Long Non-Coding RNA LINC01089 Enhances the Development of Gastric Cancer by Sponging miR-145-5p to Mediate SOX9 Expression

Background: Long non-coding RNAs (lncRNAs) have potential regulatory effects in oncogenesis. Previous studies showed that several lncRNAs could participate in the progression of gastric cancer (GC). However, the specific biological mechanisms in GC are still unclear. We analyzed an lncRNA microarray of GC and selected LINC01089 for study.

Methods: LINC01089 expression in GC was tested by qRT-PCR. GC cell proliferation was assessed using CCK-8 and EdU assays. Cell invasion was assessed using the Transwell assay. A dual-luciferase reporter gene assay and bioinformatics assay were performed to detect potential targets of LINC01089. Additionally, RNA immunoprecipitation and Western blot assays were performed to clarify their interactions and roles in the regulation of GC progression.

Results: High LINC01089 expression was observed in GC cells. LINC01089 overexpression notably expedited cell migration, proliferation, and invasion. LINC01089 positively regulated SOX9 expression by competitively binding to microRNA (miR-145-5p).

Conclusion: LINC01089 competitively binds to miR-145-5p to mediate SOX9 expression. LINC01089 may participate in the progression of GC.

Keywords: epigenetics, lncRNA, ceRNA, proliferation, migration

Introduction
Gastric cancer (GC) is the 5th leading cancer and the 3rd most prevalent cause of cancer-related death globally. The progression of GC is a complex multi-step process, which includes multiple genetic and epigenetic variations. Environmental factors, such as Helicobacter pylori infection, are also important for GC. The prognosis of GC has improved with the recent improvements of surgical techniques, radiotherapy, chemotherapy, and targeted molecular therapy. Nevertheless, the clinical outcomes for advanced and metastatic GC patients remain depressing. Since there is no targeted therapy for GC, it is of paramount importance to characterize molecular targets or candidate biomarkers to diagnose and predict GC. This goal remains hampered by the incomplete knowledge of the biological mechanism and pathogenesis of GC development. Thus, it is necessary to detect carcinogenic signal pathways and novel treatment targets for GC.

Long non-coding RNAs (lncRNAs) are non-coding RNAs with over 200 base pairs. Most lncRNAs have a poly A tail that is incapable of being translated into proteins, as with mRNA. LncRNAs used to be considered as “transcriptional noise” or “dark matter”
without any biological function. However, many dynamically expressed lncRNAs have been identified in whole genome transcriptome analysis. Many of these lncRNAs participate in various biological activities. For example, lincRNA-RoR manages the reprogramming of human-induced pluripotent stem cells. 15 \textbf{MYOSLID} is a recently discovered lncRNA that depends on serum response factors and may amplify the differentiation of vascular smooth muscle. 16 Antisense non-coding RNA is an lncRNA in the INK4 locus, which is related to the severity, inflammation, and risks in Crohn’s disease, and on the efficacy of infliximab in the treatment of this disease. 17 In addition, the diabetes mellitus-induced lncRNA Dmtn3os may regulate inflammation and macrophage functions through nuclear mechanisms. 18 Recent evidence has implicated lncRNA as a major regulatory factor in GC. TEA Domain Transcription Factor 4 modulated lncRNA MXN1-AS1 enhances GC progression partially by suppressing B-cell translocation gene 2 and activating B-cell lymphoma 2. 19 The SOX2-overlapping transcript lncRNA enhances GC progression by sponging microRNA (miR)–194-5p from AKT serine/threonine kinase 2. 20 Gastric juice lncRNA has been utilized as a marker to screen for GC. 21 However, the roles of LINC01089 in GC remain unclear.

We presently verified high LINC01089 expression in GC cells and tissues. Overexpression of LINC01089 accelerated GC cell migration, invasion, and proliferation. The involvement of LINC01089 in GC progression involved competitive binding to miR-145-5p to mediate SOX9 expression. The collective results implicate LINC01089 as having a relatively crucial role in GC pathogenesis.

**Materials and Methods**

**Clinical Tissue Samples**

Fifty pairs of GC tissues and normal tissues were extracted from pathologically confirmed cases of GC in Tongde Hospital of Zhejiang Province, China. None of the patients underwent preoperative chemotherapy or radiotherapy. All patients provided informed consent for tissue acquisition and analysis. Immediately after extraction, all samples were frozen in liquid nitrogen until required. The acquisition and storage of tissues was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province. All population-related studies were performed based on the World Medical Association Declaration of Helsinki, and signed informed consent forms were obtained from all participants. The correlations between LINC01089 expression levels and the clinicopathological parameters of GC patients are presented in Table 1.

| Features                  | Number | LINC01089 Expression | P value |
|---------------------------|--------|----------------------|---------|
|                           | (n=50) | High (25) | Low (25) |         |
| **Gender**                |        |           |          |         |
| Male                      | 38     | 20        | 18       | 0.7416  |
| Female                    | 12     | 5         | 7        |         |
| **Age (years)**           |        |           |          |         |
| <60                       | 20     | 11        | 9        | 0.7733  |
| ≥60                       | 30     | 14        | 16       |         |
| **Tumor size(cm)**        |        |           |          |         |
| ≤5                        | 23     | 7         | 16       | 0.0222  |
| >5                        | 27     | 18        | 9        |         |
| **Lymphatic metastasis**  |        |           |          |         |
| N0                        | 17     | 2         | 15       | 0.0072  |
| N1-3                      | 23     | 13        | 10       |         |
| **TNM stage**             |        |           |          |         |
| I and II                  | 19     | 2         | 17       | 0.0011  |
| III and IV                | 21     | 13        | 8        |         |

Note: P <0.05 was considered significantly significant.

**Cell Culture and Transfection**

GC cell lines (BGC-823, MKN45, AGS, and MGC-803) and the GES-1 normal human gastric mucosal cell line were acquired from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 medium (Gibco, Shanghai, China) containing 1% streptomycin-penicillin (Sigma-Aldrich, Shanghai, China) and 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA). Cell culture experiments were conducted in a humidified atmosphere containing 20% O2 or 1% O2 at 37°C. The construction of LINC01089 overexpression plasmids, LINC01089 small interfering RNA (siRNA), miR-145-5p inhibitors, and miR-145-5p mimics were performed at GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Valencia, CA, USA) according to the manufacturer’s instructions. After 48–72 h of culture, the cells were washed, collected, and transfected for further experiments.
RNA Extraction and qRT-PCR
RNAiso Plus (Takara Bio, Shiga, Japan) was employed for the extraction of total RNA from AGS and BGC-823 cells as per the product manual. The PrimeScript RT-PCR kit (Takara Bio) was applied for reverse transcription following the manufacturer’s instructions. The Fast Real-time PCR 7500 System (Applied Biosystems, Foster City, CA, USA) was used for real-time qPCR along with the SYBR-green PCR Master Mix. The qPCR was performed using triplicate 10-mL reaction volumes. The ACTB gene was used as an internal control. The primer sequences were: as LINC01089 forward 5’-GTGTCGTGGAGTCGGCAA and reverse 5’-AGCAGAGAGAGGGGTACA-3’; miR-145-5p forward 5’-ACACTCCAGCTGGGTCCCTAGGACCCTTTT-3’ and reverse 5’-CTCAACTGTTCTGTTGGGGTG-3’. The 2^{ΔΔCt} method was used to calculate the relative mRNA expression.

Cell Proliferation Assays
After transfection for 24 h, cells were seeded in 96-well plates. At day 1, 2, 3, 4, 5, the cell viability was measured using the Cell Counting Kit (CCK8, Dojindo, Japan) following the manufacturer’s instructions. A multifunctional microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) was utilized for absorbance measurement at 450 nm after 1 h of incubation.

For the 5-ethynyl-2’-deoxyuridine (EdU) assay, cells were cultured in the EdU reagent for 2 h, fixed for 15 min in 4% paraformaldehyde, and stained using EdU staining as described by the manufacturer.

Transwell Cell Invasion and Migration Assays
The Transwell chambers (Corning, Corning, NY, USA) used for invasion assay were Matrigel pre-coated (BD Biosciences, Franklin Lakes, NJ, USA). After suspending cells in serum-free medium at a cell density of 1.0 × 10^5/ mL, the Transwell chambers were placed in a 24-well plate. The apical and basolateral chambers contained 200 μL suspension and 500 μL medium containing 10% FBS, respectively. Forty-eight hours later, the chambers were removed, and the cells were fixed using 5% paraformaldehyde for 20 min. The fixed cells were stained using 0.1% crystal violet. The invading cells in five randomly selected fields for each filter were enumerated using a light microscope (Olympus, Tokyo, Japan).

Subcellular Distribution
Extraction of cytoplasmic and nuclear RNA was conducted using a PARIS Kit (Life Technologies, Carlsbad, CA, USA). The total RNA in each fraction was determined using qRT-PCR. Internal references were U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for the nucleus and cytoplasm, respectively.

Dual-Luciferase Reporter Gene Assay
The construction of mutant-type plasmids (SOX9 MUT, LINC01089 MUT) and wild-type plasmids (SOX9 WT, LINC01089 WT) was performed. After seeding HEK293T cells in 24-well plates, Lipofectamine 2000 was used to co-transfect with 50 nM miR-145-5p mimics or negative control and wild- or mutant-type plasmids. The plasmid to pRL-SV40 ratio was 80 ng/5 ng. A dual-luciferase reporter assay kit (Promega, Madison, WI, USA) was used to detect luciferase intensities.

RNA Immunoprecipitation (RIP) Assay
Magna Nuclear RIP™ Nuclear RNA-Binding Protein Immunoprecipitation Kit (Millipore, Bedford, MA, USA) was used for the RIP assay. Cells were lysed in complete RIPA buffer by adding RNase inhibitor and protease inhibitor cocktail. After conjugation with human anti-Argonaute RNA-induced silencing complex (RISC) Catalytic Component 2 (AGO2) antibody or IgG control (Millipore), the cell extracts were incubated with RIP buffer containing magnetic beads. Protein digestion was performed to obtain immunoprecipitated RNAs. The purified RNAs were quantified by qRT-PCR. The anti-miR-145-5p and anti-LINC01089 used in the RIP assay were from Abcam (Cambridge, MA, USA).

Western Blot
After extracting and quantifying protein samples using the BCA method, protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to a membrane, which was blocked using 5% defatted milk. Membranes were incubated with the rabbit anti-human IgG antibody for SOX9 and GAPDH (abcam, Shanghai, China) as its primary antibody, followed by incubation with appropriate HRP-conjugated secondary antibodies (Beyotime, Nantong, China). Bands were visualized by chemiluminescence.
Mouse Xenograft Model
Eight female BALB/c nude mice (6 weeks old; 18–22 g in weight) were purchased from the Model Animal Research Center of Nanjing University. BGC-823 cells (1 \times 10^6) transfected with LINC01089 short hairpin RNA (shRNA) or negative control (sh-NC) were injected subcutaneously into BALB/c nude mice. Tumor volumes were calculated every 7 days as (length \times width^2)/2. At 4 weeks post-injection, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg) and then sacrificed by 10% formalin perfusion fixation of the central nervous system. Tumor tissues were isolated and weighed. The experiments were approved by the Ethics Committee of Tongde Hospital of Zhejiang Province, and were performed following the NIH guidelines on animal welfare.

Statistical Analyses
All statistical analyses were conducted using SPSS 20.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 6.0 (GraphPad, La Jolla, CA, USA). Quantitative data are reported as mean ± standard deviation (SD). A nonparametric test was applied for non-normal data and the t-test was conducted for the analysis of measurement data. \(P < 0.05\) indicated statistical differences.

Results
LINC01089 Expression in GC
The GSE109476 microarray indicated that LINC01089 was highly expressed in GC tissues. Differentially expressed lncRNAs were determined following the criteria of a log2 fold-change \(>1.2\) or \(<-1.2\) and \(P < 0.05\). The differentially expressed lncRNAs in GC tissues included LINC01089 is shown in Figure 1A. qRT-PCR revealed high LINC01089 expression in the 50 pairs of GC tissue samples and MKN45, MGC-803, BGC-823, and AGS GC cells (Figure 1B and C). Particularly, among the GC cell lines used for subsequent experiments, LINC01089 expression was highest in BGC-823 cells and lowest in AGS cells. High expression of LINC01089 was associated with a poorer survival rate (Figure 1D).

LINC01089 Functions in GC Cells
qRT-PCR was performed to verify the transfection efficacy of the LINC01089 overexpression vector and LINC01089 siRNA in GC cells (Figure 2A). Moreover, the CCK-8 assay revealed that GC cell proliferation was remarkably reduced by LINC01089 downregulation and accelerated by LINC01089 overexpression (Figure 2B), similar to the data of the EdU experiment (Figure 2C and D). The Transwell assay revealed that the migration and invasion of GC cells were enhanced by LINC01089 overexpression and decreased by LINC01089 downregulation (Figure 2E–H). The collective findings indicated that LINC01089 might play regulatory roles in the proliferation, migration, and invasion of GC cells.

LINC01089 is Targeted by miR-145-5p
The biological functions of lncRNAs depend on their subcellular distribution. Presently, cytoplasmic and nuclear fractions of GC cells were obtained, with GAPDH and U6 as the respective controls to verify the cellular location of LINC01089. qRT-PCR revealed LINC01089 in the cytoplasmic fractions of BGC-823 and AGS cells (Figure 3A). The data supported the idea that the involvement of qRT-PCR also revealed the reduced expression of miR-145-5p in GC cells (Figure 3B). In addition, the expression levels of miR-145-5p and LINC01089 were negatively related in GC tissues (Figure 3C). Meanwhile, the expressions of miR-145-5p and LINC01089 were also negatively related in GC tissues from TCGA dataset (Supplementary Figure 1A). High sequence matching was identified between miR-145-5p and LINC01089 3’-untranslated region by starBase prediction. The construction of pGL3-LINC01089 WT and pGL3-LINC01089 MUT was performed according to these binding sequences (Figure 3D). Notable downregulation of luciferase activity was found in HEK293T cells after co-transfection with LINC01089 WT and miR-145-5p mimics. However, the activity was unchanged following co-transfection with LINC01089 MUT and miR-145-5p mimics (Figure 3E). MiRNAs are distributed in the cytoplasm, which is a component of the RISC containing Ago2. Ago2 is required for miRNA-mediated gene silencing. Presently, we analyzed whether LINC01089 and miR-145-5p contained the same RISC and performed an RIP assay in AGS and BGC-823 cells. LINC01089 and miR-145-5p were enhanced in Ago2 immunoprecipitate versus IgG immunoprecipitate controls (Figure 3F). The findings supported the involvement of LINC01089 in the progression of GC via posttranscriptional regulation and a role of LINC01089 in the regulation of signaling in the progression of GC by sponging miR-145-5p.

LINC01089 Regulates SOX9, a Target Gene of miR-145-5p
To explore the potential roles of miR-145-5p in GC progression, target genes of miR-145-5p were screened via
bioinformatics prediction. SOX9 was identified and further analyzed (Figure 4A). miR-NC or miR-145-5p mimics were selected to co-transfect HEK293T cells with constructed luciferase plasmids (pGL3-SOX9 MUT and pGL3-SOX9 WT, respectively). Luciferase activity of the MUT reporter was unchanged, while activity in the WT reporter group was repressed (Figure 4B). The findings implicated SOX9 as a candidate miR-145-5p target. Since miR-145-5p itself can also target other genes,20,21 we performed assays to ascertain whether SOX9 was regulated by any of the other targets. SOX9 was not regulated by any of the other targets of miR-145-5p in the GC tissues (Supplementary Figure 2). qRT-PCR revealed a notable enhancement of RNA levels of SOX9 in GC tissues versus normal tissue samples (Figure 4C). Additionally, Western blot analysis obtained identical protein levels (Figure 4D). The expressions of miR-145-5p and SOX9 were

Figure 1 Characteristics and expression of LINC01089 in GC cells and tissues. (A) Heatmap shows the differential expression IncRNAs in GC tissues adjacent normal tissue samples from GSE96964 Microarray. (B) LINC01089 expression in GC tissues and adjacent normal tissue samples (n= 50). (C) LINC01089 expression in GC cell lines (MKN45, MGC-803, BGC-823 and AGS) and normal human gastric mucosal cell line GES-1 as determined by qRT-PCR. (D) Kaplan–Meier analysis for overall survival of GC patients stratified by LINC01089 expression. *P value < 0.05, **P value < 0.01 and ***P value < 0.001.
negatively correlated (Figure 4E). However, a positive correlation existed between the expression of SOX9 and LINC01089 (Figure 4F). What is more, analyses of TCGA datasets show that the level of SOX9 is negatively related to miR-145-5p and positively related to LINC01089 (Supplementary Figure 1B and C).

To determine whether LINC01089 regulated SOX9 expression by targeting miR-145-5p, we measured SOX9 expression after adjusting the content of LINC01089 and miR-145-5p. The transfection efficacy of miR-145-5p inhibitor and miR-145-5p mimics was verified (Figure 5A). Subsequently, upregulated SOX9 expression was identified in BGC-823 cells after transfection with miR-145-5p inhibitor using both Western blotting and qRT-PCR. This effect was reversed after co-transfection with LINC01089 siRNA (Figure 5B...
The inhibitory effects of SOX9 expression were identified after transfection with miR-145-5p mimics in AGS cells. The inhibition was reversed by cotransfection with the LINC01089 overexpression plasmid (Figure 5C and E). The LINC01089 WT overexpression plasmid and corresponding mutant overexpression plasmid were used to transfect AGS cells prior to the determination of SOX9 expression. Western blot and qRT-PCR analyses revealed that LINC01089 WT overexpression upregulated SOX9 expression, whereas LINC01089 MUT had no disruptive effect on the base pairing between LINC01089 and miR-145-5p (Figure 5F and G). The collective findings indicated that LINC01089 is able to directly bind to miR-145-5p to positively regulate SOX9 expression.

**LINC01089/miR-145-5p Axis Regulates GC Cell Behavior**

We next determined whether miR-145-5p exerted any impact on the proliferation and invasion of BGC-823
and AGS cells. Both features were remarkably enhanced via miR-145-5p downregulation in BGC-823 cells versus controls, which was partially reversed by treatment with LINC01089 siRNA (Figure 6A, C and E). Overexpression of miR-145-5p lessened the proliferation and invasion of AGS cells, which were partially reversed by overexpression of LINC01089 (Figure 6B, D and F).

**Downregulation of LINC01089 Inhibits GC Tumor Growth in vivo**
Tumor growth in vivo was suppressed by LINC01089...
downregulation, and BGC-823 cells transduced from sh-LINC01089 grew slowly after implantation (Figure 7A). In addition, the average volume and weight of xenografts from cells transduced from sh-LINC01089 were markedly lower than those from sh-NC cells (Figure 7B and C). The findings indicated that LINC01089 siRNA inhibited the growth of GC tumors in vivo.

**Discussion**

GC is one of the most prevalent digestive system carcinomas. LncRNA has been reported as a regulator of many cellular processes, especially in tumors. For example, LINC01089 has been identified as a contributor to cell proliferation and migration. LncRNA MALAT1 enhances the occurrence, glycolysis, and invasion of...
multiple myelomas by the miR-1271-5p/SOX13 axis.\textsuperscript{24} Our use of the GC lncRNA microarray with the GPL24530 platform identified high LINC01089 expression in GC tissues. LINC01089 promoted the proliferative, migration, and invasive capacities of BGC-823 and AGS cells. These results imply that LINC01089 may be a treatment target for GC patients pending further research.

LncRNAs may bind to miRNAs and regulate their functions, which might be valuable in treatments.\textsuperscript{25,26} One study demonstrated that the NKILA lncRNA can downregulate miRNA-21 to repress proliferation and enhance the apoptosis of CSCC cells.\textsuperscript{27} In another study, lncRNA IGFL2-AS1 was shown to be a ceRNA for ARPP19 regulation, as it was competitively bound to miR-802 in GC.\textsuperscript{28} In our study, miR-145-5p bound to LINC01089 and was expressed at low levels in GC cell lines. Importantly, in cell function tests, the proliferation and invasion of GC cells were limited by overexpressed miR-145-5p. However, miR-145-5p mimics were partially reversed by LINC01089 OE. MiR-145-5p has been reported to be involved in the pathological processes of GC.\textsuperscript{20,29} For

![Figure 6](https://example.com/figure6.png)

**Figure 6** LINC01089 regulates cell functions by miR-145-5p. (A, B) Proliferation of BGC-823 and AGS cells as determined by CCK-8 assay. (C, D) Proliferation of BGC-823 and AGS cells as determined by EdU assay. (E, F) Invasion of BGC-823 and AGS cells post different transfections. All the data were from three individual experiments and reported as mean ± SD. *P value < 0.05, **P value < 0.01, OE: overexpression, si: siRNA, inh: inhibitor.
instance, circDUSP16 enhances the oncogenesis and invasion of GC by sponging miR-145-5p.

Next, we confirmed that SOX9 was a downstream target of miR-145-5p and that LINC01089 adjusted the expression of SOX9 by competitively binding miR-145-5p. SOX9 can promote the pathophysiological process of tumors, including GC, glioblastoma, breast cancer, and pancreatic adenocarcinoma.

There are some limitations to this research. First, the number of samples should be increased. Second, more experiments are needed to prove the functions of SOX9 in GC cells. Third, LINC01089 may regulate the development of GC in many ways. We identified only one. Fourth, the in vivo tumor formation assay could also be conducted by adding the variable miR-145-5p inhibitor/mimics, helping to more thoroughly validate the findings. Finally, quantify the results and analyzing them kinetically would provide a more detailed understanding of the proposed regulation relationship. We intend to address these limitations in future studies.

Conclusion

LINC01089 is a ceRNA that regulates SOX9 expression by sponging miR-145-5p, thereby regulating the progression of GC. LINC01089 may have value in the diagnosis of GC and as a treatment target.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Disclosure

The authors declare no competing interests for this work.

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Figure 7 LINC01089 regulates GC in vivo. (A) Xenograft tumors. (B) Tumor volumes in both LINC01089 knockdown and control groups measured at an interval of seven days. (C) Tumor weights measured after tumor dissection. *P value < 0.05. sh: shRNA.
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