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Knockdown of lncRNA SNHG4 suppresses gastric cancer cell proliferation and metastasis by targeting miR-204-5p

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Long non-coding RNAs (lncRNAs) have been identified as critical regulators in gastric cancer (GC) progression. However, whether lncRNA small nucleolar host gene 4 (SNHG4) functions in GC development remains unknown. In this study, the bio-functional role of SNHG4 and its potential mechanism on GC progression were systematically dissected. To investigate the role of SNHG4 in GC, we silenced SNHG4 using short hairpin RNAs (shRNAs) to perform loss-of-function assays. The results showed that SNHG4 expression in GC cells was at a higher level compared to normal gastric mucosal epithelial cells. Knockdown of SNHG4 dramatically suppressed proliferation, migration and invasion, and blocked cell cycle progression of GC cells. Moreover, knockdown of SNHG4 upregulated microRNA-204-5p (miR-204-5p) expression, whereas downregulated ribonucleotide reductase subunit M2 (RRM2) expression in GC cells. Dual-luciferase reporter assay results showed that miR-204-5p was a direct target of SNHG4. Additionally, knockdown of SNHG4 suppressed GC tumorigenesis in xenograft mouse models. Taken together, these data demonstrated that knockdown of SNHG4 suppressed GC development by targeting miR-204-5p.

Key words: SNHG4, miR-204-5p, gastric cancer, proliferation, metastasis

Gastric cancer (GC) is one of the most aggressive and prevalent malignancies worldwide with an estimation of 1,033,701 new cases and 782,685 cancer-related deaths in 2018 [1]. Among all populations, the burden of GC is very high in countries from Asia, Latin America, and Central and Eastern Europe [2]. Despite some advances that have been made in revealing the epidemiology, pathology, and therapeutic options of GC, it remains one of the biggest threats of cancer mortality [3]. The reason for the high mortality of GC may be that the diagnosis of GC patients frequently occurs at an advanced stage, which severely impedes the effectiveness of therapy [4]. Hence, investigations on the available screening approaches for early GC remain critical for preventing and treating GC.

It has been reported that the accumulation of genetic and epigenetic alternations is a major incentive for cancer pathogenesis [5]. Non-coding RNAs (ncRNAs), such as long non-coding RNAs (lncRNAs) and microRNAs (miRNAs), have been confirmed to affect the progression, growth, and spread of cancers [6–8]. Among them, small nucleolar host gene 4 (SNHG4) is a novel lncRNA that belongs to the SNHGs family, members of which have been documented to play critical roles in regulating tumorigenesis [9–11]. Recent studies have identified that SNHG4 functions as an important carcinogenic molecule in several cancers. For instance, Xu et al. reported that SNHG4 facilitates tumor growth by sponging miR-224-3p and is regarded as a poor prognostic factor in human osteosarcoma [12]. Li et al. demonstrated that SNHG4 acts as a competing endogenous RNA (ceRNA) for sponging miR-148a-3p, thereby upregulating c-Met expression, to promote cervical cancer progression [13]. However, the role of SNHG4 in GC development has not been elucidated.

Emerging evidence has demonstrated that lncRNAs and miRNAs interact with each other to regulate tumorigenesis and metastasis [14]. By using an online database for preliminary examination, we found that there is the binding potential between SNHG4 and miR-204-5p. miR-204-5p has been reported to function in retarding GC progression previously [15, 16]. Recently, Wu et al. demonstrated that

SNHG4 promotes tumorigenesis and invasion by acting as a ceRNA to sponge miR-204-5p and upregulate runt-related transcription factor 2 (RUNX2) expression in renal cell carcinoma [17]. Nevertheless, whether SNHG4 affects the GC progression by regulating miR-204-5p is still unknown. In this study, we investigated the effect of SNHG4 on the GC progression. Mechanically, we demonstrated that SNHG4 affects the GC progression by regulating miR-204-5p expression.

Materials and methods

Ethics statement. The animal experiments in this work were approved by the Experimental Animal Ethics Committee of the Changchun University of Chinese Medicine (approval No. 2020217). All procedures were carefully performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Cell lines and cell culture. Human gastric mucosal epithelial cell line GES-1 (Cat. No. ZQ0905), human gastric cancer cell lines AGS (Cat. No. ZQ0240), HGC-27 (Cat. No. ZQ0192), NCI-N87 (Cat. No. ZQ0060), and MKN-45 (Cat. No. ZQ0457), and human embryonic kidney epithelial cell line 293T (Cat. No. ZQ0033) were all obtained from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd (China). GES-1 cells were cultivated in Dulbecco's modification of Eagle's medium (DMEM; Gibco Inc., USA) supplemented with 10% fetal bovine serum (FBS; Zhejiang Tianhang Biotechnology Co., Ltd., China) and 1% penicillin-streptomycin solution. AGS cells were cultivated in F12K medium (Zhongqiao Xinzhou) supplemented with 10% FBS. NCI-N87 cells were cultivated in RPMI-1640 medium (Gibco) supplemented with 10% FBS. HGC-27 and MKN-45 cells were cultivated in RPMI-1640 medium supplemented with 20% FBS. 293T cells were cultivated in DMEM supplemented with 10% FBS. All cells were cultured at 37 °C in a humidity-controlled atmosphere with 95% air and 5% CO_2.

Quantitative real-time PCR (qRT-PCR). Total RNAs in cells and xenograft tumor tissues were isolated using a high-purity total RNA rapid extraction kit (Wuxi BioTeke Co., Ltd., China). RNAs were reversely transcribed into cDNAs using M-MLV reverse transcriptase (Takara Bio Inc., Japan). qRT-PCR was conducted with Taq HS Perfect Mix (Takara) and SYBR Green (BioTeke) in a fluorescence quantitative PCR instrument (Exicycler 96; Bioneer Corporation, South Korea). The reaction conditions were as follows: initial at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 40 s. The results were analyzed using the $2^{-\Delta\Delta CT}$ method. β-actin was used as an endogenous control for SNHG4 and mRNAs. U6 was used as an endogenous control for miR-204-5p. The primers were as follows: SNHG4 (forward: 5'-GGCTAGAGTACAGTGGCTCG-3'; reverse: 5'-GCAAATCGCAAGGTCAGG-3'); ribonucleotide reductase subunit M2 (RRM2; forward: 5'-GGGATGAATTGCACTCTAA-3'; reverse: 5'-TCCTCTGATACTCGCCTACT-3'); β-actin (forward: 5'-GGCACCCAGCACAATGAA-3'; reverse: 5'-TAGAAGCATTTGCGGTGG-3'); miR-204-5p (forward: 5'-TTCCCTTTGTCATCCTATGCCT-3'; reverse: 5'-GCAGGGTCCGAGGTATTC-3'); U6 (forward: 5'-GCTTCGGCAGCACATATACT-3'; reverse: 5'-GCAGGGTCCGAGGTATTC-3'). This test was independently performed in triplicate.

Cell transfection. For knockdown of SNHG4, short hairpin RNAs (shRNAs) targeting human SNHG4 (NR_003141.3) and scramble control (NC) were designed, synthesized, and cloned into pRNAH1.1 vector (Nanjing Jinsirui Biotechnology Co., Ltd., China). Sequences for shRNAs were as follows: shNC (5'-GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTT-3'); shSNHG4-1 (5'-GGCTCCACTAGGACACACAGATTTCAAGAGAATCTGTGTGTCCTAGTGGAGCTTTTT-3'); shSNHG4-2 (5'-GGAATGACATCTACCTCCATCATTCAAGAGATGATGGAGGTAGATGTCATTCTTTTT-3'). HGC-27 and MKN-45 cells at a confluence of 80% were transfected using Lipofectamine™3000 transfection reagent (Invitrogen Corporation, USA) according to the manufacturer's instructions. G418 (100 μg/ml, NanJing KeyGene Biotech Co., Ltd., China) was used to screen stable transfection clones. Transfection efficacy was evaluated using qRT-PCR.

For overexpression or knockdown of miR-204-5p, hsa-miR-204-5p mimics, or hsa-miR-204-5p inhibitor and their NCs (Shanghai GenePharma Co., Ltd., China) were transiently transfected into cells using Lipofectamine™ 3000 transfection reagent according to the manufacturer's instructions. hsa-miR-204-5p mimics, hsa-miR-204-5p inhibitor, and their NC sequences were as follows: hsa-miR-204-5p mimics (5'-UUCCCUUUGUCAUCCUAUGCCU-3' and 5'-GCAUAGGAUGACAAAGGGAAUU-3'); mimics NC (5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'); hsa-miR-204-5p inhibitor (5'-AGGCAUAGGAUGACAAAGGGAA-3'); inhibitor NC (5'-CAGUACUUUUGUGUAGUACAA-3').

Cell counting kit-8 assay. Cell viability of the transfected cells was evaluated using cell counting kit-8 (CCK-8; Sigma-Aldrich Corporation, USA) assay according to the manufacturer's instructions. Optical density (OD) values at 570 nm were detected using a microplate reader (ELX-800; BioTek Instruments, Inc., USA). This test was independently performed in triplicate.

Flow cytometry. Flow cytometry was performed to monitor the distribution of the cell cycle. Briefly, stably transfected cells were seeded, washed, fixed, and then incubated with a cell cycle detection kit (Shanghai Beyotime Biotechnology Co., Ltd., China). Cell cycle distribution was examined using a flow cytometer (NovoCyte; ACEA Biosciences Inc., USA) and analyzed using NovoExpress. This test was independently performed in triplicate.

Western blot. Total proteins in cells and xenograft tumor tissues were isolated using powerful radio-immunoprecipi-

tation assay (RIPA) lysis solution (Beyotime) with phenylmethanesulfonyl fluoride (PMSF; Beyotime). The concentrations of proteins were determined by a bicinchoninic acid (BCA) assay kit (Beyotime). The same number of proteins were electrophoresed and then transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Fisher Scientific Inc., USA). After blocking with 5% bovine serum albumin (BSA; Biosharp, China), the membranes were probed with cyclin D1 antibody (Cat. No. A19038, Abclonal Technology, China), cyclin-dependent kinase 6 (CDK6) antibody (Cat. No. A1545, Abclonal), matrix metallopeptidase 2 (MMP2) antibody (Cat. No. 10373-2-AP, Proteintech Group Inc., China), matrix metallopeptidase 9 (MMP9) antibody (Cat. No. 10375-2-AP, Proteintech), RRM2 antibody (Cat. No. A3424, Abclonal), and β-actin antibody (Cat. No. 60008-1-Ig, Proteintech). After washing with Tris buffer solution containing Tween-20 (TBST) three times, the membranes were coupled with goat anti-rabbit IgG (Cat. No. SA00001-2, Proteintech) or goat anti-mouse IgG (Cat. No. SA00001-1, Proteintech). Enhanced chemiluminescence (ECL) solution (7Sea Biotech, China) was used to visualize the blots. Densitometric analysis was conducted by the ImageJ software. This test was independently performed in triplicate.

Wound healing assay. Cell migration was evaluated using a wound healing assay. Briefly, cells at a proper confluence were treated with 1 μg/ml mitomycin-C (Sigma) in a serum-free medium for 1 h. For the investigation of miR-204-5p function, NC inhibitor or miR-204-5p inhibitor was transiently transfected into the stably transfected cells and incubated for 24 h before the cells were treated with mitomycin-C. Subsequently, a scratch was made with a 200 μl pipette tip. The images were photographed at the same position at 0 h and 24 h after the scratch was established under an inverted phase-contrast microscope (IX53; Olympus Corporation, Japan). This test was independently performed in triplicate.

Transwell assay. Cell invasion was evaluated using Transwell assay. Briefly, stably transfected cells were seeded in the upper chamber of the Matrigel-coated Transwell chamber (Corning Inc. USA), while a medium containing 30% FBS was added to the lower chamber. Twenty-four hours after cells were incubated, the chamber was fixed, stained with crystal violet (Amresco Inc., USA), and then counted under an inverted phase-contrast microscope. This test was independently performed in triplicate.

Dual-luciferase reporter gene assay. The wild-type (wt) or mutant-type (mut) sequences of human SNHG4 were cloned into pmirGLO (Jinsirui) luciferase reporter vectors. The luciferase reporter vectors and miR-204-5p mimic or NC mimic were co-transfected using Lipofectamine™3000 transfection reagent according to the manufacturer's instructions. Forty-eight hours after transfection, luciferase activities were measured using a dual-luciferase assay kit (KeyGene). Relative luciferase (Firefly luciferase/Renilla luciferase) activity was calculated.

Xenograft tumor model. Six-week-old female BALB/c nude mice were purchased from Beijing HFK Bioscience Co. Ltd. (China) and fed with standard food and water in the specific pathogen-free house under a humidity-controlled atmosphere with 12 h light/12 h dark cycle at a controlled temperature. After bred adaptively for a week, mice were randomly and equally allocated to four groups (n=6 mice/group). A total of 5×10^6 stably transfected (shNC and shSNHG4-2) HGC-27 and MKN-45 cells were subcutaneously injected into the right armpit of each nude mouse according to the group information. Twenty-five days after injected, the mice were euthanized by cervical dislocation, and the xenograft tumor tissues were carefully removed. The tumor tissues were immediately photographed under a digital camera. After the tumor tissues were weighed, a part of each tumor tissue was fixed with 4% paraformaldehyde for histopathological examinations and the rest was stored at −80 °C for examination.

Immunohistochemistry. The fixed tumor tissues were embedded in paraffin and sectioned into 5 μm thick pieces. Then the paraffin-embedded tissue sections were dewaxed, rinsed, and rehydrated. After the antigen retrieval was performed, the sections were immunostained with anti-Ki67 (Abclonal) at 4 °C overnight. After washing with phosphate-buffered solution (PBS) twice, the sections were covered with goat anti-rabbit IgG (Thermo Fisher) for 30 min. Diaminobenzidine (DAB) chromogen solution (Beijing Solarbio Science & Technology Co., Ltd., China) was added for color development. The staining results were observed under a light microscope (BX53; Olympus).

Statistical analysis. Data were analyzed using GraphPad Prism software. Significant differences between the two groups were calculated by Student's t-test. Significant differences among the groups (three or more) were calculated by one-way analysis of variance (one-way analysis of variance). A p-value <0.05 was considered statistically significant.

Results

lncRNA SNHG4 was upregulated in human GC cells. First, the expressions of lncRNA SNHG4 in human GC cell lines (AGS, HGC-27, NCI-N87, and MKN-45) and a human normal gastric mucosal epithelial cell line (GES-1) were assessed. Its expressions in HGC-27, NCI-N87, and MKN-45 cells were significantly higher than that in GES-1 cells, while its expression in AGS cells was higher only on average, but with no significance (Figure 1A). Based on this expression pattern, we chose the two highest SNHG4 expression cell lines HGC-27 and MKN-45 to verify the role of SNHG4 on GC progression. Subsequently, stable SNHG4 knockdown HGC-27 and MKN-45 cells were constructed using shRNA (Figures 1B, 1C) to investigate the biological function of SNHG4.

Knockdown of lncRNA SNHG4 inhibits GC cell proliferation *in vitro*. To explore the effect of SNHG4 in GC

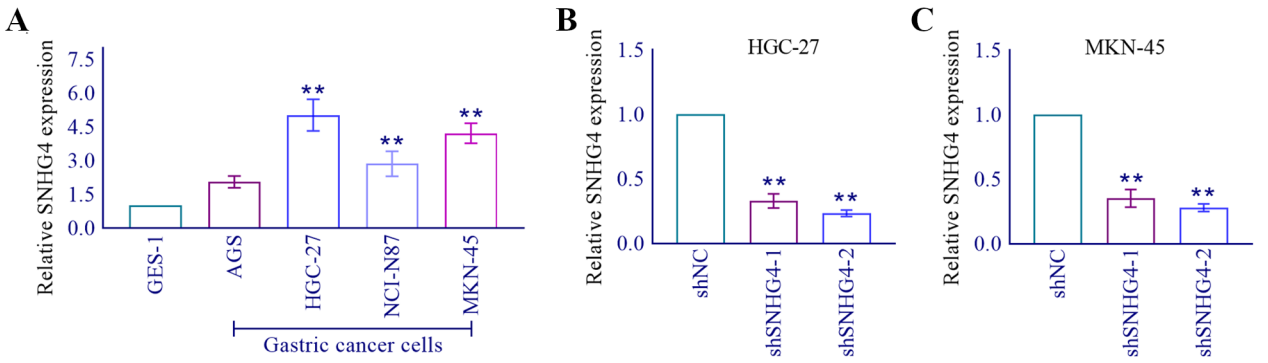

Figure 1. Expressions of SNHG4 in human GC cell lines and transfection efficiency of shRNAs targeting SNHG4. A) The relative SNHG4 expressions in human gastric mucosal epithelial cell line GES-1 and human GC cell lines AGS, HGC-27, NCI-N87, and MKN-45 were detected using qRT-PCT. B, C) The transfection efficiencies of two shRNAs targeting SNHG4 in HGC-27 cells (B) and MKN-45 cells (C) were detected using qRT-PCR. **p<0.01

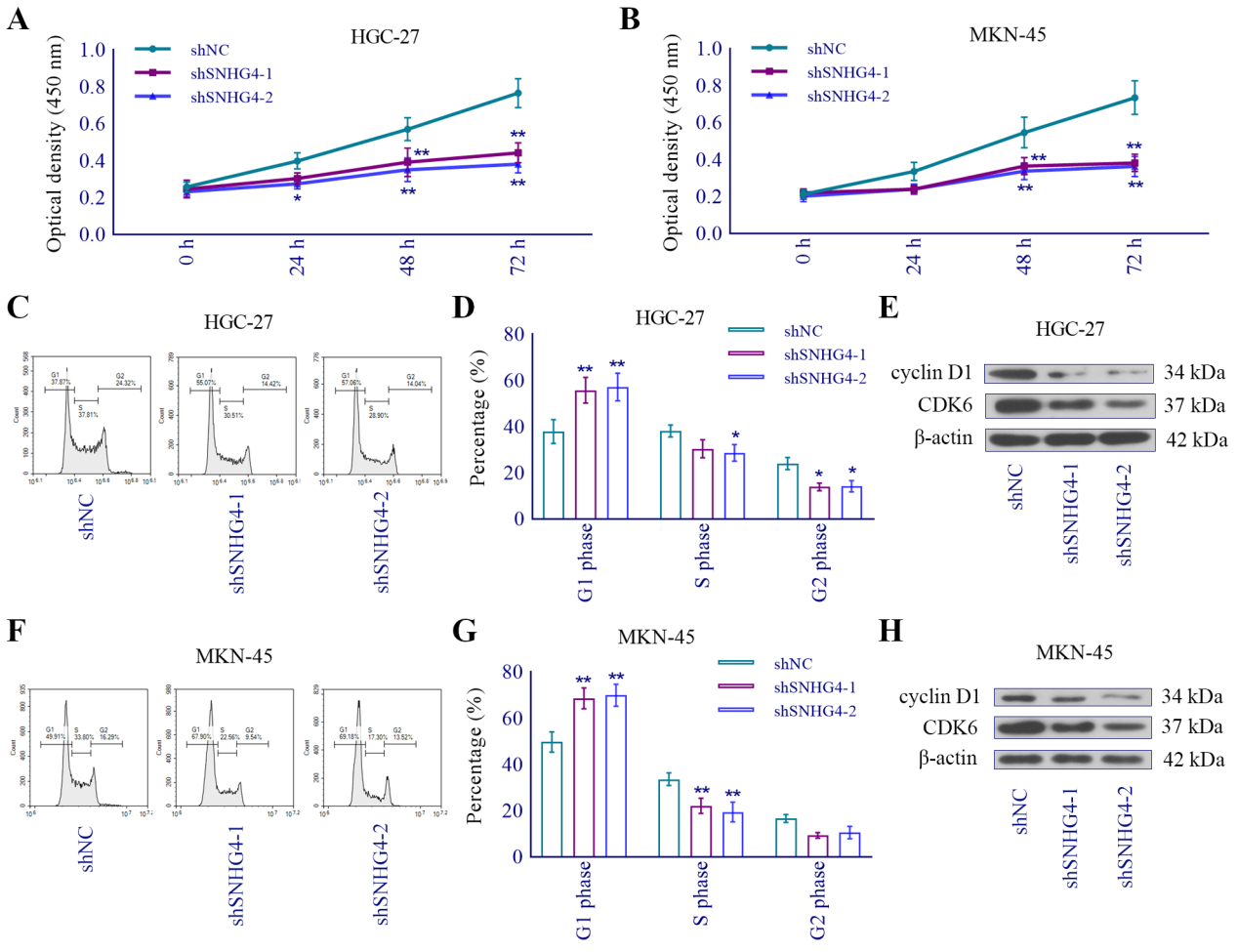

Figure 2. Knockdown of SNHG4 inhibits GC cell proliferation *in vitro*. A, B) Knockdown of SNHG4 inhibits cell proliferation in HGC-27 cells (A) and MKN-45 cells (B), as assessed by the CCK-8 assay. C, D) Knockdown of SNHG4 increases the cell numbers in the G1 phase and decreases the cell numbers in the S phase and G2 phase in HGC-27 cells, as assessed by flow cytometry. E) Knockdown of SNHG4 decreases cyclin D1 and CDK6 expressions in HGC-27 cells, as assessed by western blot. F, G) Knockdown of SNHG4 increases the cell numbers in the G1 phase and decreases the cell numbers in the S phase and G2 phase in MKN-45 cells, as assessed by flow cytometry. H) Knockdown of SNHG4 decreases cyclin D1 and CDK6 expressions in MKN-45 cells, as assessed by western blot. *p<0.05; **p<0.01

cell proliferation, CCK-8 assay and flow cytometry were applied to assess GC cell viability and cell cycle distribution, respectively. The results of the CCK-8 assay showed that the optical density values at 450 nm in shSNHG4-transfected GC cells were significantly lower than that in shNC-transfected GC cells (Figures 2A, 2B). The results of flow cytometry presented that the number of cells in the G1 phase was significantly higher, whereas in S phase and G2 phase were much lower in shSNHG4-transfected GC cells than that in the shNC-transfected GC cells (Figures 2C, 2D, 2F, and 2G). Additionally, the expressions of cyclin D1 and CDK6 were also found downregulated in shSNHG4-transfected GC cells compared with shNC-transfected GC cells (Figures 2E, 2H).

Knockdown of lncRNA SNHG4 inhibits GC cell metastasis *in vitro*. To explore the effect of SNHG4 in GC cell metastasis, a wound-healing assay and Transwell assay were used to assess GC cell migration ability and invasion ability, respectively. As shown in Figure 3, knockdown of SNHG4 remarkably inhibited the migration rate and invasion rate of GC cells compared to its vector control. Furthermore, knockdown of SNHG4 affected the expressions of metastasis-related proteins, including MMP2 and MMP9, in GC cells.

miR-204-5p is a target miRNA of SNHG4. By using the bioinformatics prediction software (http://starbase.sysu.edu.cn/index.php), we predicted that miR-204-5p potentially bound to SNHG4. Hence, we detected miR-204-5p expression in several GC cell lines and also in shSNHG4-transfected GC cells. The results showed that miR-204-5p expression was significantly lower in GC cell lines (AGS, HGC-27, NCI-N87, and MKN-45) compared to a normal gastric mucosal epithelial cell line (GES-1) (Figure 4A). Also, miR-204-5p expression was significantly upregulated in shSNHG4-transfected GC cells compared to shNC-transfected GC cells (Figures 4B, 4C). In addition, the results of dual-luciferase reporter gene

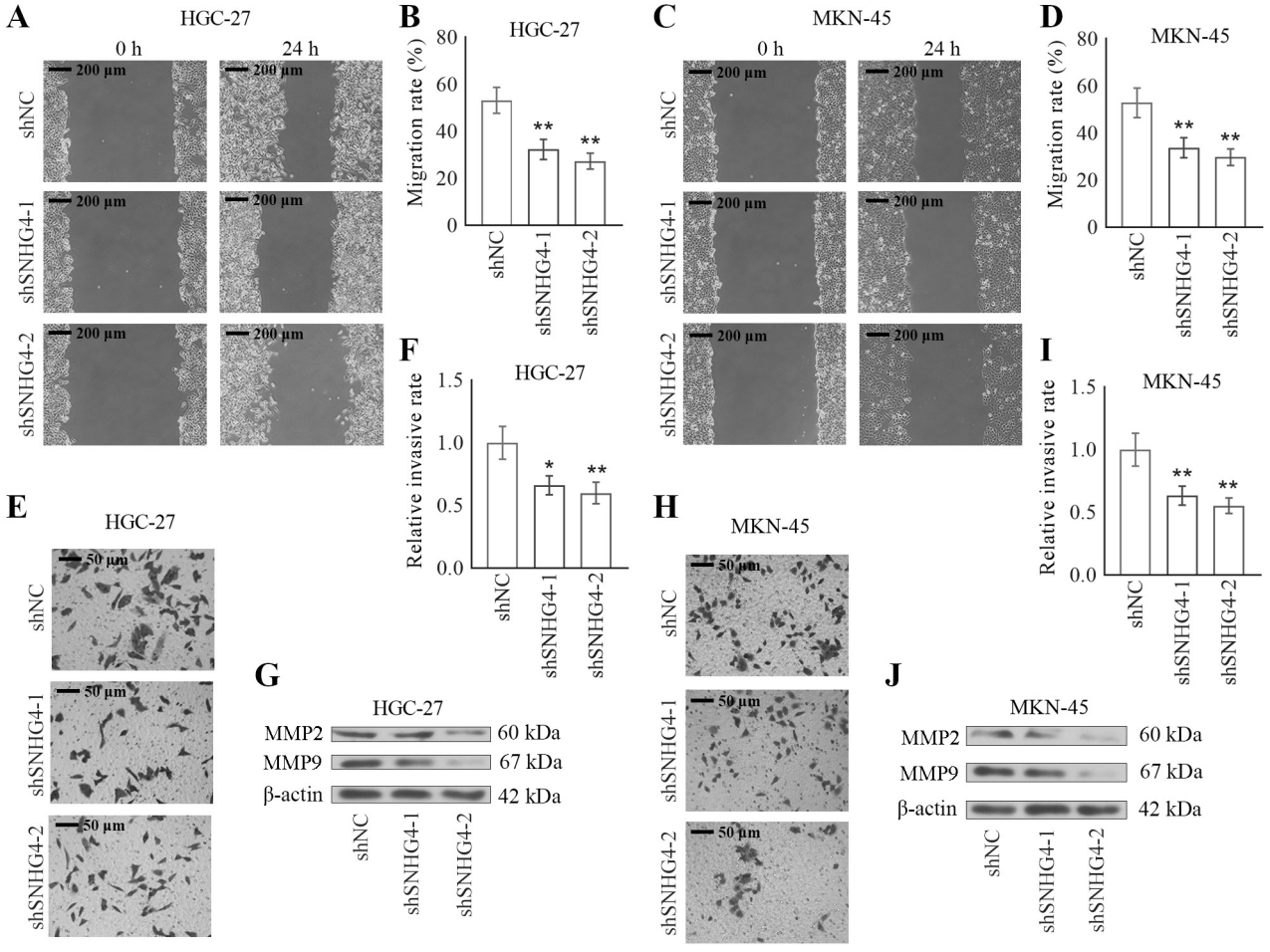

Figure 3. Knockdown of SNHG4 inhibits GC cell metastasis *in vitro*. A, B) Knockdown of SNHG4 inhibits cell migration in HGC-27 cells, as assessed by the wound-healing assay. C, D) Knockdown of SNHG4 inhibits cell migration in MKN-45 cells, as assessed by the wound-healing assay. E, F) Knockdown of SNHG4 inhibits cell invasion in HGC-27 cells, as assessed by the Transwell assay. G) Knockdown of SNHG4 decreases MMP2 and MMP9 expressions in HGC-27 cells, as assessed by western blot. H, I) Knockdown of SNHG4 inhibits cell invasion in MKN-45 cells, as assessed by the Transwell assay. J) Knockdown of SNHG4 decreases MMP2 and MMP9 expressions in MKN-45 cells, as assessed by western blot. *p<0.05; **p<0.01

assay validated that miR-204-5p mimic dramatically reduced the luciferase activity of wt-SNHG4, while had no influence on the luciferase activity of mut-SNHG4 (Figures 4D, 4E). Furthermore, we detected the expression of RRM2, which is a known target gene of miR-204-5p. We found that RRM2 was downregulated in shSNHG4-transfected GC cells compared to shNC-transfected GC cells (Figures 4F–4I). These results indicated that SNHG4 may sponge miR-204-5p and upregulate RRM2 expression in GC cells.

miR-204-5p inhibition promotes cell proliferation and metastasis in SNHG4-knockdown GC cells. To further investigate whether SNHG4 affects GC development by regulating miR-204-5p, the miR-204-5p inhibitor was added in shSNHG4-transfected GC cells to block the inhibitory effect of miR-204-5p on GC progression. As shown in Figure 5, compared to the NC inhibitor treatment, miR-204-5p inhibitor treatment dramatically promoted cell proliferation, migration, and invasion abilities in shSNHG4-transfected HGC-27 and MKN-45 cells.

Knockdown of lncRNA SNHG4 inhibits the GC cell growth *in vivo*. Furthermore, we investigated the bio-functional role of SNHG4 in tumorigenesis *in vivo*. The results showed that in the xenograft tumor mice model, the growth of shSNHG4-transfected GC cells was obviously slower compared to shNC-transfected GC cells, as reflected by a significant decrease in tumor volume and tumor weight (Figures 6A–6C, 6J–6L). Consistent with the *in vitro* results, the expressions of SNHG4 and RRM2 was found downregu-

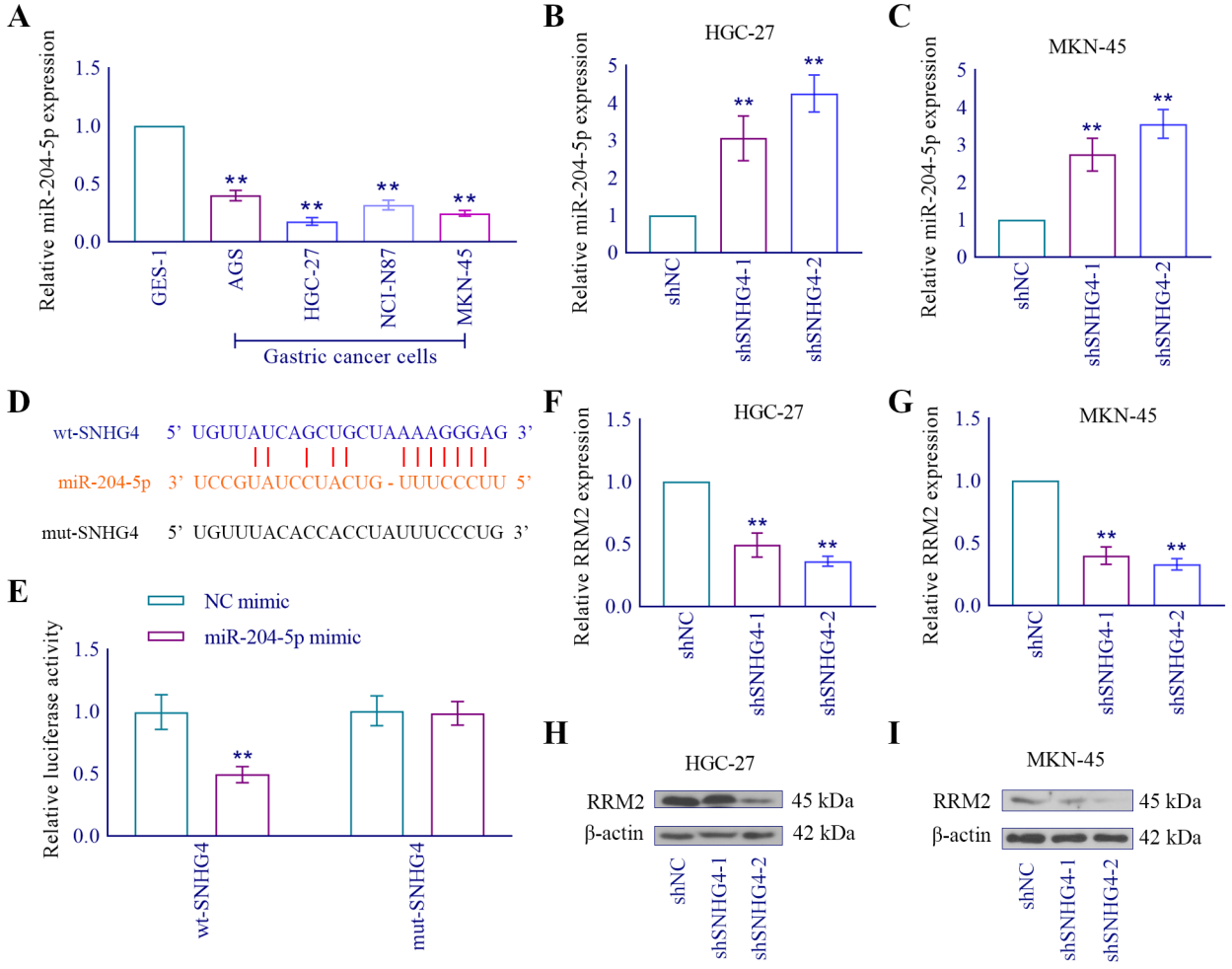

Figure 4. miR-204-5p is a target miRNA of SNHG4. A) The relative miR-204-5p expressions in human gastric mucosal epithelial cell line GES-1 and human GC cell lines AGS, HGC-27, NCI-N87, and MKN-45 were detected using qRT-PCT. B, C) Knockdown of SNHG4 increases miR-204-5p expressions in HGC-27 cells (B) and MKN-45 cells (C), as detected by qRT-PCR. D) Schematic illustration of the putative targeting site of miR-204-5p and SNHG4. E) The luciferase activities were detected by dual-luciferase reporter gene assay after co-transfection of wild-type (wt)-SNHG4 or mutant-type (mut)-SNHG4 and NC mimic or miR-204-5p mimic. F, G) Knockdown of SNHG4 decreases RRM2 expressions in HGC-27 cells (F) and MKN-45 cells (G), as detected by qRT-PCR. H, I) Knockdown of SNHG4 decreases RRM2 expressions in HGC-27 cells (H) and MKN-45 cells (I), as assessed by western blot. **p<0.01

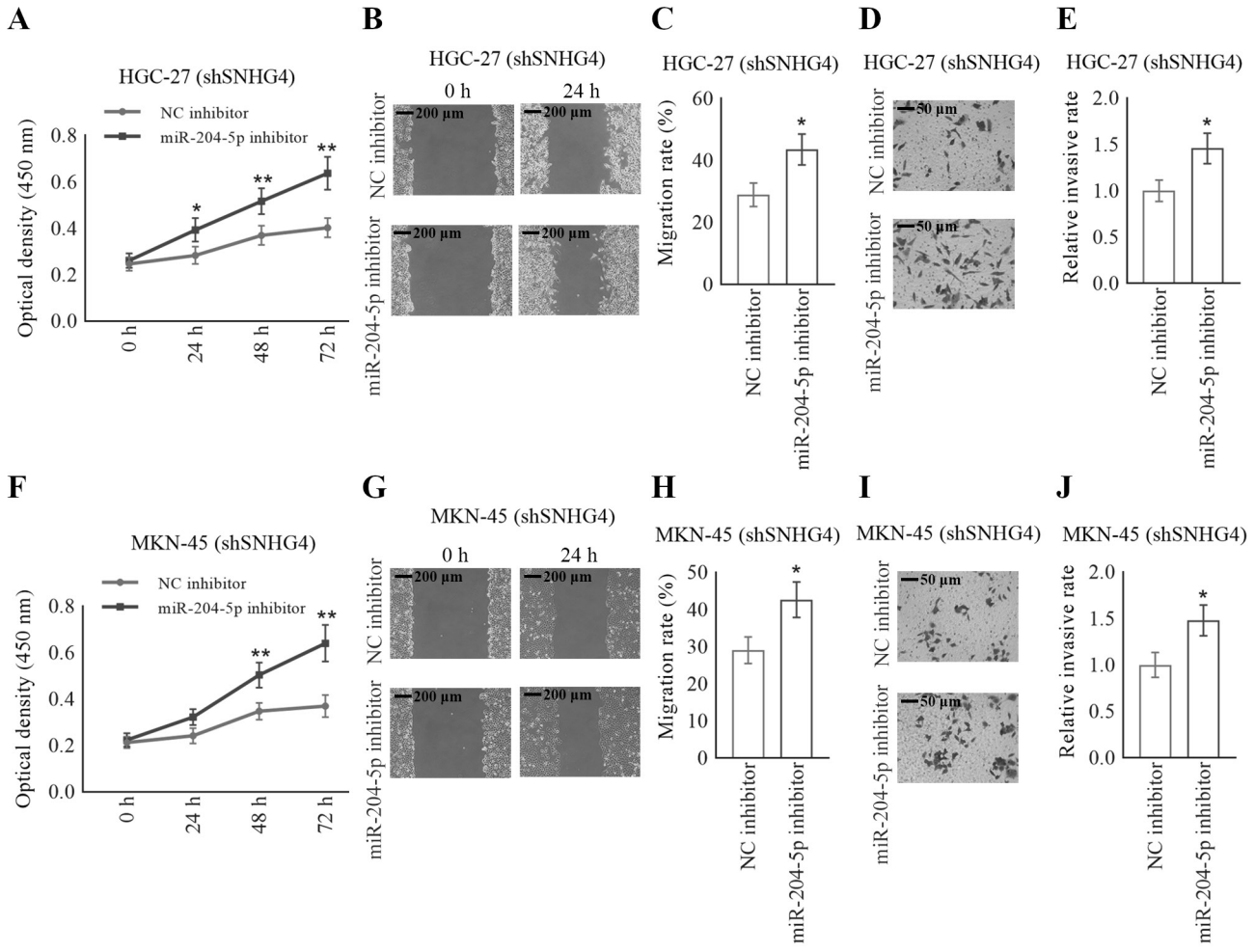

Figure 5. miR-204-5p inhibition promotes cell proliferation and metastasis in SNHG4-knockdown GC cells. A) miR-204-5p inhibition promotes cell proliferation in SNHG4-knockdown HGC-27 cells, as assessed by the CCK-8 assay. B, C) miR-204-5p inhibition promotes cell migration in SNHG4-knockdown HGC-27 cells, as assessed by the wound-healing assay. D, E) miR-204-5p inhibition promotes cell invasion in SNHG4-knockdown HGC-27 cells, as assessed by the Transwell assay. F) miR-204-5p inhibition promotes cell proliferation in SNHG4-knockdown MKN-45 cells, as assessed by the CCK-8 assay. G, H) miR-204-5p inhibition promotes cell migration in SNHG4-knockdown MKN-45 cells, as assessed by the wound-healing assay. I, J) miR-204-5p inhibition promotes cell invasion in SNHG4-knockdown MKN-45 cells, as assessed by the Transwell assay. *p<0.05; **p<0.01

lated, while the expression of miR-204-5p was found upregulated in the isolated tumor tissues (Figures 6D–6G, 6M–6P) of mice in the shSNHG4 groups. Similarly, the expressions of a proliferation marker Ki67, and two metastasis markers MMP2 and MMP9, were found downregulated in shSNHG4-transfected GC cells (Figures 6H, 6J, 6Q, and 6R). These results showed that the knockdown of lncRNA SNHG4 successfully inhibited the GC cell growth *in vivo*.

Discussion

Until now, GC is still the leading cause of cancer-related death in both sexes worldwide, especially in Eastern Asia [18]. Despite some advances that have been made in GC treatment, including surgical resection and chemotherapy, the mortality of advanced GC patients still stays high due to the poor prognosis and recurrence. Notably, emerging evidence has revealed that lncRNAs, a pivotal group of ncRNAs, are intensely involved in the occurrence, metastasis, and recurrence of GC, and might become compelling therapeutic targets for GC therapy. A novel lncRNA, SNHG4, has been documented as an important regulator in several types of tumor carcinogenesis and progression recently. However, the bio-functional role and underlying mechanism of SNHG4 in GC progression remain unclear.

In the present study, we found that SNHG4 was highly expressed in GC cell lines. To further investigate the role of SNHG4 in GC progression, we constructed two stable SNHG4-knockdown GC cell lines for the loss-of-function exploration. We found that the knockdown of SNHG4

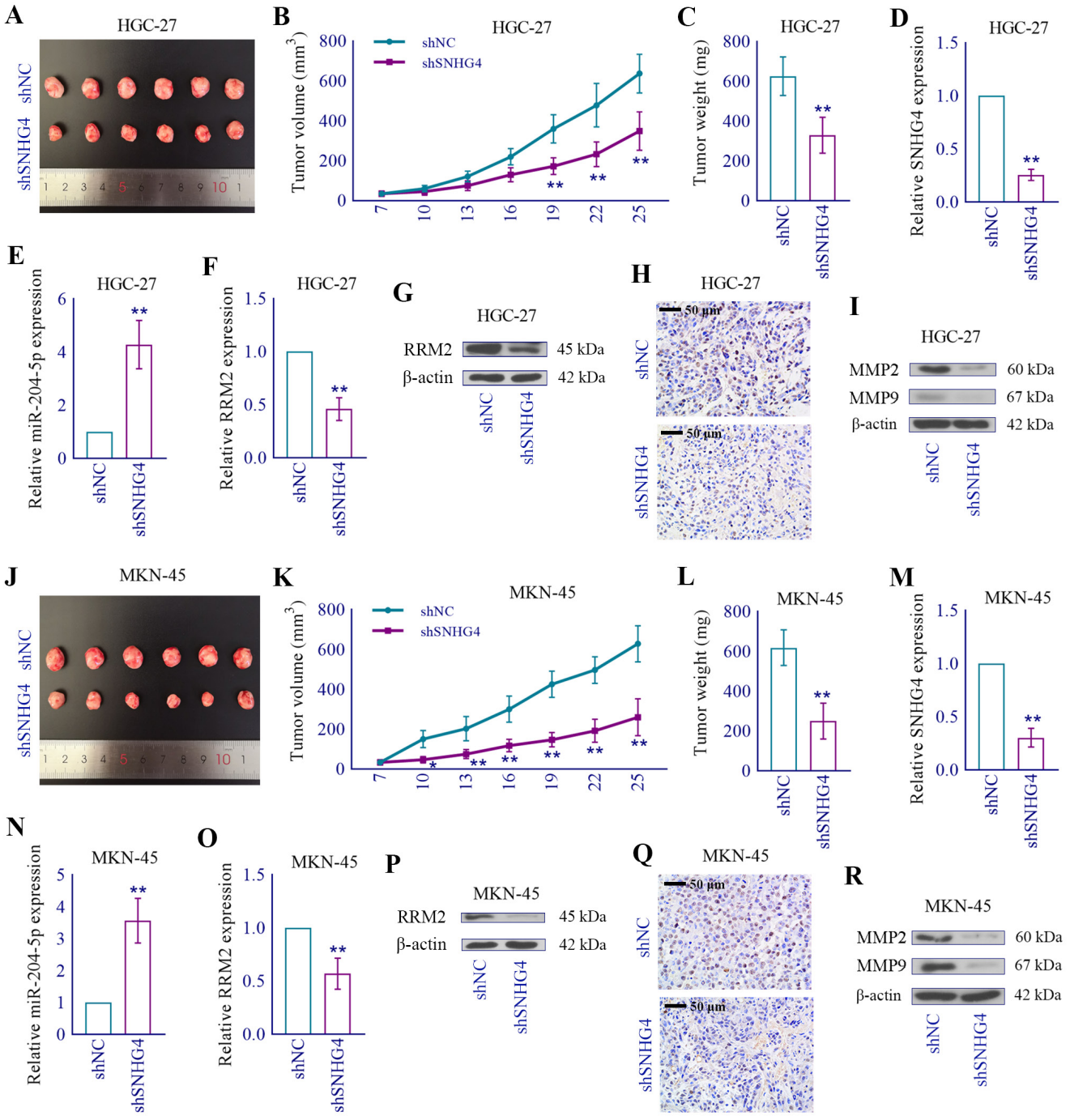

Figure 6. Knockdown of lncRNA SNHG4 inhibits GC cell growth *in vivo*. Mice were inoculated with stable shNC or shSNHG4 transfected HGC-27 or MKN-45 cells. A, J) Tumor images in mice inoculated with stable shNC or shSNHG4 transfected HGC-27 cells (A) and MKN-45 cells (J). B, K) Knockdown of SNHG4 decreases tumor volumes in a xenograft tumor model of HGC-27 cells (B) and MKN-45 cells (K). C, L) Knockdown of SNHG4 decreases tumor weights in a xenograft tumor model of HGC-27 cells (C) and MKN-45 cells (L). D, M) The relative SNHG4 expression in mice inoculated with stable shNC or shSNHG4 transfected HGC-27 cells (D) and MKN-45 cells (M), as assessed by qRT-PCR. E, N) Knockdown of SNHG4 increases miR-204-5p expression in a xenograft tumor model of HGC-27 cells (E) and MKN-45 cells (N), as assessed by qRT-PCR. F, O) Knockdown of SNHG4 decreases RRM2 expression in a xenograft tumor model of HGC-27 cells (F) and MKN-45 cells (O), as assessed by qRT-PCR. G, P) Knockdown of SNHG4 decreases RRM2 expression in a xenograft tumor model of HGC-27 cells (G) and MKN-45 cells (P), as assessed by western blot. H, Q) Knockdown of SNHG4 decreases Ki67 expression in a xenograft tumor model of HGC-27 cells (H) and MKN-45 cells (Q), as assessed by immunohistochemistry. I, R) Knockdown of SNHG4 decreases MMP2 and MMP9 expressions in a xenograft tumor model of HGC-27 cells (I) and MKN-45 cells (R), as assessed by western blot. *p<0.05; **p<0.01

reduced cell viability and induced cell cycle arrest, accompanied by a decrease in the expressions of G1 checkpoint protein Cyclin D1, and its catalytic subunit CDK6 [19, 20]. Also, we found that SNHG4 knockdown reduced the expression of Ki67, a well-known proliferation marker that predicts cancer progression [21], indicating that SNHG4 knockdown successfully inhibited the proliferation capacity of GC cells.

Metastasis, a biological cascade that ultimately results in the widespread dissemination of cancer cells into multiple organs, is the main incentive of cancer mortality [22]. GC metastasis also has no exception [23]. It has been reported that the high level of SNHG4 was associated with lymph node metastasis in patients with prostate cancer [24], and also the distant metastasis in osteosarcoma patients [25]. Migration and invasion are two vital initial steps of cancer metastasis [26]. It has been illustrated that matrix metallopeptidases are important enzymes that function in degrading basement membrane type IV collagen [27], which is thought as the first step for cancer invasion and metastasis [28]. Consistent with these notions, our results showed that SNHG4 knockdown effectively suppressed GC cell migration and invasion abilities, accompanied by the decrease of two key matrix metallopeptidases MMP2 and MMP9, indicating that SNHG4 knockdown effectively inhibited the metastasis capacity of GC cells.

Moreover, recent evidence has revealed that lncRNAs regulate tumorigenesis by acting as ceRNAs to specific miRNAs. miRNAs are also a group of ncRNAs that play functional roles in regulating multiple biological processes through translational repression or mRNA degradation [29, 30]. By using online bioinformatics prediction software, we found that there is the binding potential between SNHG4 and miR-204-5p. miR-204-5p has been documented as a tumor-suppressive miRNA in GC via downregulating ubiquitin-specific peptidase 47 (USP47) and RAB22A [16]. Also, miR-204-5p was reported to inhibit GC cell proliferation and metastasis by targeting epidermal growth factor receptor (EGFR) [15]. A recent study reported that SNHG4 promotes tumorigenesis and invasion by sponging miR-204-5p in renal cell carcinoma [17]. Consistently, by performing rescue experiments and a dual-luciferase reporter gene assay, we demonstrated that SNHG4 affects GC proliferation and metastasis by regulating miR-204-5p in this work.

Notably, it has been documented that miR-204-5p affected the development of varied human tumors progression by regulating diverse genes such as runt-related transcription factor 2 (RUNX2) [17], human epidermal growth factor receptor 2 (HER-2) [31], high mobility group B1 (HMGB1) [32], and RRM2 [33]. Among them, RRM2 is a catalytic subunit of ribonucleotide reductase that is intensely involved in DNA synthesis [34]. Its oncogenic effects in GC progression have been identified as previously reported [35–37]. Importantly, research has indicated that inhibiting RRM2 may be a potential therapeutic strategy to overcome the resistance of chemotherapy [38]. Consistently in our work, we found that in SNHG4-knockdown GC cells, RRM2 presented a lower level. These results implied that SNHG4 may regulate GC progression by sponging miR-204-5p and un-regulating RRM2 expression.

Regrettably, in this work, there appear to be some deficiencies. This study lacks the support of the measurement of SNHG4 expression in clinical samples. Also, the regulatory correlation between SNHG4 and miR-204-5p needs to be further explored in GC. Despite it is hard for us to make better improvements in these aspects due to the existing constraints, the in-depth investigations are still worthy of our further research.

In the current study, we demonstrated that the knockdown of SNHG4 dramatically suppressed the proliferation and metastasis on GC cells via regulating miR-204-5p. Our findings first demonstrated that SNHG4 may serve as an oncogene in GC development and indicated that SNHG4 might become a potential therapeutic target in GC treatment.

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