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LINC00707 promotes cell proliferation and invasion of colorectal cancer via miR-206/FMNL2 axis

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Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs) have been verified to participate in the regulation of colorectal cancer (CRC). However, the role of LINC00707 in CRC still remains unknown. Here, we aim to study the role of LINC00707 in CRC.

PATIENTS AND METHODS: LINC00707 expression in 97 pairs of CRC tissues and adjacent normal tissues was determined by the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). LINC00707 overexpression or knockdown in SW620 or HCT116 cells was achieved by lentivirus transfection. The proliferation and cell circle progression of established cells were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Cell invasion and migration abilities were studied by transwell assay. Dual-luciferase assay and Western blot was used to verify the underlying mechanism of LINC00707 in CRC. Nude mice were obtained to identify the in vivo function of LINC00707 in CRC.

RESULTS: LINC00707 was significantly over-expressed in CRC tissues and cell lines. Up-regulation of LINC00707 promoted cell proliferation, cell cycle progression, invasion, and migration of SW620 cells. Conversely, down-regulation of LINC00707 reduced cell growth and metastasis of HCT116 cells. MiR-206 was verified as a direct target of LINC00707, and its function was inhibited by LINC00707. FMNL2 was a target for miR-206 in CRC cells. Meanwhile, LINC00707 promoted tumor growth of CRC *in vivo*.

CONCLUSIONS: LINC00707 was up-regulated in CRC tissues and cells, which promoted cell proliferation and metastasis via sponging miR-206 to increase FMNL2 expression. This might provide a novel target for the biological treatment of CRC.

Key Words:
LINC00707, Proliferation, Invasion, MiR-206, FMNL2, CRC.

Introduction

Colorectal cancer (CRC) is one of the most common tumors worldwide. In China, colorectal cancer is one of the most common lethal tumors, and its incidence is rising[1-3]. Tumor metastasis is the most important cause of death in CRC patients[4]. In the past few decades, many studies have been conducted to explore the metastasis mechanism of CRC, but its molecular mechanism has not yet been clearly understood.

Long non-coding RNA (lncRNA) is a transcribed RNA sequence with a length of more than 200 nt without protein-coding function[5]. Studies[6,7] have shown that lncRNA plays an important role in the various activities of cells, including cell growth, differentiation, tumor development, and metastasis. Abnormal expression of lncRNA could be found in various tumors. For example, lncRNA AFAP1-AS1 acts as an oncogene in non-small cell lung cancer *via* accelerating cell migration. MEG3 promotes the ubiquitination of enhancer of zeste homolog 2 (EZH2) to regulate LATS2, thus inhibiting cell growth and metastasis in gallbladder cancer. LncRNA GCAWKR accelerates development of gastric cancer *via* scaffolding KAT2A and WDR5. Also, lncRNA FAL1 promotes the proliferation, invasion, and epithelial-mesenchymal transition (EMT) of NSCLC cells *via* regulating PTEN/AKT signal pathway. LncRNA GIHCG accelerated tumor progression by epigenetically regulating miR-200b/a/429 in hepatocellular carcinoma[8-12]. However, the function of LINC00707 in CRC has not been reported.

In this study, LINC00707 was highly expressed in colorectal cancer cell lines and tissues. *In vitro* experiments further validated the role of

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LINC00707 in proliferation, cell cycle, and metastasis of CRC cells. Our study provides evidence for the use of LINC00707 as a new early diagnostic indicator and therapeutic target for CRC.

Patients and Materials

CRC Tissues

The clinical specimens of 97 CRC patients admitted to the People's Hospital of Liaocheng from May 2016 to December 2017. Tumor tissues and adjacent tissues were surgically removed and stored in liquid nitrogen. None of the patients received anti-tumor therapy such as radiotherapy or chemotherapy before surgery, and the final diagnosis was confirmed by routine pathological examination. The sample collection was approved by the patients. The study was approved by the People's Hospital of Liaocheng Ethics Committee.

Cells and Culture

Human colorectal cell lines SW620, SW620, SW480, HCT116, and human colorectal normal epithelial cell line NCM460 were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). All cells were cultured according to cell culture instructions using Roswell Park Memorial Institute-1640 (RPMI-1640) medium or Dulbecco's Modified Eagle Medium-High (DMEM-H; Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin.

Cell Transfection

Lentivirus for overexpressing or interfering LINC00707 and their control groups (LINC00707, control, shRNA-LINC00707, shRNA-NC) were synthesized by Genepharma (Shanghai, China). When the cell density reached 70%, cells were transfected with the negative control, shRNA-LINC00707 or shRNA-NC using polybrene (Genepharma, Shanghai, China) following the manufacturer's instructions.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

After transfection for 48 h, SW620 or HCT116 cells were lysed with TRIzol (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted and reverse transcribed into complementary deoxyribose nucleic acid (cDNA) as a template. QRT-PCR was carried out by ABI7500 (BD Sciences, Franklin Lakes, NJ, USA) using SYBR green (TaKaRa, Dalian, China). The reaction conditions were: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 10 s, and annealing at 60°C for 30 s, for a total of 35 cycles. The experiment was repeated 3 times. Tissue and xenograft RNA extraction was also lysed by TRIzol. The primer sequences used in the experiment were as follows: FMNL2: Forward Primer: 5'-GCTATGAACCTACCTCCTGACA-3', Reverse Primer: 5'-AACACGCCGTCTGAATTTCTT-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward Primer 5'-GGAGCGAGATCCCTCCAAAAT-3', Reverse Primer: 5'-GGCTGTTGTCATACTTCTCATGG-3'.

Cell Counting Kit-8 (CCK-8) Assay

Stably transfected SW620 or HCT116 cells were suspended in RPMI-1640 medium and plated into 96-well plate at a density of 3000 cells /100 μL per well. After cell culture for 0, 24, 48, 72 h, a total of 10 μL of CCK8 reagent (Dojindo, Kumamoto, Japan) was added into the well. The absorbance of 490 nm was detected after 2 h culture in the dark. The experiment was repeated for at least three times.

Cell Cycle Detection

The established cells were digested with 0.25% trypsin, centrifuged 3 times (1000 r/min×5 min) in 4°C pre-cooled phosphate-buffered saline (PBS), and the supernatant was discarded. Next, cells were treated with 0.011% RNase and 0.5% propidium iodide (PI; Vazyme, Nanjing, China) for 20 min at 4°C. Flow cytometry was used to detect the DNA content of each cycle of the cells.

Transwell Assay

Transwell assay was performed to detect invasion and metastasis of CRC cells. Cells were digested, washed twice with PBS, and resuspended in serum-free medium at the cell density of 1×10^5 cells/mL. The basement membrane of the upper chamber was pre-coated with Matrigel (BD, Franklin Lakes, NJ, USA). A total of 150 μL of cell suspension was added into the upper chamber, and 500 μL of medium containing 20% FBS was added to the bottom chamber. After 48 h of culture, the chamber was taken out, rinsed with sterile PBS, and carefully treated with a cotton swab. Cells in the inner layer of the membrane

were wiped off. Cells on the bottom surface were fixed with 95% ethanol for 20 min and stained with crystal violet solution. The stained cells were counted under an inverted microscope and photographed. Five fields of view were randomly selected in each sample for cell counting. The cell migration assay was conducted with the same procedures except for Matrigel pre-coating.

Dual-Luciferase Assay

The luciferase reporter vector was co-transfected with miR-206 overexpressed SW620 cells. Transfection prl-TK was used as the standard internal quality control. After 36 h of transfection, the cells were harvested. The luciferase activity of SW620 cells was detected according to the Promega luciferase activity assay kit (Promega, Madison, WI, USA). Relative luciferase activity was calculated. The experiment was repeated three times.

Western Blot

Transfected cells were collected, lysed using radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China), and total protein concentration was determined by bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). After loading 50 μg of protein per well, the target protein was separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using BIO-RAD (Hercules, CA, USA) vertical electrophoresis system. Primary antibody was diluted with 1:1000 combined with the corresponding target protein, and the horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibody was applied at a dilution of 1:2000. The relative expression of the protein was detected by enhanced chemiluminescence (ECL) luminescence (Thermo Fisher Scientific, Waltham, MA, USA). The primary antibodies used were as follows: anti-FMNL2 (Abcam, Cambridge, MA, USA), anti-GAPDH (CST, Danvers, MA, USA).

Xenograft Assay

Nude mice were randomly divided into two groups. The transfected HCT116 cells (shRNA-LINC00707 and shRNA-NC) in the logarithmic growth phase were collected, and the cell viability was detected at over 90%. 0.1 mL of cell suspension containing 1×10^6 cells was injected into the ventral side of each nude mouse. After 5 weeks, the mice were sacrificed, and tumors were isolated. The tumor was weighed and stored in liquid nitrogen for later use.

Immunohistochemistry (IHC)

Following the instructions for the immunohistochemical SP kit (Elabscience®, Wuhan, China). FMNL-positive cells were observed under a fluorescence microscope (×400). The antibodies used in IHC were as follows: anti-FMNL2 (Abcam, Cambridge, MA, USA).

Statistical Analysis

According to the analysis, Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used. Measurement data were expressed as mean ± standard deviation (SD). The comparison between the two groups was analyzed using the *t*-test. $p<0.05$ was considered statistically significant.

Results

LINC00707 Expressed Significantly Higher in CRC Tissues and Cells

QRT-PCR was used to detect the expression of LINC00707 in 97 human colorectal cancer (CRC) tissues and matched normal tissues. Moreover, LINC00707 expression in CRC cell lines SW620, HCT116, SW620, SW480, as well as human colorectal normal epithelial cell line NCM460, was examined as well. The expression level of LINC00707 was significantly higher in CRC tissues and cell lines (Figure 1A, 1B). The results showed that LINC00707 might promote CRC development and progression

To further explore the role of LINC00707 in CRC, this study first constructed LINC00707 stably overexpressed cell line and its control cell line (LINC00707 and control) using the SW620 cell line with low expression of LINC00707. LINC00707 stable silencing cell line and its control cell line (shRNA-LINC00707 and shRNA-NC) were constructed using the colorectal cancer cell line HCT116 with higher expression of LINC00707. The cell line was successfully constructed as confirmed by qRT-PCR (Figure 1C, 1D).

LINC00707 Regulated Cell Proliferation and Cell Cycle of CRC Cells

CCK8 assay and cell cycle detection were performed to evaluate the function of LINC00707 in the proliferation of CRC cells. As shown in Figure 2A, 2B, the overexpression of LINC00707 significantly promoted cell proliferation of SW620 cells compared with the control group. However, knockdown of LINC00707 reduced cell growth of HCT116 cells compared with the negative control.

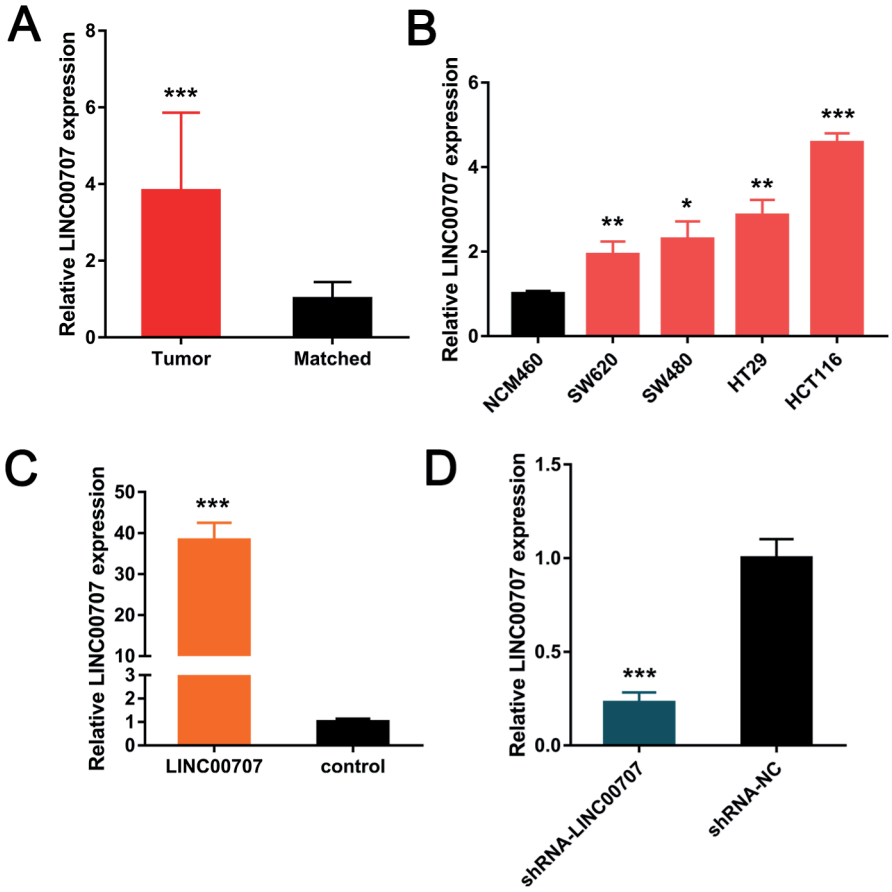

Figure 1. LINC00707 was increased in CRC tissues and cell lines. ***A,*** Analysis of the expression level of LINC00707 in 97paires of CRC tissue samples and adjacent normal tissue samples. ***B,*** Analysis of LINC00707 expression level in CRC cell lines (SW620, SW480, HCT116, HT29) and human normal colorectal epithelial cell line NCM460. ***C,*** Expression of LINC00707 in Lentivirus-LINC00707 treated SW620 cells and its control (LINC00707 *vs.* control). ***D,*** Expression of LINC00707 in shRNA-LINC00707 treated HCT116 cells and its control (shRNA-LINC00707 *vs.* shRNA-NC). *p<0.05, **p<0.01, ***p<0.001.

Furthermore, up-regulated LINC00707 accelerated cell transition from G1 phase to S phase, but down-regulation of LINC00707 inhibited the G1 to S transition (Figure 2C, 2D). All these data indicated that LINC00707 could promote cell proliferation and cell circle progression of CRC cells.

Regulation of LINC00707 Affected Cell Metastasis in CRC

To determine the influence of LINC00707 in cell invasion and migration, we employed the transwell assay. Transwell invasion assay confirmed that the number of SW620-LINC00707 cells penetrating through the membrane was more than SW620-control cells. However, the number of HCT116-shRNA-LINC00707 cells permeating through the membrane was significantly less than that of HCT116-shRNA-NC cells (Figure 3A). Transwell migration assay showed that the migration ability of SW620-LINC00707 cells was stronger than the control cells, while HCT116-shRNA-LINC00707 cells showed lower migration ability (Figure 3B). This confirmed that LINC00707 increased cell invasion and migration of CRC cells.

LINC00707 Function as an Oncogene Via Targeting MiR-206/FMNL2 Axis in CRC

Furthermore, to study the mechanism of LINC00707 in CRC, we explored several databases and found miR-206 as a potential target of LINC00707 in CRC. The binding site of LINC00707 for miR-2206 was shown in Figure 4A. Next, we employed dual-luciferase assay to verify our assumption. The relative lucifer-

ase activity of the wild-type LINC00707 3'-UTR markedly decreased, but no difference in the mutant-type group was found (Figure 4B). The expression of miR-206 in the experimental SW620 cells and HCT 116 cells was measured by qRT-PCR. A significant decrease was found in LINC00707 up-regulated SW620 cells, but a remarkable increase in LINC00707 down-regulated HCT116 cells compared to relative control group (Figure 4C, 4D). Next, we explored the underlying molecule of miR-206 in CRC using bioinformatics analysis and found FMNL2 as a direct target for miR-206. FMNL2 directly targeted the 3'-UTR of miR-206 (Figure 4E). Similarly, the luciferase activity of wild-type FMNL2 3'-UTR decreased significantly than the mutant-type group, indicating the direct binding relationship between the miR-206 and FMNL2 (Figure 4F). Furthermore, we detected the protein level in the established cells. Up-regulation of LINC00707 promoted the expression of FMNL2, while down-regulation of LINC00707 reduced the FMNL2 protein level (Figure 4G). All these suggested LINC00707 promoted *in vitro* proliferation and metastasis of CRC cells *via* regulating miR-206/FMNL2 axis in CRC.

LINC00707 Promoted CRC Tumor Growth in vivo

As we have explored the influence of LINC00707 in CRC *in vitro*, we designed the xenograft model using experimental HCT116 cells injecting into the nude mice. The xenografts of the shRNA-LINC00707 group grew markedly smaller than the shRNA-NC group (Figure 5A). The weight of xenografts in the knockdown group was lower than the negative control group (Figure 5B). Using qRT-PCR, we detected the expressions of LINC00707, miR-206, and FMNL2 in the xenografts. The expressions of LINC00707 and FMNL2 decreased but miR-206 expression increased in the shRNA-LINC00707 group com-

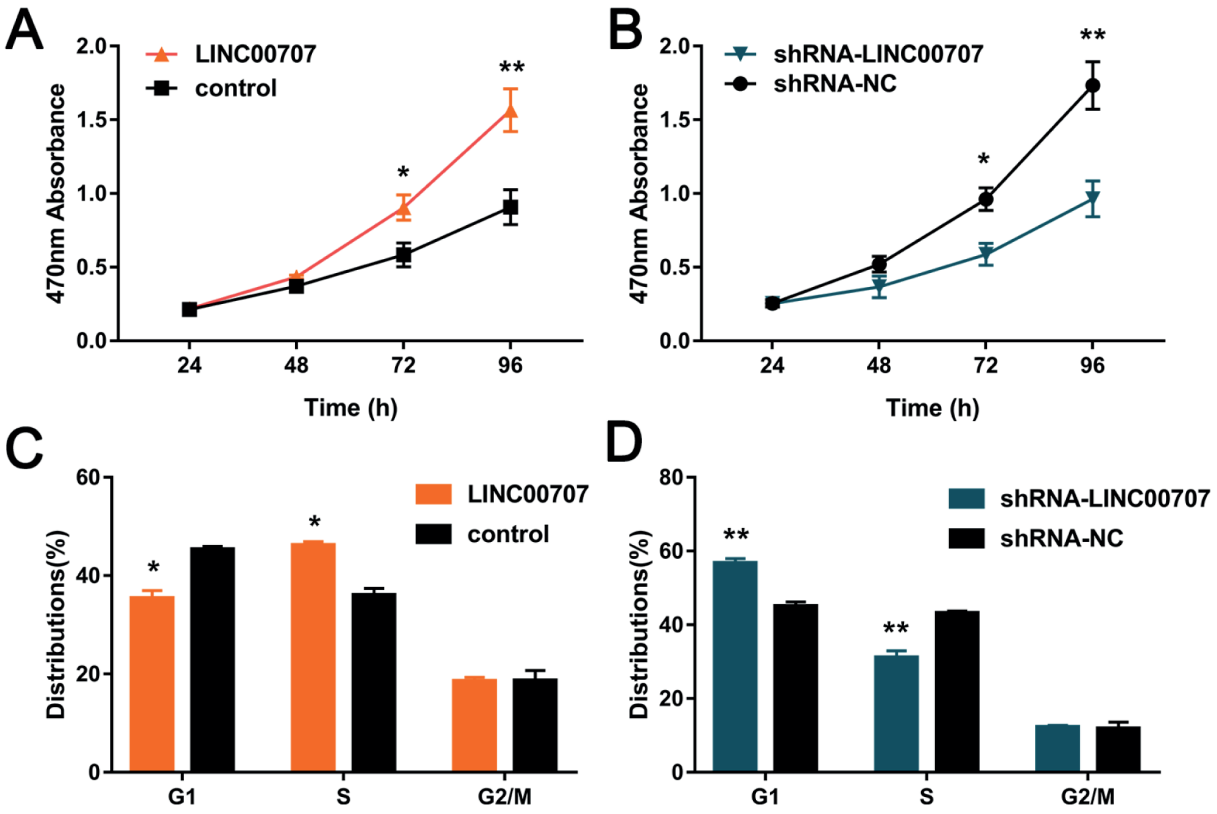

Figure 2. LINC00707 affected the proliferation and cell circle of CRC cells. ***A, B,*** CCK8 assay was performed to determine the proliferation of SW620 (***A***) or HCT116 (B) cells treated with LINC00707 or shRNA-LINC00707 compared to each negative control. ***C, D,*** Colony formation analysis was performed to determine the cell growth of SW620 (***C***) or HCT116 (***D***) cells transfected with LINC00707 or shRNA-LINC00707, respectively. *p<0.05, **p<0.01, ***p<0.001.

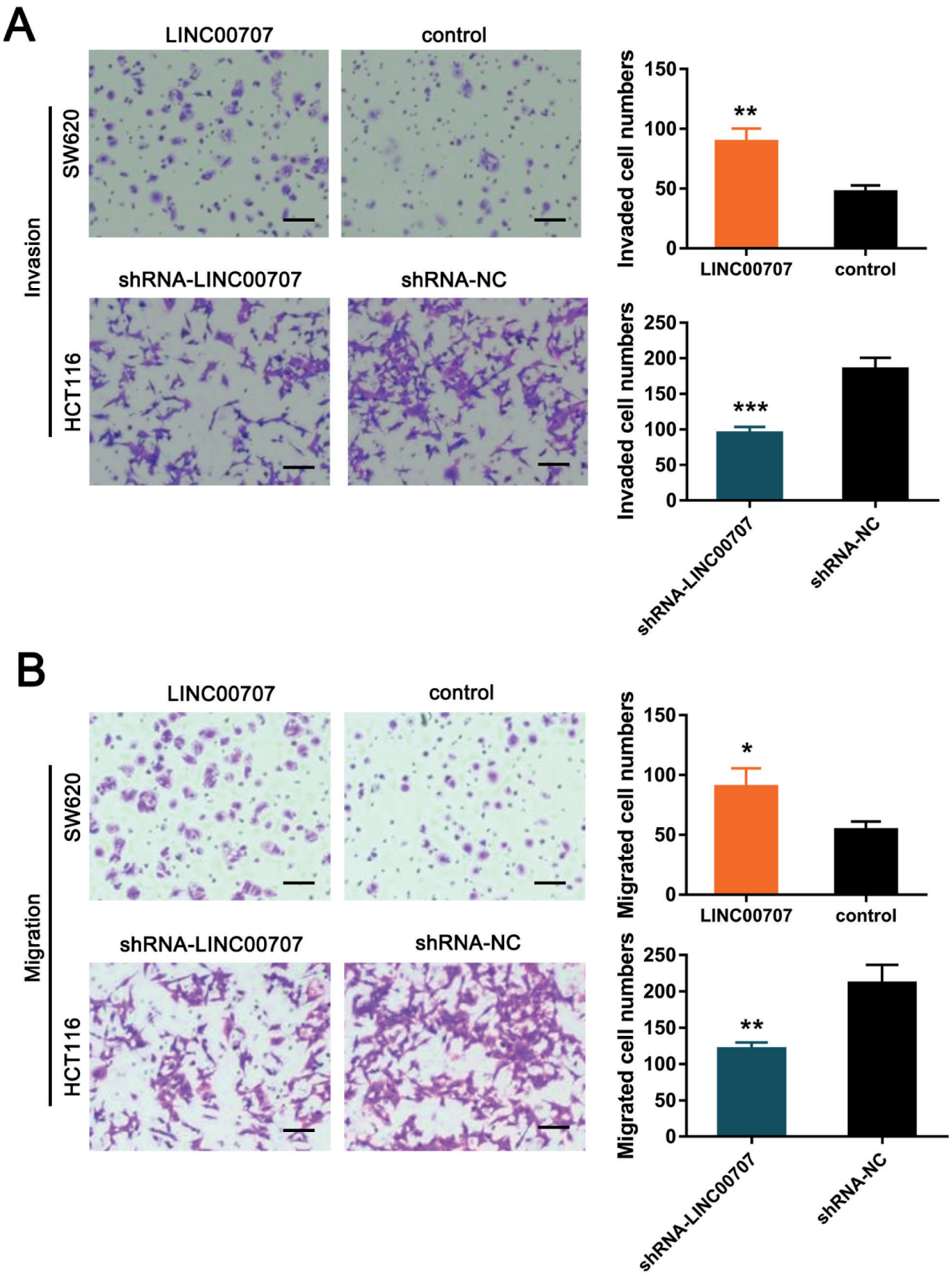

Figure 3. LINC00707 effected the invasion and migration of CRC cells. ***A,*** Transwell invasion assay was used to detect the invasion ability of LINC00707 treated SW620 cells or shRNA-LINC00707 treated HCT116 cells. ***B,*** Migration assay was used to detect the migration ability of LINC00707 treated SW620 cells or shRNA-LINC00707 treated HCT116 cells. Data are presented as the mean ± SD of three independent experiments. Magnification 200×. *p<0.05, **p<0.01, ***p<0.001.

pared to the shRNA-NC group (Figure 5C, 5D, 5E). IHC showed that FMNL2 protein expression was significantly reduced in the xenografts with FMNL2 knockdown (Figure 5F). These results

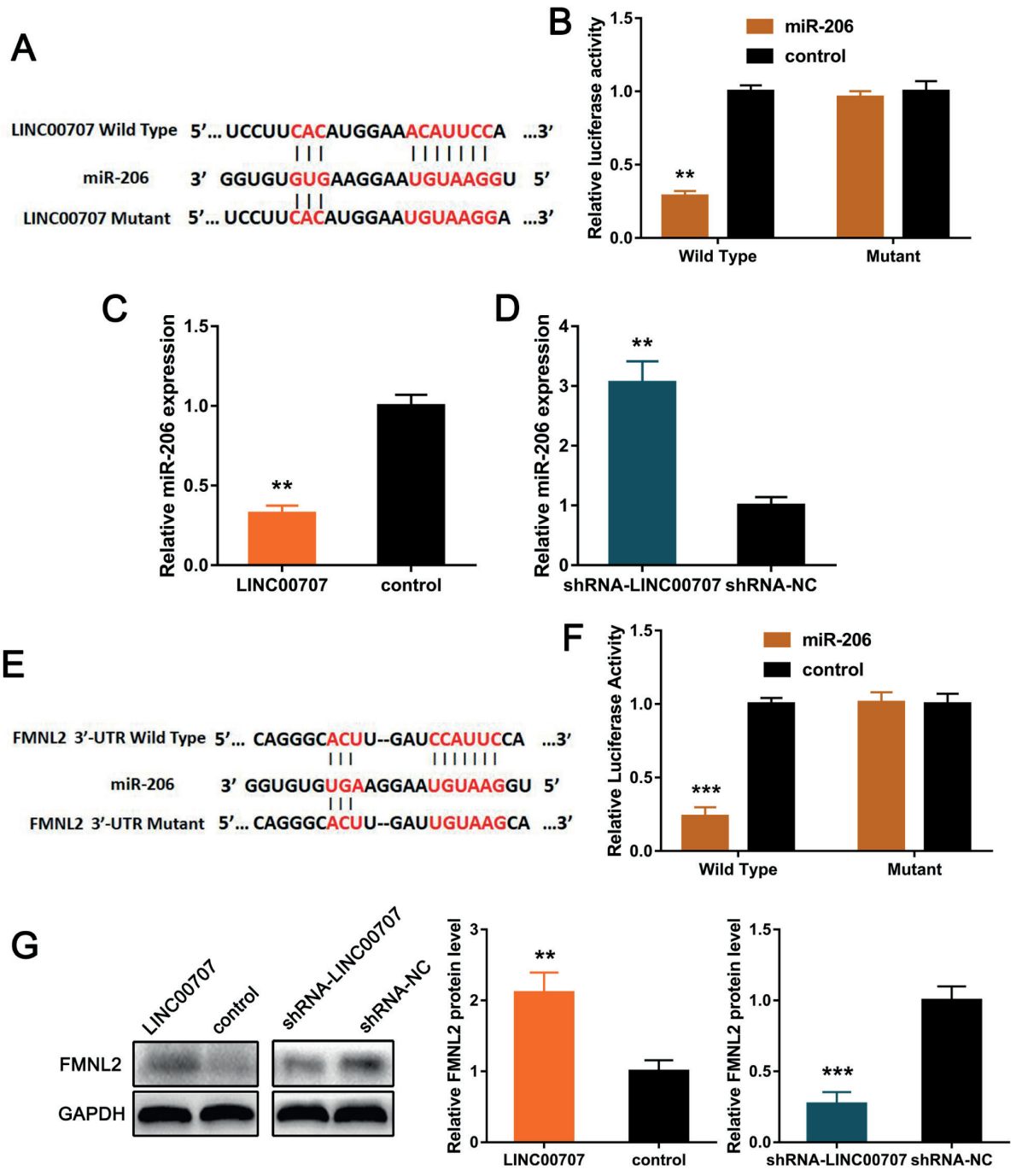

Figure 4. LINC00707 sponged miR-206 to promote FMNL2 expression. ***A,*** The predicted binding sites of miR-206 in the 3'-UTR of LINC00707. ***B,*** Dual-luciferase reporter assay was used to determine the binding site of miR-206 in the 3'-UTR of LINC00707. ***C, D,*** Expression level of miR-206 established CRCSW620 or HCT116 cells. ***E,*** The predicted binding sites of miR-206 in the 3'-UTR of FMNL2. ***F,*** Dual-luciferase reporter assay was used to determine the binding site of miR-206 in the 3'-UTR of FMNL2. ***G,*** Levels of FMNL2 and GAPDH protein measured by western blotting in LINC00707 over-expressed SW620 cells and LINC00707 knockdown HCT116 cells. The relative protein level of FMNL2 was compared to GAPHD. Data are presented as the mean ± SD of three independent experiments. *$p< 0.05$, **$p< 0.01$, ***$p<0.001$.

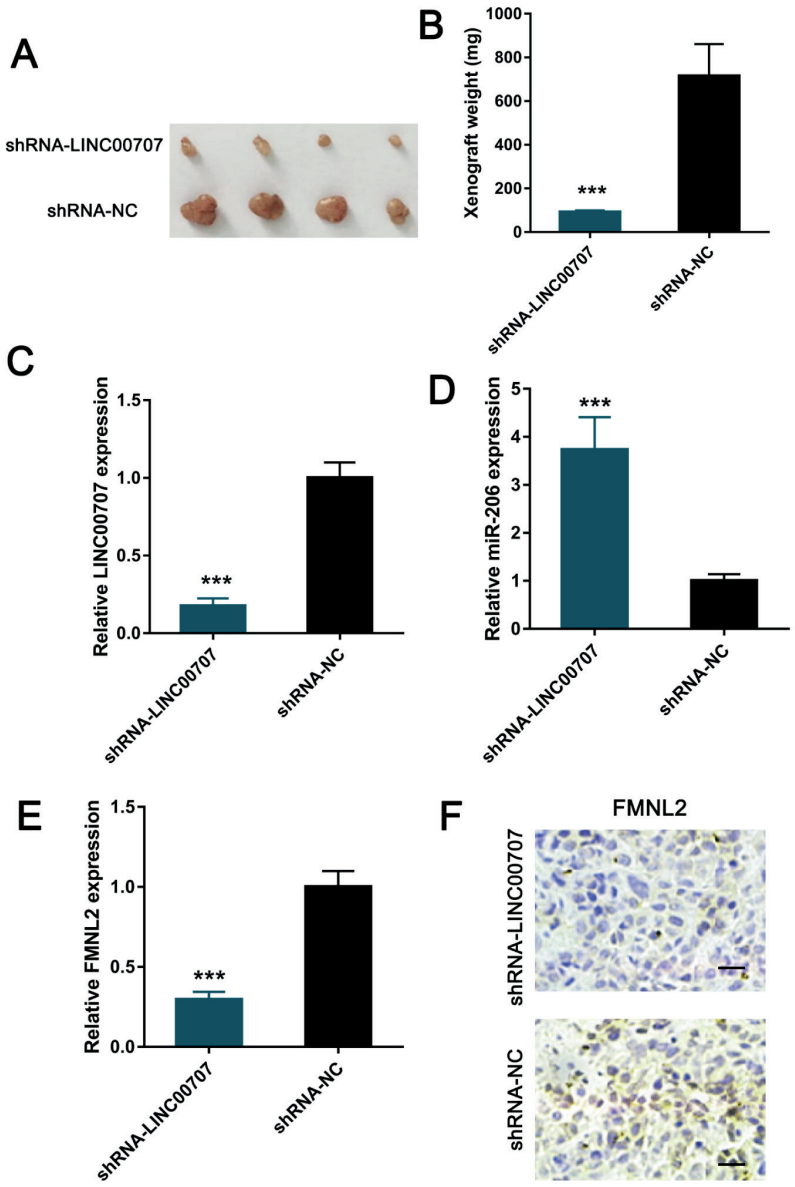

Figure 5. Inhibition of LINC00707 inhibited CRC cell growth *in vivo*. ***A,*** Xenografts of shRNA-LINC00707 or shRNA-NC treated HCT116 cells. ***B,*** Analysis of the weight of xenografts. ***C,*** Expression of LINC00707 in xenografts. ***D,*** Expression of miR-206 in xenografts. ***E,*** Expression of FMNL2 mRNA in xenografts. ***F,*** IHC showed the expression of FMNL2 protein in xenografts. Magnification 400×. Data are represented as the mean ± SD of three replicates. *p<0.05, **p<0.01, ***p<0.001.

indicated that LINC00707 knockdown could inhibit tumor growth of CRC *in vivo via* regulating miR-206/FMNL2 axis.

Discussion

Although many advances have been made in the mechanisms of the development and progression of colorectal cancer (CRC) in the past few decades, and its radical treatments include surgical treatment, chemotherapy, and molecular targeted therapy developed quickly, the outcomes of the 5-year survival remains unoptimistic[13-15]. Many CRC patients have lymph node metastasis or distant metastasis at the time of treatment, which affects the prognosis of patients. In addition, CRC has a high heterogeneity, and the treatment options vary greatly between patients in different tumor stages, so the incidence of drug resistance

in molecular targeted therapy is high[16,17]. Therefore, revealing the molecular mechanism of the occurrence and progression of CRC is important for developing early diagnostic indicators and therapeutic targets

LncRNA is a newly discovered non-coding RNA in recent years. It has attracted a lot of attention since its discovery, and its function has been continuously revealed. LncRNA could affect the biological behavior of tumors by regulating genes and miRNA expressions, and it participates in various processes such as chromosomal remodeling, transcriptional regulation, and RNA degradation[6,7,13]. In addition, due to its tissue specificity, it is significantly more sensitive in disease diagnosis than DNA, protein-encoding RNA, and protein markers. A number of studies[18-23] have reported the function of lncRNAs in CRC, such as lncRNA NEAT1, SNHG5, MAFG-AS1, SLCO4A1-AS1, UCA1. Therefore, further exploration of the function of lncRNA in CRC is not only important for revealing the mechanism of colorectal carcinogenesis and development, but also may provide evidence for finding new diagnostic indicators and therapeutic targets for CRC.

LINC00707 has been reported to promote several cancer progressions. It activates ERK/JNK/AKT pathway to promote progression of hepatocellular carcinoma. In lung adenocarcinoma, it enhances cell proliferation and migration *via* regulating Cdc42 expression. LINC00707 functions as a signature of tumor prognosis in melanoma[24-26]. However, the expression and function of LINC00707 in CRC have not been discovered before.

We found that LINC00707 expression in colorectal normal epithelial cells and adjacent tissues was significantly lower than CRC cells and tissues, suggesting that LINC00707 is also a tumor-promoting gene in CRC. *In vitro* cell experiments further confirmed that high expression of LINC00707 promoted the proliferation and metastasis of CRC cells. On the contrary, interference with the expression of LINC00707 significantly inhibited the growth and metastasis of CRC cells. The above experiments confirmed that LINC00707 could promote the proliferation, cell cycle progression, and metastasis of colorectal cancer cells.

Furthermore, we confirmed miR-206/FMNL2 as direct targets for LINC00707 in CRC. LINC00707 could sponge miR-206 to promote the expression of FMNL2. MiR-206 functioned as an anti-cancer miRNA in several tumors including breast cancer, pancreatic adenocarcinoma, clear-cell renal cell carcinoma, gastric cancer, and ERα-positive endometrioid adenocarcinoma[27-31]. In CRC, miR-206 could play a role as an independent prognostic factor and suppress tumor growth and metastasis *via* targeting TM4SF1, NOTCH3[32,33]. Here, FMNL2 was recognized as an oncogene in CRC. It promotes CRC cell proliferation, cell circle, and progression *via* different pathways including NF-κB pathway and Rho/ROCK pathway[34-38]. We verified LINC00707 inhibited the role of miR-206 and increased FMNL2 level to promote proliferation and metastasis of CRC cells.

In vivo experiment found that knockdown of LINC00707 markedly slowed down the growth of CRC xenograft tumors, which increased the miR-206 level but reduced FMNL2 level. This was similar to the results *in vitro*.

Conclusions

Our study for the first time identified that LINC00707 was over-expressed in CRC tissues and cell lines. It promoted proliferation and metastasis of CRC cells *in vitro* and tumor growth of CRC *in vivo via* sponging miR-206 to up-regulate FMNL2 expression. LINC00707 may serve as a novel target for the further diagnosis and treatment for CRC.

Conflict of interest

The authors declare no conflicts of interest.

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