

# LncRNA LINC00689 Promotes the Progression of Gastric Cancer Through Upregulation of ADAM9 by Sponging miR-526b-3p

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**Introduction:** Increasing studies have demonstrated that noncoding RNAs, including miRNAs and lncRNAs, have vital roles in mediating cancer progression. However, the expression features and biological functions of LINC00689 in gastric cancer (GC) remain largely unknown. This study was designed to investigate the functions of LINC00689, miR-526b-3p and ADAM9 as well as their interactions in GC.

**Methods:** Real time PCR(RT-PCR) was used to detect the expression of LINC00689, miR-526b-3p and ADAM9 in both GC tissues or cell lines. Gain- and loss- of functions of assays were conducted to verify the role of LINC00689, miR-526b-3p and ADAM9 in GC development. Cell proliferation were determined by CCK8 assay and transwell assay and scratch wound-healing assay were used to test cell invasion and migration. Further, the relationships between LINC00689 and miR-526b-3p, miR-526b-3p and ADAM9 were predicted by bioinformatics analysis and then proved by Luciferase reporter assay and RNA Immunoprecipitation(RIP) assay.

**Results:** We found that LINC00689 was upregulated in GC tissues and positively correlated with advanced tumor stage and tumor size, while miR-526b-3p was downregulated. Furthermore, gain- and loss-of-function experiments revealed that LINC00689 promoted the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) of GC cells, while miR-526b-3p had the opposite effects. The underlying mechanisms indicated that LINC00689 functioned as a competing endogenous RNA (ceRNA) by sponging miR-526b-3p in GC cells. Further investigations confirmed that ADAM9 was a direct target of miR-526b-3p and positively modulated the progression of GC.

**Conclusion:** Our study suggests that LINC00689 functions as a novel oncogenic lncRNA in the development of GC by promoting ADAM9 expression through suppression of miR-526b-3p.

**Keywords:** gastric cancer, LINC00689, miR-526b-3p, ADAM9, competing endogenous RNA

## Background

Gastric cancer (GC) remains the fourth most common cancer and third leading cause of cancer-related death worldwide.<sup>1</sup> Despite advances in gastrectomy and chemotherapy, patient long-term survival rates are still unsatisfactory due to recurrence and metastasis.<sup>2</sup> Therefore, it is of great significance to explore the potential biological mechanisms of GC for better therapeutic intervention of the disease.

Long noncoding RNAs (lncRNAs) belong to a class of RNAs that are more than 200 nucleotides in length and do not encode proteins due to a lack of open reading

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frames. Recently, a number of studies have demonstrated the crucial roles of lncRNAs in regulating cancer biological processes such as cell proliferation, invasion, differentiation and apoptosis.<sup>3,4</sup> For instance, LINC00152, one member of lncRNAs, has been found to be upregulated in multiple cancers, including hepatocellular carcinoma,<sup>5</sup> esophageal squamous cell carcinoma<sup>6</sup> and gastric cancer.<sup>7</sup> Moreover, knockdown of LINC00152 exerted significant anticancer effects in these tumors. Additionally, lncRNAs such as HOTAIR, HULC, MALAT2, H19, GHET1, and GACAT3 have been demonstrated to have oncogene activities, while LEIGC, GAS5, and FER1L4 are thought to be tumor suppressors.<sup>8</sup> LINC00689 is a novel lncRNA located on 7q36.3 with a length of 4082 bp. Moreover, LINC00689 has been found to promote the growth, metastasis and glycolysis of glioma cells.<sup>9</sup> However, the role of LINC00689 in GC is much less explored.

MiRNAs are a class of highly conserved small single-stranded noncoding RNAs. Accumulating evidence has revealed that miRNAs are involved in the occurrence and progression of cancer. For example, miR-21 was observed to be overexpressed in oral squamous cell carcinoma (OSCC) compared to controls, and the increased expression of miR-21 was also associated with tumor size, metastasis and local invasion of OSCC.<sup>10</sup> In terms of GC, previous reports have also shown that miRNAs play important roles in multiple tumor-related biological processes, including differentiation, proliferation, metastasis and apoptosis, by altering the expression of oncogenes.<sup>11</sup> miR-526b-3p expression was significantly downregulated in glioma and was involved in regulating the proliferation, invasion, and migration of glioma cells.<sup>12</sup> Nevertheless, the function of miR-526b-3p in GC remains to be studied.

A disintegrin and metalloproteinase domain (ADAM) belongs to a class of cell surface proteins with a unique structure possessing both potential adhesion and protease domains.<sup>13</sup> As a kind of metalloendopeptidase, the members of this protein family cleave many proteins including occluding, tight junction protein 1 and E-cadherin, thus inducing decreased cell connectivity.<sup>14,15</sup> More importantly, dysregulated ADAMs such as ADAM10<sup>16</sup> and ADAM12<sup>17</sup> play a role in gastric cancer. ADAM9 is another member of ADAMs family and also involved in several biological processes such as myogenesis, fertilization, cell migration, inflammatory response, proliferation, and cell-cell interactions.<sup>18</sup>

Recently, a new regulatory mechanism has been identified between ncRNAs and the targeted genes, namely, the lncRNA-miRNA-mRNA network. By this mechanism,

lncRNAs function as competing endogenous RNAs (ceRNAs) to sponge miRNAs, thereby modulating the derepression of miRNA targets and imposing an additional level of posttranscriptional regulation.<sup>19</sup> In the present research, we first attempted to explore the expression, clinical importance, functions and potential mechanisms of LINC00689 in GC. Our results demonstrated that LINC00689 functions as a ceRNA to regulate the expression of ADAM metalloproteinase domain 9 (ADAM9) through competition for miR-526b-3p, thus playing an oncogenic role in gastric pathogenesis. Overall, this study aims to provide new insight into the diagnosis and treatment of gastric cancer.

## Methods

### Clinical Samples

Forty-one matched GC tissues and adjacent noncancer tissues were obtained from Beijing Shijitan Hospital and diagnosed as gastric cancer by professional pathologists. None of the cases underwent any chemical or radiotherapy prior to the excision of the cancer. The study was reviewed and approved by the Research Ethics Board of Beijing Shijitan Hospital, Capital Medical University. All individuals were formally informed for the purpose of using the samples, and all patients signed consent forms.

### Cell Line Culture

The human gastric carcinoma cell lines MGC-803, MKN45, SUN-5, SGC-790, and AGS and the immortalized gastric epithelial cell line GES-1 were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) in 5% CO<sub>2</sub> at 37 °C.

### Cell Transfection

Negative control (NC), miR-526b-3p mimics, miR-526b-3p inhibitor, sh-NC, sh-LINC00689, si-ADAM9, pcDNA3.1 plasmid and pcDNA3.1-LINC00689 were all obtained from Genepharma (Shanghai, China). The cell suspension was prepared by 0.25% pancreatin digested AGS or MGC-803 cells and complete medium. Then, the cells were incubated in six-well plates (1×10<sup>6</sup> cells/well) for 18–24 h. Three hours before transfection, GC cells at 80–90% confluence were incubated with fresh medium without serum or antibiotics. Transfection was performed

using Lipofectamine 2000 (~ 0.6 µg Lipofectamine reagent/1 µg DNA, Life Technologies).

### CCK8 Assay

Transfected AGS and MGC-803 cells in the logarithmic growth phase were seeded in a 96-well plate ( $1 \times 10^3$  cells/well) to incubate for 1 d, 2 d, 3 d and 4 d in an incubator. Then, 10 µL of CCK8 reagent (Beyotime Biotechnology, Shanghai, China) was added into each well, and the incubation was continued for 1 hr in an incubator. Then, the absorbance of each well was measured at a wavelength of 450 nm with a microplate reader. Each experiment was performed in triplicate, and each measurement was also performed in triplicate.

### Transwell Assay

To investigate cell invasion ability, cells ( $5 \times 10^4$  cells/well) were seeded onto the upper layer of plates with an 8 µm pore membrane in serum-free DMEM. The upper chamber was precoated with BD Matrigel Matrix (BD Biosciences). DMEM supplemented with 20% FBS was added into the lower chambers. After incubation at 37°C for 24 h, cells on the upper surface were removed, and the invasive cells on the lower surface were stained with 0.5% crystal violet (Beyotime, China) for 30 min at room temperature. Cells in five randomly chosen fields were counted under a microscope (Olympus Optical, Tokyo, Japan).

### Scratch Wound-Healing Assay

The migration abilities of GC cells were measured by a scratch wound-healing assay. Stably transfected GC cells ( $1 \times 10^6$ ) were plated onto 6-well plates and scraped with a pipette tip to generate uniform wounds after the cells were confluent. Each well was washed three times with PBS to remove floating cells and then cultured in medium with low serum. The initial distance (0 h) and the distances traveled by cells 24 h after scratching were detected microscopically at a magnification of 200× for each group.

### Quantitative Real-Time PCR (RT-PCR)

Total RNA was isolated by TRIzol reagent (Invitrogen), after which RNA concentration was measured using a Nanodrop 2000 (Thermo, Waltham, MA, USA). For mRNA and lncRNA detection, RNA was reverse transcribed into cDNA using the ReverTra Ace qPCR RT Kit (Toyobo, Japan). The TaqMan MicroRNA Reverse Transcription kit (ABI, Foster City, CA, USA) was used for miRNA reverse transcription. qRT-PCR was conducted using SYBR Premix Ex Taq II

(Takara). GAPDH was used as an endogenous control for lncRNAs and mRNAs, and U6 was used for miRNAs. The primers are as follows. LINC00689 (Forward: 5'-GCTCACTGCAACCTCTGTCT-3'; Reverse: 5'-CACCAGGAACATCCAGGACC-3'); ADAM9 (Forward: 5'-AATGATGGAAGAGGCGGAGG-3'; Reverse: 5'-TGAGGTCTGTTGAAAGCCTGG-3'); GAPDH (Forward: 5'-GGGTCTTATGACCACTGTCC-3'; Reverse: 5'-GTAAGCTTCCCATTGAGCTCAG-3'); miR-526b-3p (Forward: 5'-GCGCTCTTGAGGGAGCACT-3'; Reverse: 5'-TACGTTCCATAGTCTACCA-3'); U6 (Forward: 5'-GCTTCGGCAGCACATATACTAAAAT-3'; Reverse: 5'-CGCTTCACGAATTTGCGTGCAT-3'). The reaction process for PCR was initial denaturation (94°C, 2 min), 30 cycles of denaturation (94°C, 30 s), primer annealing (54°C, 30 s), and primer extension (72°C, 1 min), and final extension (72°C, 10 min). The expression levels of lncRNAs, mRNAs and miRNAs were calculated by the  $2^{-\Delta\Delta CT}$  method. Each experiment was performed in triplicate, and each measurement was also performed in triplicate.

### Western Blot Assay

Cells and tissues were lysed with RIPA buffer (Beyotime, Shanghai, China), and protein concentration was quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by SDS-PAGE and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The primary antibodies used in this study included anti-E-cadherin (1:1000; ab40772, Abcam, Cambridge, MA, USA), anti-Vimentin (1:1000; ab92547, Abcam, Cambridge, MA, USA), anti-ADAM9 (1:1000; ab186833, Abcam, Cambridge, MA, USA), and anti-GAPDH (1:2000; Abcam) as the control. The membranes were incubated at 4°C overnight, then incubated with HRP-conjugated secondary antibody and visualized using ECL detection reagent (Millipore).

### Luciferase Reporter Assay

The fragment from LINC00689 containing the predicted miR-526b-3p binding site was amplified by PCR and cloned into a pmir-GLO Expression Vector (Promega, Madison, WI, USA) to form the LINC00689-wild-type reporter vector (LINC00689-WT) containing a luciferase reporter gene. The corresponding mutant was created by mutating the miR-526b-3p seed sequence binding site, which was named LINC00689-MT. MGC-803 cells were cotransfected with the wild-type fragments and miR-526b-3p mimic using Lipofectamine 2000 (Invitrogen). To test the relationship between miR-526b-3p and ADAM9, the 3'-UTR of ADAM9 containing the binding site of miR-526b-3p was

cloned into the pGL3 vector (Promega). The cells were transfected with miR-526b-3p mimics or mimics-ctrl and ADAM9-wild-type (ADAM9-WT) or ADAM9-mutant type (ADAM9-MT) plasmids. A luciferase reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega).

## RNA Immunoprecipitation (RIP)

The binding relationship between LINC00689 and miR-526b-3p was identified by RIP assay through a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) according to the manufacturer's protocol. MGC-803 cells at the stage of 80% confluence were collected and lysed using RIP lysis buffer. Then, Ago2 immunoprecipitation was performed using an anti-Ago2 antibody (Abcam, Cambridge, UK). The immunoglobulin G (IgG) antibody was used as a negative control. After that, the immunoprecipitated RNA was isolated, and the abundance of LINC00689 and miR-526B-3p in bound fractions was determined by RT-PCR assay.

## Statistical Analysis

All data are quantified as the mean  $\pm$  SD. All statistical analyses were performed with SPSS 17.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) or two-tailed Student's *t*-test was applied to determine the group differences. The association between LINC00689 and clinicopathological parameters was analyzed by Pearson's chi-square test. Differences were considered significant at  $P < 0.05$ .

## Results

### LINC00689 Was Upregulated in GC Tissues and Cell Lines

To investigate the expression characteristics of LINC00689 in GC, we conducted RT-PCR assays to detect LINC00689 expression in 41 cases of GC tissues compared to adjacent noncancer tissues. The results showed that LINC00689 was significantly upregulated in GC tissues ( $p < 0.05$ , Figure 1 A). Furthermore, the analysis of the association between LINC00689 expression and the clinical features of GC patients indicated that upregulation of LINC00689 was positively correlated with an increase in TNM stage and tumor size ( $p < 0.05$ , Figure 1B, Table 1). Besides, we analyzed the correlation between LINC00689 expression and survival of GC patients through KM-plotter (<http://kmplot.com/analysis/index.php?p=service>).

The results showed that GC patients with higher level of LINC00689 have poorer overall survival and post progression survival ( $p < 0.05$ , Figure 1C and D). Subsequently, LINC00689 expression in different cell lines (MGC-803, MKN45, SUN-5, SGC-790, and AGS) was examined, and the results demonstrated that LINC00689 expression was also distinctively elevated compared with that in the normal gastric epithelium cell line GES-1 (Figure 1E). Therefore, we speculated that LINC00689 may act as a prognostic factor of poor GC prognosis and promote GC progression.

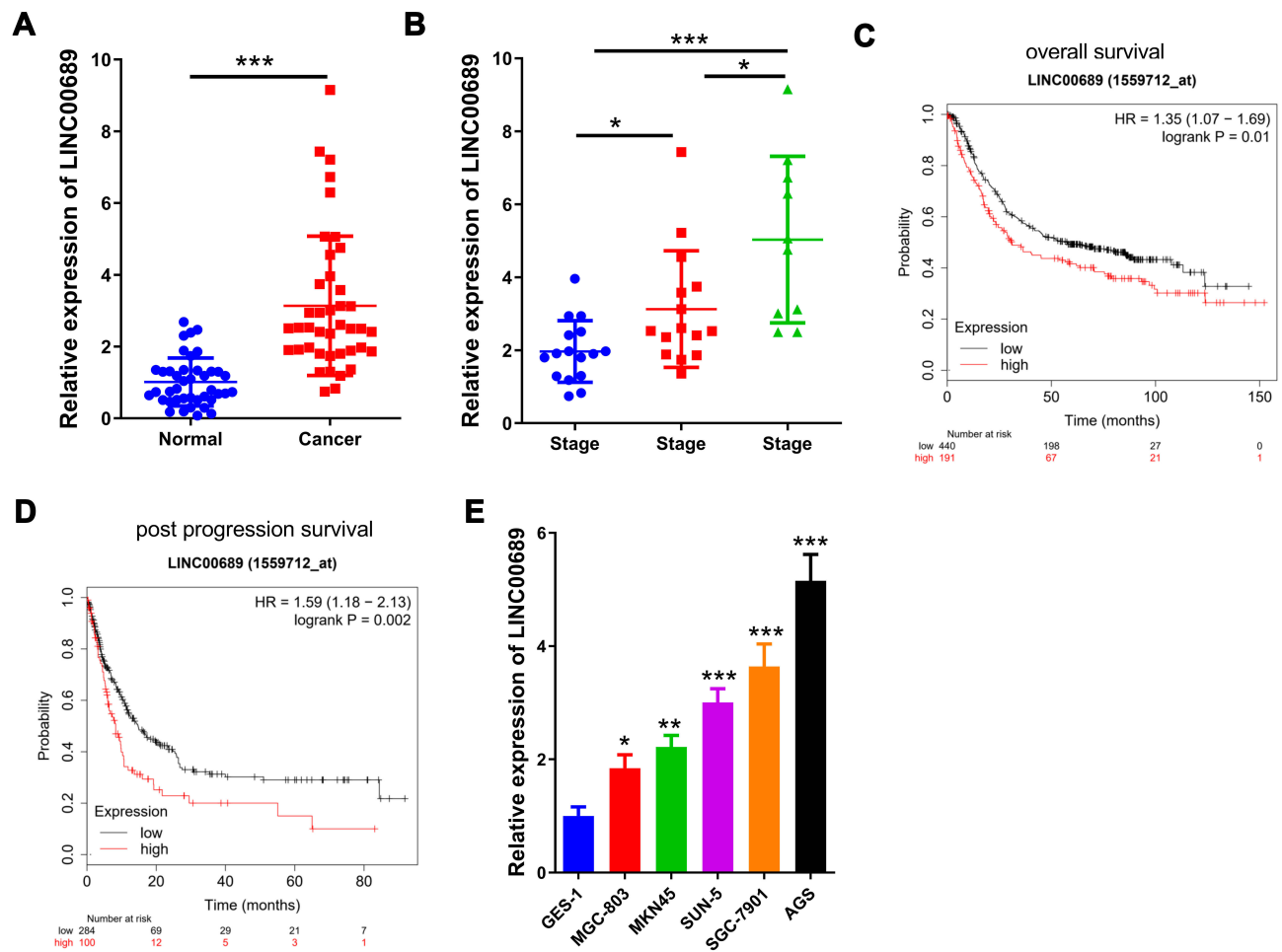
### LINC00689 Promoted the Proliferation, Migration and Invasion of GC Cells

To observe the biofunctions of LINC00689 in GC cells, we transfected the MGC-803 and AGS cell lines with functional pcDNA/LINC00689 and LINC00689-specific shRNA, respectively (Figure 2A and B). Next, CCK8 assay, Transwell assay and scratch wound-healing assay were carried out to determine the proliferation, migration and invasion of GC cells. The results showed that overexpression of LINC00689 markedly increased the proliferation, migration and invasion abilities of GC cells, while knocking down LINC00689 had the opposite effects (Figure 2C–F). It is known that, epithelial mesenchymal transition (EMT), the adoption of a mesenchymal-like phenotype by epithelial cells, is a process coopted by carcinoma cells to initiate invasion and metastasis.<sup>20</sup> Recent evidence has established that aberrant EMT activation in the human stomach is closely associated with gastric carcinogenesis and tumor progression.<sup>21</sup> Here, to further explore the mechanism of LINC00689 in affecting GC metastasis, we detected the protein levels of the epithelial marker E-cadherin and the mesenchymal marker Vimentin by Western blot. We found that LINC00689 overexpression significantly reduced the expression of E-cadherin but downregulated Vimentin expression. In contrast, inhibition of LINC00689 showed the opposite effects (Figure 2G). These results suggested that LINC00689 promoted GC progression.

### LINC00689 Targeted miR-526b-3p in GC Cells

To further explore the underlying mechanism of LINC00689 in GC, we predicted potential downstream targets of LINC00689 via StarBase (<http://starbase.sysu.edu.cn/>) and found that LINC00689 has a putative binding site for the seed sequence of miR-526b-3p (Figure 3A). Next,





**Figure 1** Expression of LINC00689 in GC tissues and cell lines. (A) The level of LINC00689 in human GC tissues and matched adjacent normal tissues was detected by RT-PCR (n = 41). \*\*\*p<0.001 vs the normal group. (B) The expression of LINC00689 was increased in patients with advanced TNM stage. (n= 16 in stage I, n=15 in stage II, n=10 in stage III). \*represents p<0.05, \*\*\*represents p<0.001. (C and D) The correlation between LINC00689 expression and overall survival (C) or post progression survival (D) of GC patients through KM-plotter (<http://kmplot.com/analysis/index.php?p=service>). (E) The expression of LINC00689 was elevated in GC cells compared with the immortalized gastric epithelium cell line GES-1. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 vs the GES-1 group.

a luciferase reporter assay and RIP assay were used to verify the targeted relationship between miR-526b-3p and LINC00689. The results showed that miR-526b-3p decreased the luciferase activity of the LINC00689 wild-type reporter construct (LINC00689-WT) but not the mutant type construct (LINC00689-MUT) (Figure 3B). Besides, the enrichment of LINC00689 and miR-526b-3p in Ago2-immunoprecipitated complex were both increased in comparison with the control immunoglobulin G (IgG)-immunoprecipitated complex (p < 0.05, Figure 3C and D), indicating that both of LINC00689 and miR-526b-3p could bind to Ago2. Moreover, the relative expression of miR-526b-3p in GC tissues and cells was tested by RT-PCR, which revealed that the expression of miR-526b-3p was downregulated (vs adjacent noncancer tissues) and negatively correlated with LINC00689 expression in GC tissues, and it was also

downregulated in GC cell lines (vs the normal cell line GES-1) (Figure 3E–G). Furthermore, LINC00689 overexpression inhibited miR-526b-3p expression, while LINC00689 knock-down increased miR-526b-3p expression (Figure 3H). Collectively, those results revealed that LINC00689 targeted miR-526b-3p in GC cells.

### miR-526b-3p Inhibited the Proliferation, Invasion and Migration of GC Cells

To explore the biological function of miR-526b-3p in GC cells, we detected the proliferation, invasion and migration capacities as well as EMT-related marker expression of GC cells after miR-526b-3p overexpression or inhibition (Figure 4A). The results showed that the proliferation, invasion and migration capacities of GC cells were inhibited by miR-526b-3p overexpression, while miR-526b-3p

**Table 1** Correlations Between the Expression of LINC00689 and Clinicopathologic Features

Clinicopathologic Features		Cases (n)	LINC00689 Expression Level		P value
			High (n=21)	Low (n=20)	
Age (years)			57.45±5.31	56.33±4.9	0.4875
Sex	Male	24	13	11	0.6537
	Female	17	8	9	
Tumor size	>5 cm	18	13	5	0.0173
	≤5 cm	23	8	15	
Differentiation	Poor	19	11	8	0.4268
	High/	22	10	12	
	Moderate				
TNM stage	I	16	4	12	0.0266
	II	15	10	5	
	III	10	7	3	
Lymph node metastasis	Yes	17	11	6	0.1459
	No	24	10	14	

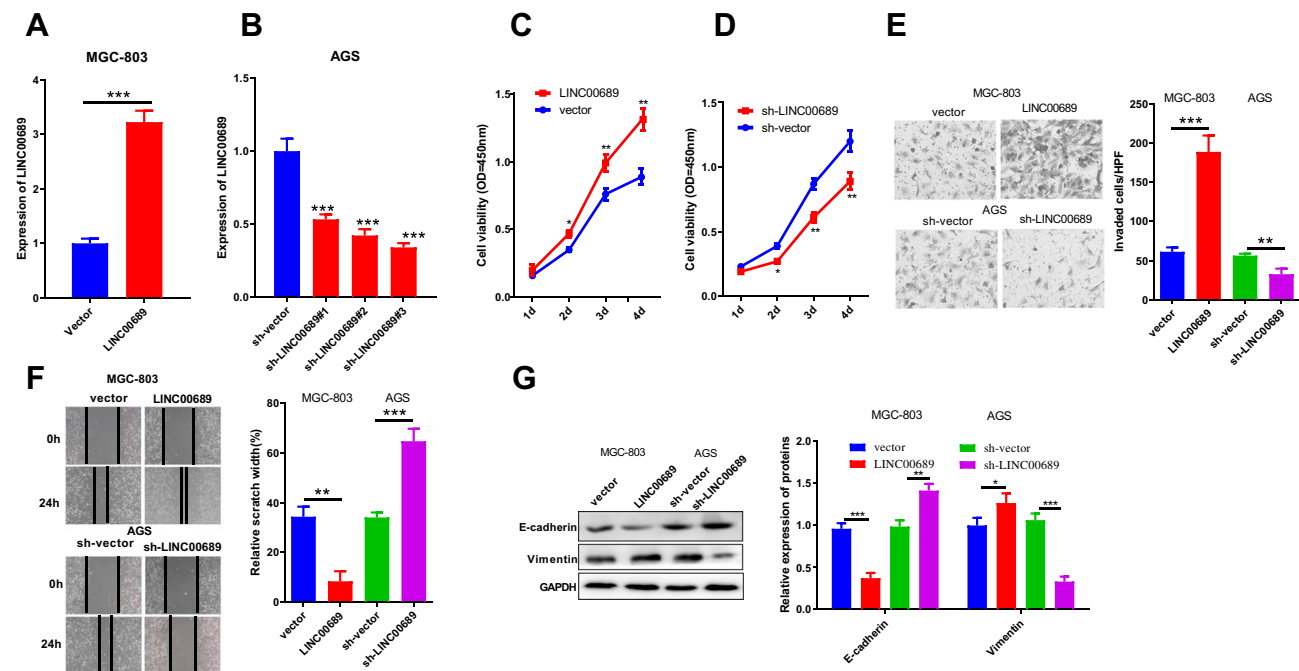
**Notes:** P value represents the significant difference value of Student's t-test or Pearson chi-square test.

knockdown promoted the proliferation, invasion and migration capacities of GC cells (Figure 4B–E). In addition, the Western blot results showed that miR-526b-3p

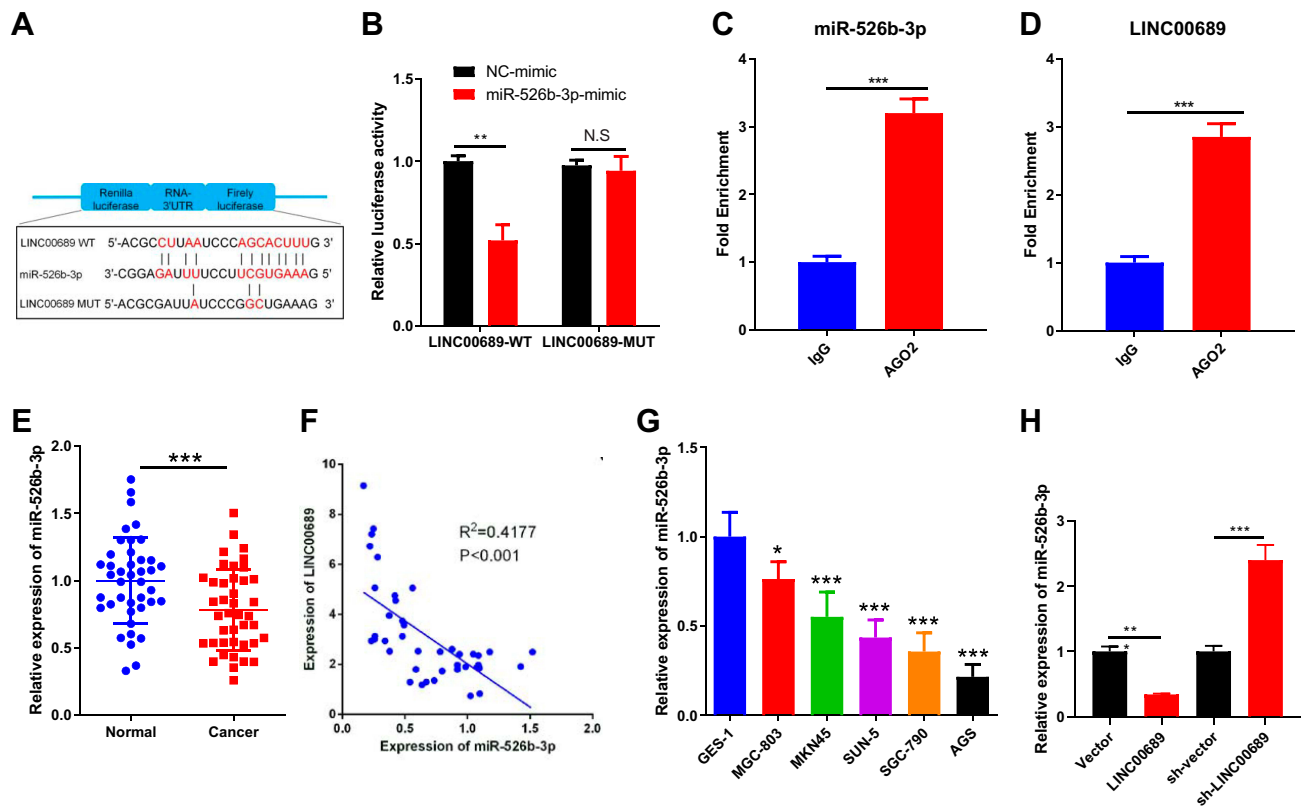
overexpression led to significant upregulation of E-cadherin expression and downregulation of Vimentin expression. Conversely, inhibition of miR-526b-3p showed the opposite effects (Figure 4F). Those results demonstrated that miR-526b-3p exerted an inhibitory role in GC development.

## ADAM9 Is a Direct Target of miR-526b-3p in GC and Promotes GC Progression

Numerous studies have confirmed that microRNAs can target the 3'UTR of the mRNA to induce posttranscriptional regulation of gene expression. To further explore the downstream target of miR-526b-3p, we conducted bioinformatics analysis through biological database including miRmap (<http://mirna.map.mbc.nctu.edu.tw/>), PicTar (<https://pictar.mdc-berlin.de/>) and Targetscan ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)). The results showed that ADAM9 was a promising target of miR-526b-3p (Figure 5A) and the targeting sites were shown in Figure 5B. Next, the luciferase reporter assay confirmed the interaction between miR-526b-3p and ADAM9 (Figure 5C). Additionally, it was found that the expression of ADAM9 in human GC tissues was significantly increased compared with that in the matched adjacent normal tissues (Figure 5D and E), which was consistent with the ADAM9 expression in GC tissues (data from TCGA database) (Figure 5F). Further



**Figure 2** LINC00689 promoted the proliferation, migration and invasion of GC cells in vitro. MGC-803 and AGS cells were transfected with LINC00689-overexpressing plasmid, sh-LINC00689 or corresponding negative controls. (A and B). RT-PCR was used to detect the expression of LINC00689; (C and D). CCK8 assay was used to detect the proliferation of GC cells. (E). Cell invasion was detected by Transwell assay. (F). Scratch wound-healing assay was used to determine cell migration. (G). Western blot was used to detect the expression of the EMT-related markers E-cadherin and Vimentin. n=three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

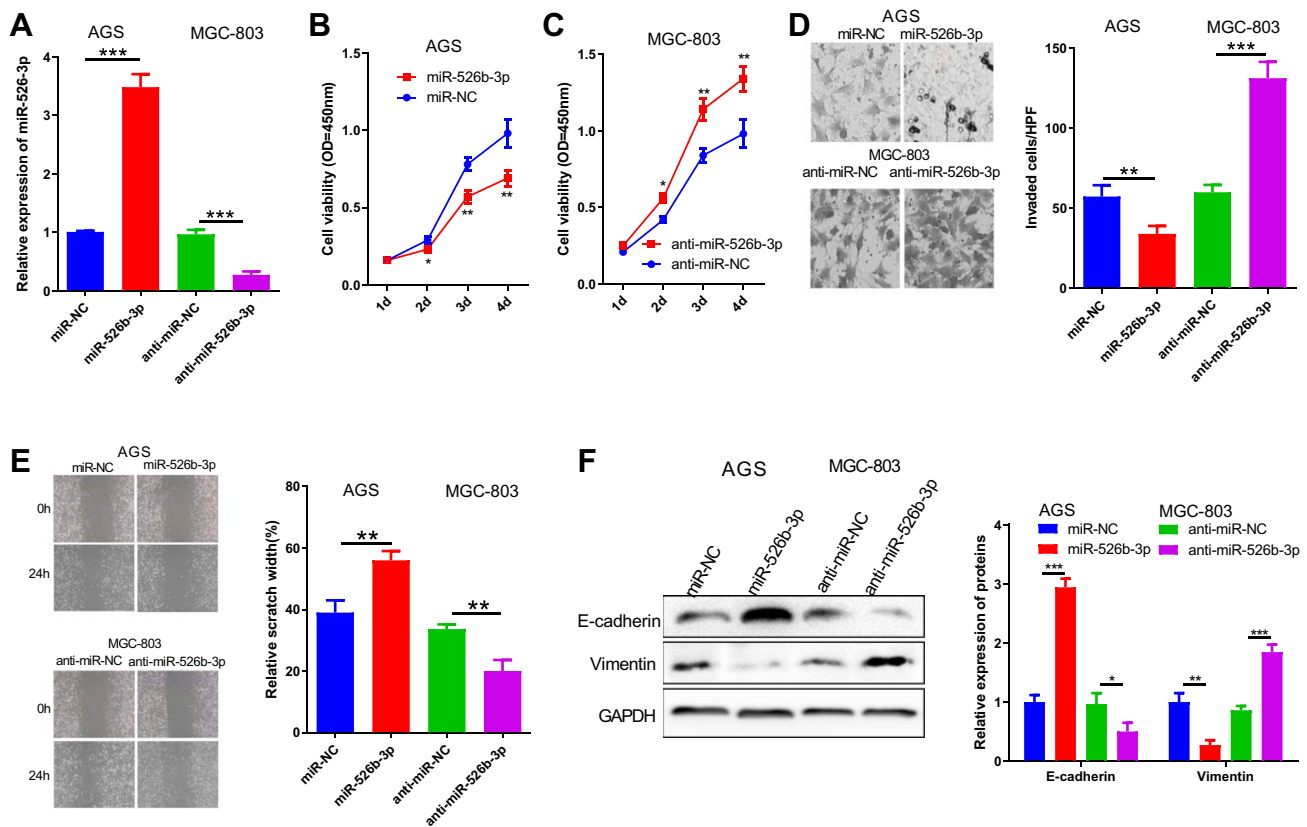


**Figure 3** miR-526b-3p is a target of LINC00689 in GC. (A). The base binding sites of miR-526b-3p and LINC00689 were predicted by StarBase (<http://starbase.sysu.edu.cn/>). (B). Dual luciferase reporter assay was conducted to verify the targeted relationship between miR-526b-3p and LINC00689, which showed that miR-526b-3p decreased the luciferase activity of the LINC00689 wild-type reporter construct (LINC00689-WT) but not the mutant type construct (LINC00689-MUT), N.S (no significance) represents  $p > 0.05$ , \*\*represents  $p < 0.01$ . (C and D). RIP assay was conducted to detect the binding between LINC00689 and Ago2 or miR-526b-3p and Ago2. (E). The expression level of miR-526b-3p was detected in GC tissues and matched adjacent normal tissues by RT-PCR ( $n=41$ ). (F). Pearson's regression analysis was used to analyze the relationship between miR-526b-3p and LINC00689. (G). Relative expression of miR-526b-3p in the GC cell lines and immortalized gastric epithelium cell line GES-1, \* $p < 0.05$ , \*\*\* $p < 0.001$  vs the GES-1 group. (H). miR-526b-3p expression was increased by inhibition of LINC00689 but inhibited upon overexpression of LINC00689, \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ .

analysis showed that ADAM9 expression was positively correlated with LINC00689 expression and negatively correlated with miR-526b-3p expression in GC tissues (Figure 5G and H). Furthermore, experiments intervening with the expression of miR-526b-3p and LINC00689 revealed that co-transfection with miR-526b-3p mimics or knockdown of LINC00689 inhibited the expression of ADAM9, which was then upregulated by inhibition of miR-526b-3p or overexpression of LINC00689 (Figure 5I and J). Furthermore, the down-regulation of ADAM9 by si-ADAM9 transfection significantly reduced the proliferation, invasion and migration capacities of GC cells (Figure 6A–E). After knocking down ADAM9, the epithelial marker E-cadherin was upregulated, and the mesenchymal marker Vimentin was downregulated. (Figure 6F). Altogether, those data indicated that ADAM9 is a direct target of miR-526b-3p in GC and promotes GC progression.

## LINC00689 Increased ADAM9 Expression by Sponging miR-526b-3p

To further investigate the LINC00689/miR-526b-3p/ADAM9 interactions in GC, we transfected miR-526b-3p mimics into LINC00689-overexpressing MGC-803 cells. RT-PCR results revealed that the transfection of miR-526b-3p mimics increased the expression of miR-526b-3p, which was partially reversed by the overexpression of LINC00689 (Figure 7A and B). In addition, we demonstrated that miR-526b-3p mimics inhibited ADAM9 expression, which was reversed by upregulation of LINC00689 (Figure 7C). In terms of biofunction changes, it was similarly found that LINC00689 dampened the inhibitory effects of miR-526b-3p on the proliferation, invasion, migration and EMT of GC cells (Figure 7D–G). Thus, we suggested that LINC00689 promoted the progression of gastric cancer through upregulation of ADAM9 by sponging miR-526b-3p (Figure 7H).



**Figure 4** miR-526b-3p inhibited the proliferation, migration, invasion and EMT process in GC cells in vitro. AGS and MGC-803 cells were transfected with miR-526b-3p mimics or inhibitors and the corresponding miRNA negative controls. (A). The expression of miR-526b-3p was detected by RT-PCR. (B and C). CCK8 assay was used to detect the proliferation of GC cells. (D). Cell invasion was detected by Transwell assay. (E). Scratch wound-healing assay was used to determine cell migration. (F). Western blot was used to detect the expression of the EMT-related markers E-cadherin and Vimentin. n=three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

## Discussion

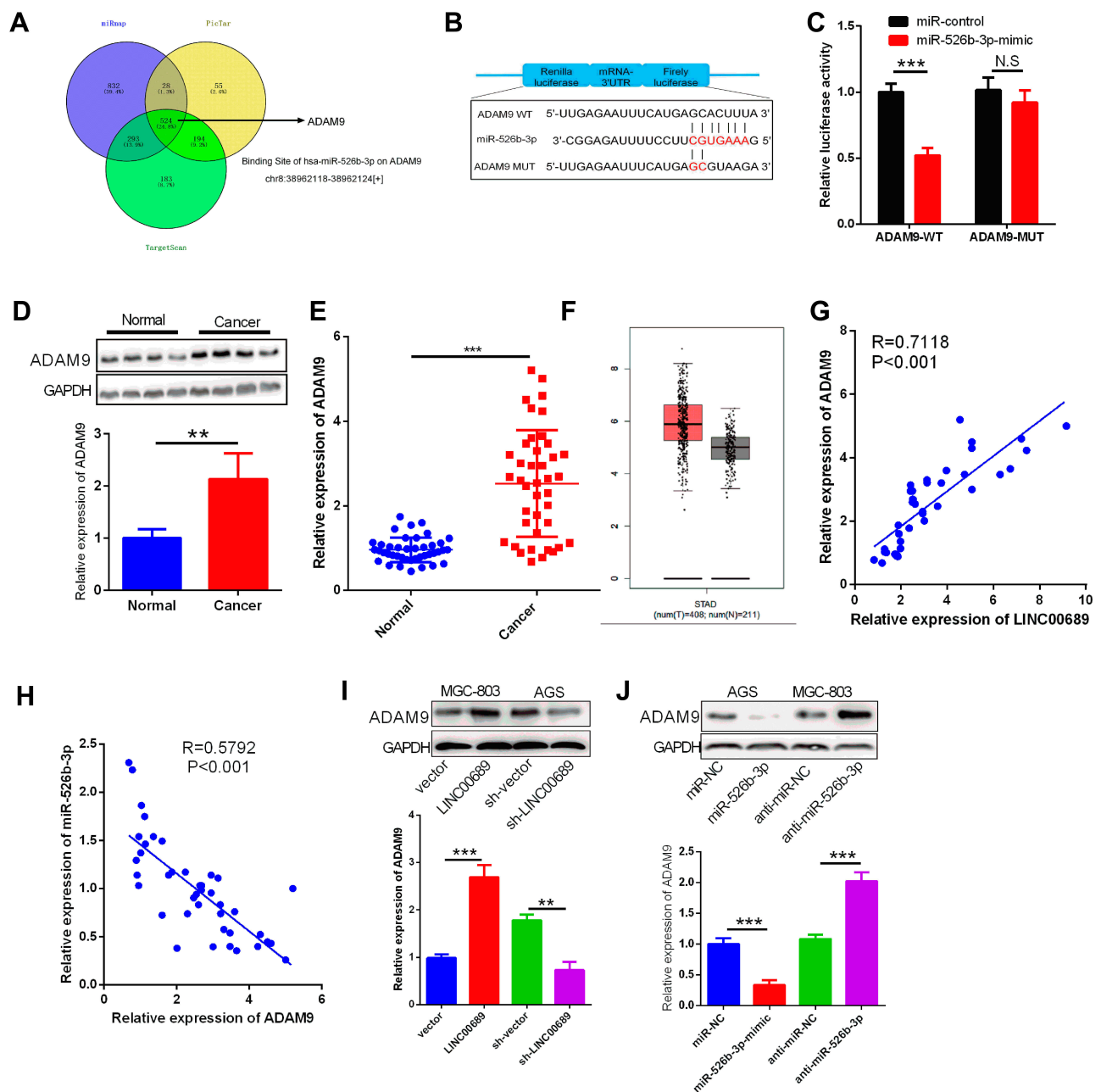
Here, our study showed that LINC00689 was upregulated in gastric cancer tissues and cell lines, and further exploration of the biological function of LINC00689 revealed that it promoted the progression of GC by targeting the miR-526b-3p/ADAM9 axis. These results indicate that LINC00689 serves as an oncogene in GC progression.

Increasing studies have revealed that a growing number of lncRNAs are aberrantly expressed in GC and play critical roles in the progression of GC by acting as either tumor suppressors or oncogenes. For instance, GNAT1-1 was downregulated in gastric cancer patients infected with *H. pylori*, and its over-expression inhibited gastric cancer growth through the Wnt/ $\beta$ -catenin signaling pathway.<sup>22</sup> MDC1-AS inhibited gastric tumorigenesis through an MDC1-dependent mechanism.<sup>23</sup> On the other hand, HOTAIR was found to be highly expressed in GC and associated with poor overall survival in GC patients, suggesting that HOTAIR might be a potentially useful independent prognostic biomarker for GC.<sup>24</sup> LINC00689 is a lncRNA located at chromosome 7q36.3. Studies have

found that it is not only an obesity susceptibility gene in Northern Han Chinese<sup>25</sup> but is also involved in the regulation of mRNA translation via posttranslational protein modification.<sup>26</sup> Notably, a recent study found that LINC00689 promoted the progression of glioma by targeting the miR-338-3p/PKM2 axis.<sup>9</sup> Similarly, our results also suggested that LINC00689 plays a carcinogenic role in GC.

As a series of small noncoding RNAs 18–25 nucleotides in length, microRNAs are found to be involved in many biological processes (e.g., cell proliferation, differentiation, apoptosis, migration and invasion) through gene repression.<sup>27</sup> Emerging roles of microRNAs with oncogenic or tumor suppressive properties in gastric tumorigenesis are discovered. For example, miR-93 expression was significantly upregulated in gastric cancer tissues and was also associated with lymph node metastasis and TNM stage. Additionally, both in vitro and in vivo studies showed that miR-93 functionally promoted the development of GC.<sup>28</sup> Likewise, miR-532 promoted gastric cancer migration and invasion by targeting NKD1.<sup>29</sup> In contrast to those effects, Yang et al demonstrated that miR-125a

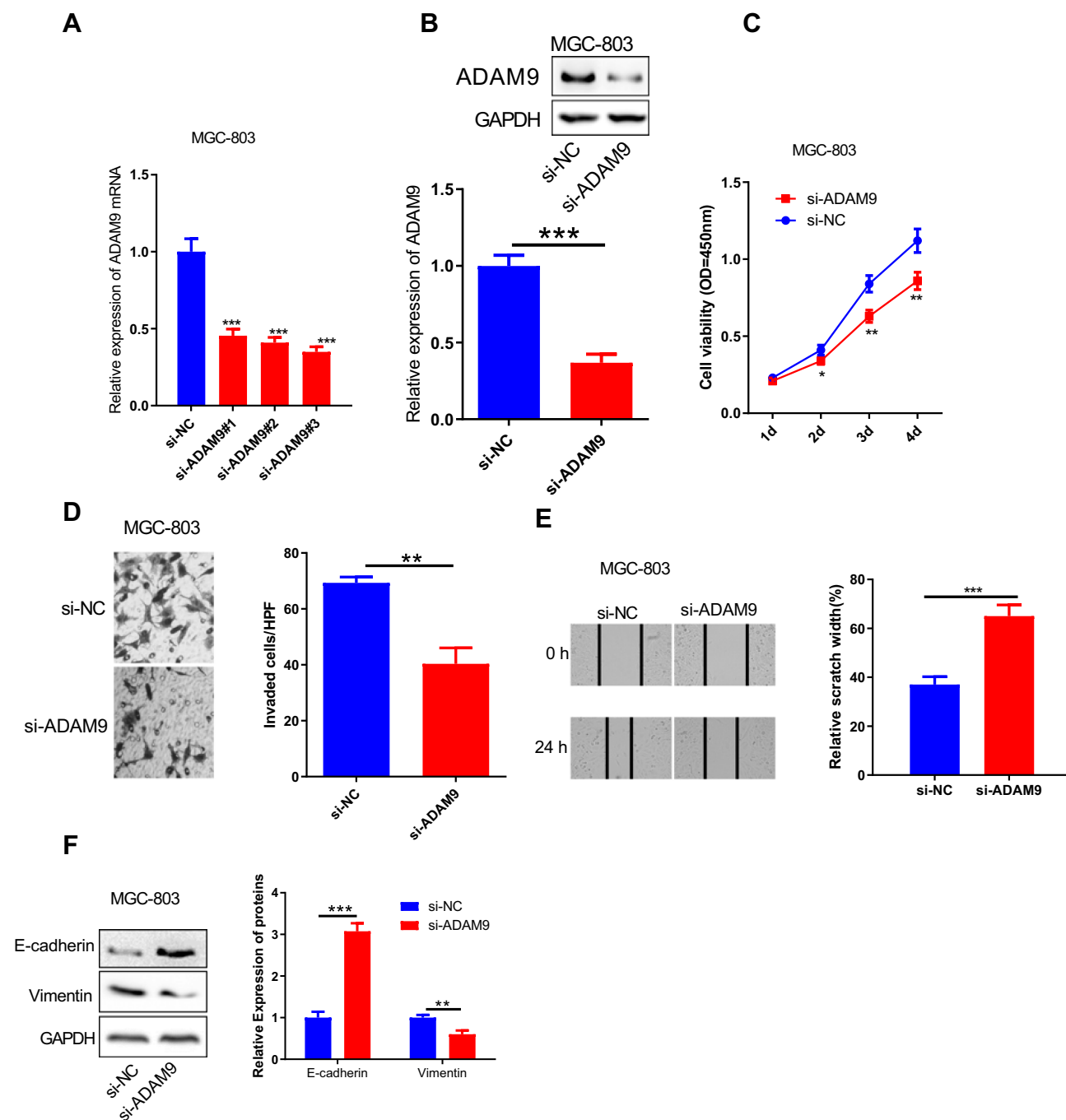




**Figure 5** ADAM9 is a direct target of miR-526b-3p in GC cells. (A). ADAM9 was a potential target of miR-526b-3p as predicted by miRmap (<http://mirnamap.mbc.nctu.edu.tw/>), PicTar(<https://pictar.mdc-berlin.de/>) and Targetscan ([http://www.targetscan.org/ver\\_72/](http://www.targetscan.org/ver_72/)). (B). miR-526b-3p and its putative binding sequences in the 3'-UTR of ADAM9. (C). The relative luciferase activity in cells cotransfected with miR-526b-3p mimics and ADAM9-WT was decreased, while that in cells cotransfected with miR-526b-3p mimics and ADAM9-MT was not significantly changed. N.S (no significance) represents  $p > 0.05$ , \*\*\* represents  $p < 0.001$ . (D). Expression of ADAM9 in human GC tissues and matched adjacent normal tissues by Western blot (n = 4). (E). The expression level of ADAM9 was detected in GC tissues and matched adjacent normal tissues by RT-PCR (n=41). (F). Data of ADAM9 expression in Stomach adenocarcinoma was downloaded from GEPIA (<http://gepia.cancer-pku.cn/>). (G and H). Pearson's regression analysis was used to analyze the relationship between ADAM9 and LINC00689 (G) or ADAM9 and miR-526b-3p (H). (I). LINC00689 overexpression increased the expression of ADAM9, and LINC00689 knockdown reduced the level of ADAM9. (J). miR-526b-3p overexpression reduced the expression of ADAM9, while miR-526b-3p knockdown had the opposite effect. N.S, \*\*, and \*\*\*represents  $p > 0.05$ ,  $p < 0.01$  and  $p < 0.001$ , respectively.

inhibited the migration and invasion of gastric cancer cells by targeting STAT3.<sup>30</sup> miR-526b-3p is a vital miRNA, and studies have corroborated that it functions as a tumor suppressor in multiple cancers, including colon cancer<sup>31</sup> and glioma.<sup>12</sup> Here, our study first found that miR-526b-

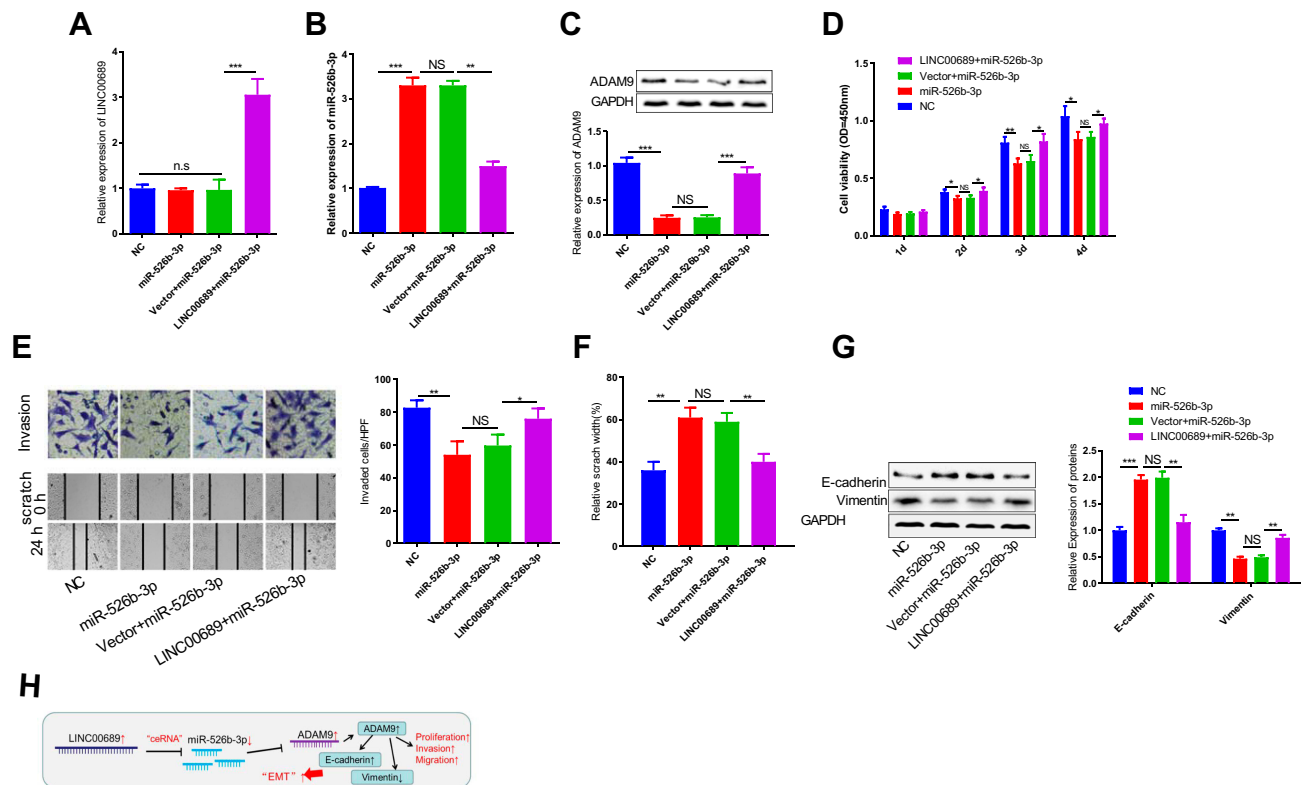
3p was downregulated in both GC tissues and cell lines. Moreover, the gain- and loss-of-function experiments confirmed that it played an anticancer role in GC, as it inhibited the proliferation, invasion, migration and EMT of GC cells.



**Figure 6** ADAM9 promotes the proliferation, migration and invasion of GC cells. **(A and B)**. RT-PCR and Western blot were used to detect the expression of ADAM9 in MGC-803 cells that were transfected with si-ADAM9 or corresponding siRNA negative vector. **(C)**. CCK8 assay was used to detect the proliferation of GC cells. **(D)**. Cell invasion was detected by Transwell assay. **(E)**. Scratch wound-healing assay was used to determine cell migration. **(F)**. Western blot was used to detect the expression of the EMT-related markers E-cadherin and Vimentin. n=three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Recently, lncRNAs have been found to act as molecular sponges to regulate miRNAs and biological functions of gastric cancer. For instance, HOTAIR functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in gastric cancer,<sup>32</sup> and Lei et al demonstrated that lnc-ATB promotes tumor progression by interacting with miR-

141-3p.<sup>33</sup> Therefore, we hypothesize that LINC00689 also served as a miRNA sponge in GC. Interestingly, our results showed that LINC00689 contained a binding site for miR-526b-3p. Inhibition of LINC00689 increased the expression of miR-526b-3p, while overexpression of LINC00689 had the opposite effect. Moreover, overexpression of LINC00689



**Figure 7** LINC00689 increased ADAM9 expression by sponging miR-526b-3p. MGC-803 cells were transfected with miR-526b-3p mimics or LINC00689 overexpression plasmid. (A and B). Expression levels of LINC00689 and miR-526b-3p were detected by RT-PCR. (C). Western blot was conducted to detect the expression of ADAM9. (D). CCK8 assay was used to detect the proliferation of GC cells. (E). Cell invasion was detected by Transwell assay. (F). Scratch wound-healing assay was used to determine cell migration. (G). Western blot was used to detect the expression of the EMT-related markers E-cadherin and Vimentin. (H). A diagram of the mechanisms of LINC00689, miR-526b-3p and ADAM9 networks in regulating the progression of gastric cancer n=three independent experiments. NS  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

largely relieved the antitumor effects of miR-526b-3p, indicating that LINC00689 may act as a sponge for miR-526b-3p in GC cells.

ADAMs (a disintegrin and metalloproteinase) comprise a family of multidomain transmembrane and secreted proteins. ADAM-10, ADAM-12, ADAM-15, and ADAM-17 are causally involved in tumor formation/progression.<sup>34</sup> Specifically, emerging studies have revealed that *ADAM9* is a crucial gene in regulating tumorigenesis. For example, ADAM9 promotes the progression of lung cancer through vascular remodeling, particularly by increasing the functions of VEGFA, ANGPT2, and PLAT.<sup>35</sup> Moreover, ADAM9 functions as a tumor promoter in GC by modulating GC cell proliferation and is directly targeted by miR-126.<sup>36</sup> Here, bioinformatics tools were used to explore the downstream targets of miR-526b-3p in GC cells. Interestingly, it was found that ADAM9 may be one of the candidate targets of miR-526b-3p. In this study, the data supported that the expression of ADAM9 in human GC tissues was significantly increased compared with that in the matched adjacent normal tissues. After knocking down ADAM9, the proliferation, invasion, migration and EMT of GC cells were

all inhibited. Moreover, luciferase reporter assays indicated that miR-526b-3p directly targeted the 3'UTR of ADAM9 mRNA, and ADAM9 was further confirmed to be positively regulated by LINC00689 and negatively regulated by miR-526b-3p. These findings suggested that ADAM9 was regulated by LINC00689/miR-526b-3p.

## Conclusions

In summary, the present study suggests that both LINC00689 could be a prognostic indicator of GC. In addition, our study provides evidence that LINC00689 enhanced the proliferation, invasion, migration and EMT of GC through upregulation of ADAM9 by sponging miR-526b-3p, thus promoting GC progression. Our data serve as a starting point for exploring additional crosstalk between lncRNAs and microRNAs and may provide a promising regimen for the treatment of GC.

## Abbreviations

ADAM9, a disintegrin and metalloproteinase 9; BSA, bovine serum albumin; ceRNA, competitive endogenous RNA; DMEM, Dulbecco's modified Eagle medium; DMSO,

dimethyl sulfoxide; EMT, epithelial mesenchymal transition; FBS, fetal bovine serum; GC, gastric cancer; lncRNAs, long noncoding RNAs; miRNAs, microRNAs; NC, negative control.

## Consent for Publication

Consent for publication has been obtained from the patients.

## Ethics Approval and Consent to Participate

In this study, all investigations and experiments were conducted with patient consent and were approved by the Ethics Committee for Clinical Research of Beijing Shijitan Hospital, Capital Medical University.

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## Disclosure

The authors declare that they have no competing interests in this work.

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