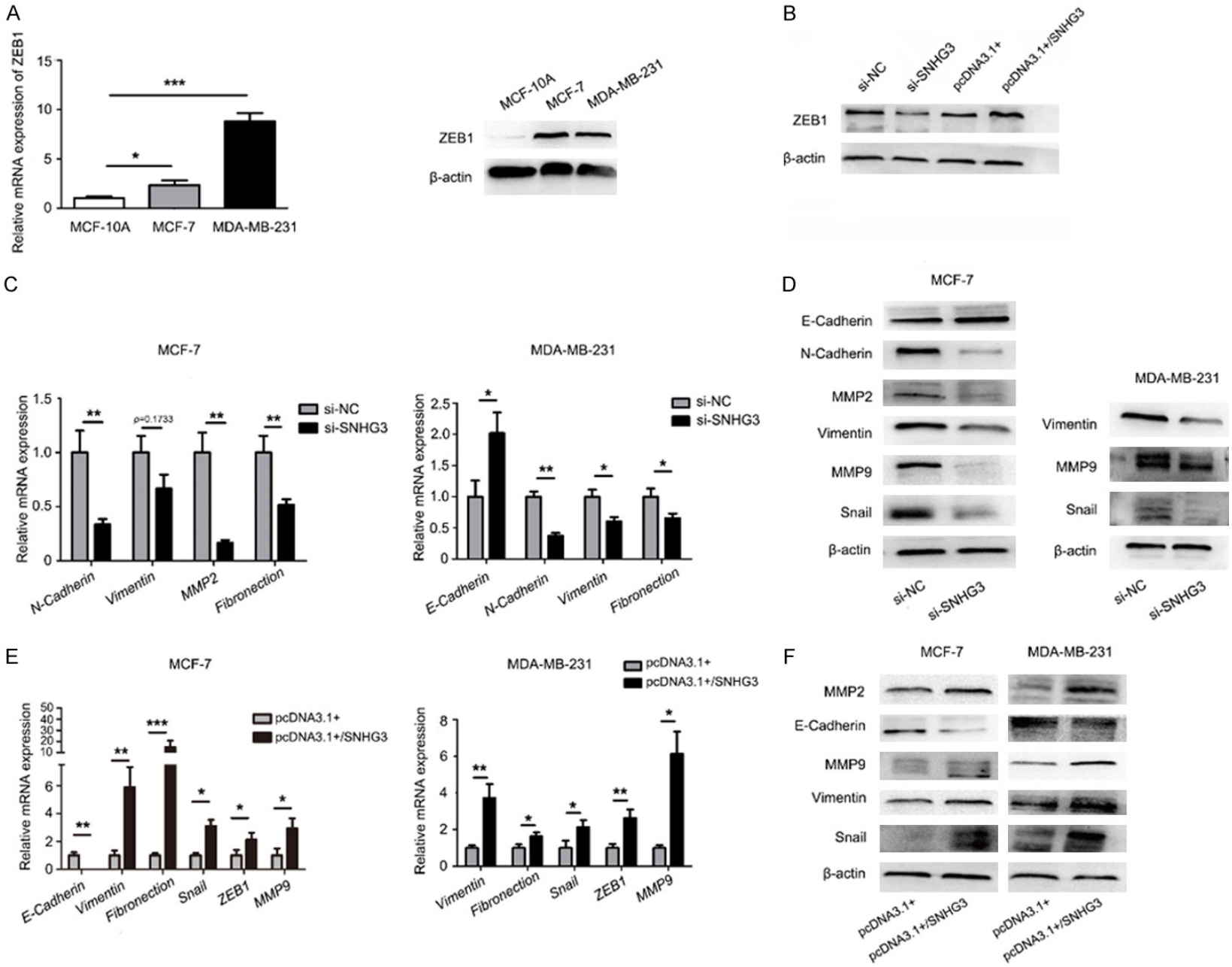
A
Relative mRNA expression of ZEB1
MCF-10A
MCF-7
MDA-MB-231
ZEB1
β-actin
B
si-NC
si-SNHG3
pcDNA3.1+
pcDNA3.1+/SNHG3
C
MCF-7
MDA-MB-231
Relative mRNA expression
si-NC
si-SNHG3
N-Cadherin
Vimentin
MMP2
Fibronection
E-Cadherin
D
E-Cadherin
N-Cadherin
MMP2
Vimentin
MMP9
Snail
β-actin
E
pcDNA3.1+
pcDNA3.1+/SNHG3
ZEB1
F

Figure 6. SNHG3 induced the EMT pathway by increasing ZEB1 to facilitate the migration and invasion of BC cells. A. Expression levels of ZEB1 in cells were detected by RT-qPCR and western blot analysis. B. Western blotting to detect the expression of ZEB1 after transfection of MCF-7 cells with si-SNHG3 and SNHG3 overexpressing. C, D. Expression levels of EMT markers in BC cells after SNHG3 knockdown quantified by western blotting and quantitative RT-qPCR. E, F. Expression levels of EMT markers in BC cells after SNHG3 overexpression measured by western blotting and quantitative RT-qPCR.

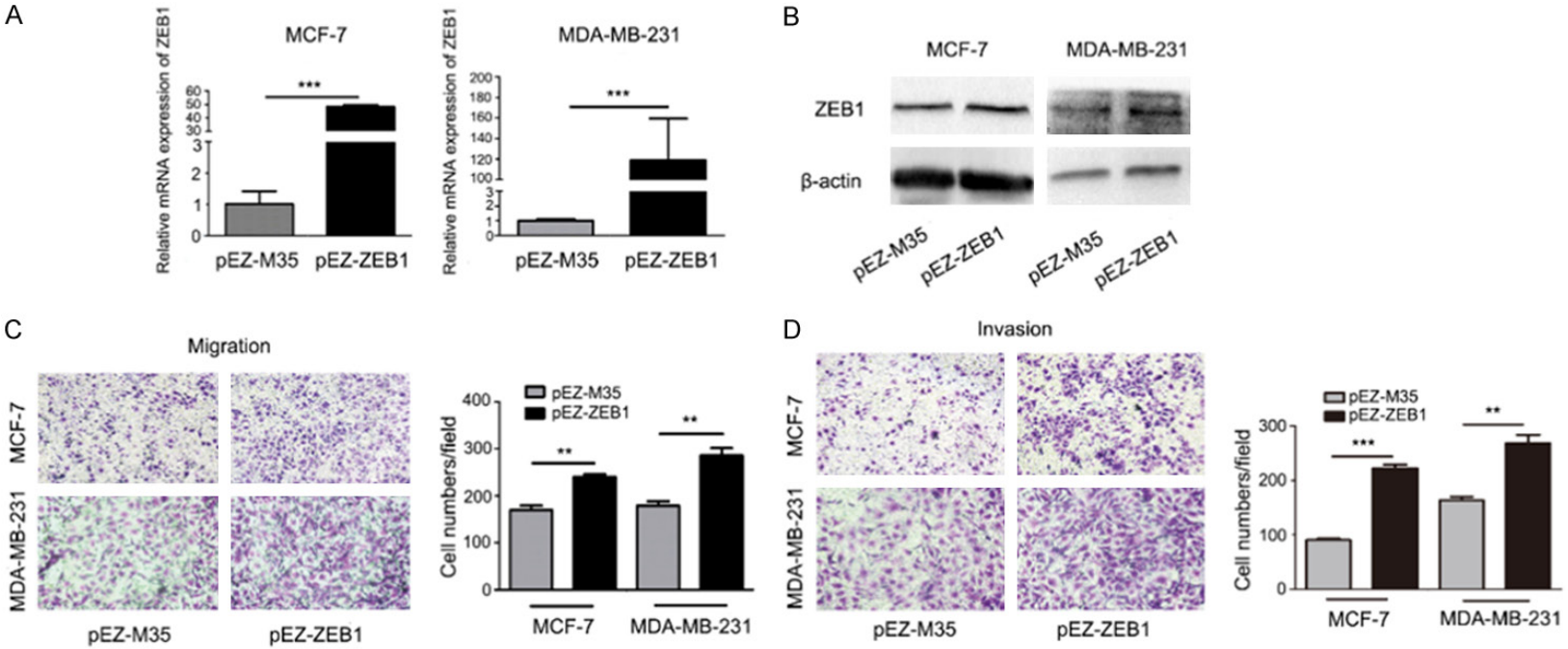

Figure 7. ZEB1 overexpression promoted BC cells migration and invasion. A, B. Expression levels of ZEB1 in cells were verified by RT-qPCR and western blotting analysis. C. Transwell migration assays to evaluate the migration of BC cells; D. Transwell invasion assays to assess invasion of BC cells. Cells were stained with 0.1% crystal violet (400 ×).

the disease remains the leading cause of cancer-related death in women [28]. Therefore, it is particularly important to explore the mechanism underlying the growth and metastasis of BC.

Increasing evidence has shown that EMT is associated with the degree of malignancy of various types of tumors, and activation of EMT signals is widely associated with invasion, metastasis, recurrence, and treatment resistance [29, 30]. ZEB1 is a core transcription factor of EMT and involved in regulating key factors during malignant cell transformations by triggering EMT. EMT confers cancer cells with a proinvasive and stem-like phenotype; it also indicates worse clinical prognosis in most human cancers. As a crucial member of the ZEB transcription factor family, it promotes E-cadherin epigenetic silencing by recruiting multiple chromatin-modifying enzymes to the E-cadherin promoter [31].

LncRNAs reportedly play important roles in regulating the pathological and physiological processes of numerous cancers and can act as either tumor suppressor genes or oncogenes in tumorigenesis, metastasis, diagnosis, and prognosis [32]. It is well-known that lncRNAs do not encode proteins, but rather regulate protein-coding genes at epigenetic, transcriptional, or post-transcriptional levels [33-36]. An increasing number of studies has demonstrated that many lncRNAs, including H19, SNHG16, NEAT1, and MALAT1, exhibit disease-associated dysregulation in BC [37-40]. Small nucleolar RNAs (snoRNAs) are the most widely characterized classes of non-coding RNAs. Most snoRNAs are encoded by host genes (SNHGs); although it was widely assumed that snoRNAs only perform cellular housekeeping functions, snoRNAs and their host genes were recently implicated in the control of cell fate and tumorigenesis [41]. This suggests that the classic functions of snoRNAs or their host genes are conducive to the development of cancer [42], which requires further analysis. SNHG3 was shown to be significantly associated with the malignant degree and poor prognosis of hepatocellular carcinoma [22]. Additionally, SNHG3 is involved in the glucose metabolism pathway of ovarian cancer and fatty acid metabolism of liver cancer. Studies have confirmed that SNHG3 can promote the development of liver cancer, colorectal cancer, and ovarian cancer [43-45]. However, the biological characteristics and mechanism of SNHG3 in BC have not been studied in depth.

In the present study, we found that SNHG3 was overexpressed in BC tissues and cell lines. Our findings showed that SNHG3 exhibited tumorigenic and pathologic effects by promoting proliferation, migration, and invasion. Mechanistically, we observed that SNHG3 induces EMT by upregulating ZEB1 and MMP-2/9 expression in BC cells. These results indicate that SNHG3, as an oncogene of BC, can be considered as a potential prognostic factor of BC. We observed that SNHG3 induced EMT by upregulation ZEB1 and MMP-2/9 expression in BC cells. These results indicate that SNHG3 acts as an oncogene in BC. Combined lncRNA expression profiling chip and RT-qPCR analyses showed that the expression of SNHG3 was significantly decreased after BMP9 treatment of BC cells. Using Oncomine and TCGA database analysis, we found that the expression of SNHG3 in patients with BC was increased compared to in the average population. Further, compared with the immortalized normal mammary epithelial cells MCF-10A, SNHG3 was highly expressed in BC cells. FISH analysis confirmed that SNHG3 was mainly present in the cytoplasm of BC cells.

Further analysis confirmed that SNHG3 was successfully overexpressed. We found that knockdown of endogenous expression of SNHG3 decreased the migration and invasion of BC cells, whereas overexpression of SNHG3 facilitated the proliferation, migration, and invasion of BC cells. SNHG3 can promote the transformation of BC cells into EMT. We observed that the expression of ZEB1 was increased after overexpression of SNHG3 and *vice versa*. In the rescue experiment, SNHG3 promoted the transformation of BC cells into EMT by binding to the miR-186-5p site to upregulate ZEB1 expression and increase cell migration and invasion. Tumorigenic experiments in nude mice showed that the tumorigenic ability of BC cells was inhibited after knockdown of SNHG3 expression in MCF-7 cells; SNHG3 knockdown inhibited the expression of ZEB1, Snail, and MMP2. Additionally, SNHG3 knockdown promoted the expression of E-cadherin and inhibited the expression of PCNA and vimentin. In summary, our study confirmed that SNHG3 promoted the proliferation, migration, and invasion

of BC cells, suggesting that SNHG3 becomes a sink for miR-186-5p and thereby modulates the decrease in ZEB1 to induce EMT. Thus, SNHG3 is a potential treatment target for BC.

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Disclosure of conflict of interest

None.

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