LINC00675 Suppresses Cell Proliferation and Migration via Downregulating the H3K4me2 Level at the SPRY4 Promoter in Gastric Cancer

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Accumulating evidence indicates that long noncoding RNAs (lncRNAs) are dysregulated in diverse tumors and take a pivotal role in modulating biological processes. In our study, a decreased expression level of LINC00675 in gastric cancer (GC) was first determined by data from The Cancer Genome Atlas (TCGA) and was identified using specimens from GC patients. Then, *in vitro* and *in vivo* functional experiments elaborated that LINC00675 could suppress cell proliferation and migration in GC. Multiple differentially expressed genes (DEGs) in LINC00675-overexpressing cells were identified through RNA sequencing analysis. An RNA-binding protein immunoprecipitation (RIP) assay was conducted to reveal that LINC00675 competitively bound with lysine-specific demethylase 1 (LSD1). A coimmunoprecipitation (coIP) assay indicated that LINC00675 overexpression may strengthen the binding of LSD1 and H3K4me2, whereas the chromatin immunoprecipitation (ChIP) assay results verified lower expression of H3K4me2 at the sprouty homolog 4 (SPRY4) promoter region. Together, our research identified that LINC00675 was remarkably downregulated in GC tissues and cells relative to nontumor tissues and cells. LINC00675 could repress GC tumorigenesis and metastasis via competitively binding with LSD1 and intensifying the binding of LSD1 and its target H3K4me2. Importantly, this contributed to attenuated binding of H3K4me2 at the promoter region of oncogene SPRY4 and suppressed SPRY4 transcription, thus suppressing GC cell proliferation and migration.

INTRODUCTION

Gastric cancer (GC) has been a severe handicap for public health since it is the fifth most common form of cancer and the third most frequent cause contributing to cancer deaths all around the world.[1,2] Due to the difficulties of detecting GC at an early age and the lack of effective treatments, the prognosis of patients with GC remains still unsatisfactory.[3,4] Multiple studies have indicated that various molecules and pathways are involved in the development of GC.[5,6]

Long noncoding RNAs (lncRNAs), which are currently regarded as critical participants in biological processes rather than "transcriptional noises," normally comprise more than 200 nucleotides and have no capacity of encoding proteins.[7,8] The involvement of lncRNAs in cancer development has declared that lncRNAs can regulate multiple oncogenes and tumor-suppressor genes to impact tumorigenesis, metastasis, and prognosis due to interaction with DNAs, RNAs, and proteins.[9,10] Recent research has focused on the involvement of lncRNAs in GC development. For example, lncRNA FOXD2-AS1 was significantly upregulated in GC and functioned as an oncogene through EphB3 downregulation mediated by interaction with enhancer of zeste homolog 2 (EZH2) and lysine-specific demethylase 1 (LSD1).[11] Xu et al.[12] found that LINC00346 could promote GC progression by acting as a competing endogenous RNA (ceRNA), which formed a molecular decoy for microRNA (miR)-34a-5p and targeted AXL, CD44, and NOTCH1 for degradation.

LINC00675, also known as TMEM238L, has been reported to be dysregulated in various kinds of cancers, including GC,[13] colorectal cancer,[14] cervical cancer,[15] glioma,[16] oral squamous cell carcinoma (OSCC),[17] esophageal squamous cell carcinoma (ESCC),[18] and pancreatic ductal adenocarcinoma (PDAC).[19] Since there has been very little research concerning the functions exerted by LINC00675 in GC and its underlying mechanisms, LINC00675 was of particular interest to us for further study.

LSD1, encoded by the gene KDM1A, is the first identified histone demethylase. In eukaryotic cells, nucleosomes are the basic units of chromosomes, which are composed of 147 bp DNA wrapping around an octamer centered on two molecules of histones H2A, H2B, H3, and H4. Each histone is formed by a folding region and an amino terminal domain, and its distinguishing feature is that it is easy to be covalently modified by methylation, acetylation, phosphorylation, ubiquitination, etc. Most of these covalent modifications of histones are reversible, whereas methylation modification of histones was considered to be an irreversible and permanent histone marker in the past. The discovery of LSD1 in 2004 challenged this point of view and paved a new way for further research on the mechanism of protein modification.[20,21] LSD1 mediates gene activation and repression via removal of the methyl group (me) from mono- or dimethylated histone H3 at lysine 4 me 1/2 (H3K4me1/2) and lysine 9 (H3K9me1/2), which are endowed with the main specificity for H3K4me2.[22,23] Evidence has shown that LSD1 is upregulated and acts as an oncogene in various kinds of tumors.[24,25] Zhang et al.[4] have revealed that LSD1 had upregulated expression in GC and positively contributed to GC development.

Sprouty homolog 4 (SPRY4), one of the four homologs the SPRY family, is one of the important genes in the process of tumor development.[26] Some of the previous studies indicated that SPRY4 acted as a tumor-suppressor gene in cancers originated from lung,[27] colon,[28] breast,[29] and esophagus,[30] whereas some others identified SPRY4 as an oncogene, such as in testicular germ cell tumor (TGCT).[31,32] However, its functional role in GC has never been studied and is worth taking further steps into exploring more.

In our research, we deciphered a critical effect of LINC00675 downregulation on GC tumorigenesis and metastasis and provided mechanistic insights into the epigenetic regulation of the downstream oncogene SPRY4 caused by LINC00675-LSD1 binding.

RESULTS

LINC00675 Is Downregulated in Human GC Tissues and Is Negatively Associated with Poor Prognosis

Based on The Cancer Genome Atlas (TCGA), the expression level of LINC00675 was initially analyzed in 375 cancer tissues and 32 normal tissues of patients with GC, and the results indicated that LINC00675 was obviously downregulated in tumor tissues compared to nontumor tissues (Figure 1A). As the functional roles and underlying mechanisms of LINC00675 in GC were still blurry, we set out to conduct further research. First, LINC00675 was verified to have no capacity of protein encoding according to Open Reading Frame (ORF) Finder analysis.[13] Then, the qRT-PCR experiment was performed to identify that compared to normal gastric cell line GES-1, LINC00675 exhibited lower expression in GC cell lines SGC-7901, BGC-823, AGS, and MGC-803 (Figure 1B). To confirm the reliability of the conclusion, we further compared LINC00675 expression levels between paired GC and nontumor tissues (n = 78) by qRT-PCR. The tumor/adjacent nontumor tissue (T/N) ratio for LINC00675 enrichment was examined, and LINC00675 was shown to have decreased the expression level in 73.1% (57 of 78 paired) of GC tissues (p < 0.001; Figure 1C).

We then divided enrolled GC patients into LINC00675 high-expression (n = 21) and low-expression groups (n = 57) according to whether it was upregulated or downregulated relative to nontumor tissues and further analyzed clinicopathologic characteristics of GC patients. It was revealed that the LINC00675 expression level was associated with tumor size (p = 0.029), invasion depth (p = 0.003), Tumor Node Metastasis (TNM) stages (p = 0.01), lymphatic metastasis (p = 0.04), and regional lymph nodes (p = 0.005), whereas it was not correlated to age, gender, tumor location, histologic differentiation, and distant metastasis (p > 0.05) (Table 1).

To evaluate the relationship between LINC00675 expression and the prognosis of GC patients, we employed online Kaplan-Meier analysis (https://kmplot.com/analysis/) and explored that the low-expression level of LINC00675 in GC patients was correlated to poor clinical outcomes. The low-expression cohort presented the median overall survival (OS) as 28.13 months and the median first time to progression (FP) as 17.2 months, whereas the high-expression cohort presented the median OS as 85.6 months and the median FP as 74.9 months. As shown in Figure 1D, low expression of LINC00675 could predict a poor prognosis in GC. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway clustering illustrated that pathways related to cancer cell apoptosis and adhesion were most markedly involved in GC cell dysregulation upon LINC00675 overexpression (Figure 1E).

To explore the mechanism that contributed to the low expression of LINC00675 in GC, we treated GC cells with 5-aza-2′-deoxycytidine (AzaD) in different concentrations and found that LINC00675 expression was boosted after AzaD treatments (Figure 1F). AzaD, also known as decitabine, could activate silenced genes by promoter demethylation. However, no CpG islands were found in the promoter region of LINC00675 by searching the database. Since AzaD could make influences on gene expression in multiple ways, some of which were independent of DNA demethylation at promoter regions, repressive histone modifications may also be targets of AzaD and finally result in gene upregulation.[33] Accumulating evidence suggested that AzaD could induce overexpression of genes by decreasing H3K9 methylation levels, especially H3K9me2. The chromatin immunoprecipitation (ChIP) assay demonstrated that after AzaD treatments, the expression level of H3K9me2 at the LINC00675 promoter region was significantly downregulated, which may result in the overexpression of LINC00675 (Figure 1G).[34–36]

Taken together, LINC00675 was remarkably downregulated in GC tissues and cells, and a low-expression level of LINC00675 predicted poor prognosis in GC.

LINC00675 Suppresses GC Cell Proliferation and Migration In Vitro and In Vivo

To evaluate the functional roles of LINC00675 in GC progression, LINC00675-overexpressing SGC-7901 and BGC-823 cells were
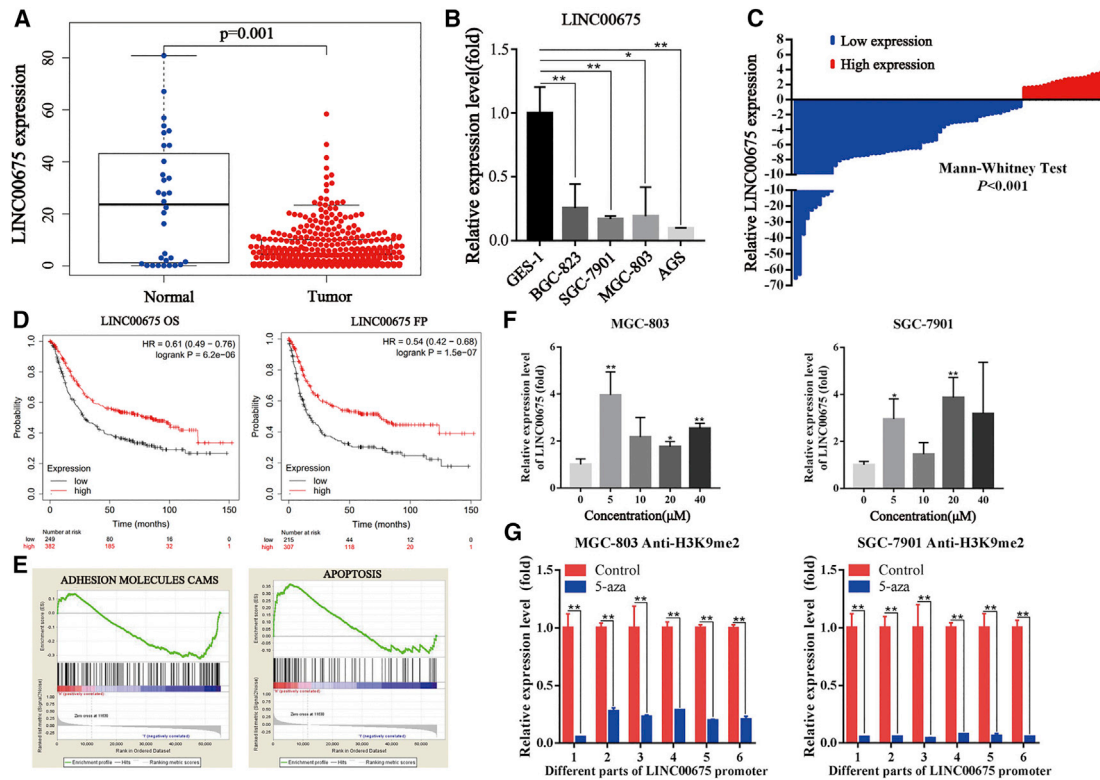
Figure 1. LINC00675 Is Downregulated in Human GC Tissues and Cells and Is Negatively Associated with Poor Prognosis

(A) The expression level of LINC00675 was analyzed in TCGA. LINC00675 was downregulated in GC tissues compared to nontumor tissues. (B) LINC00675 was downregulated in GC cell lines SGC-7901, BGC-823, AGS, and MGC-803 compared to normal gastric cell line GES-1. (C) LINC00675 was detected in 78 pairs of GC tissues and normal tissues by qRT-PCR. The levels of LINC00675 in GC tissues were significantly lower than those in nontumor tissues. (D) Online Kaplan-Meier analysis showed that GC patients with lower expression of LINC00675 had shorter median OS and FP times, which predicted poor prognosis. (E) KEGG analysis illustrated that LINC00675 was involved in cancer cell apoptosis and adhesion pathways. (F) After treating GC cells with AzaD in different concentrations, the expression of LINC00675 was upregulated. (G) After treating GC cells with AzaD, ChIP assay results showed that the expression level of H3K9me2 at the LINC00675 promoter region was downregulated. *p < 0.05, **p < 0.01.

constructed. The transfection efficacy of LINC00675-overexpressing plasmid (pcDNA-LINC00675) was shown in Figure S1. Cell Counting Kit-8 (CCK-8) assays were performed in which LINC00675 overexpression remarkably inhibited GC cell proliferation (Figure 2A). The same results could be seen in colony-formation assays (Figure 2B). Furthermore, we conducted Transwell migration assays and illustrated that LINC00675 acted as a suppressor of GC cell migration (Figure 2C). The effect of LINC00675 on GC cell apoptosis was also tested by flow cytometry, suggesting that overexpression of LINC00675 could significantly promote the apoptosis of SGC-7901 and BGC-823 cells, respectively (Figure 2D). Overall, we speculated that LINC00675 inhibited GC tumorigenesis and metastasis *in vitro*.

To evaluate the functional role of LINC00675 in GC tumorigenesis *in vivo*, BGC-823 cells transfected with LINC00675-overexpressing lentivirus and empty vectors were injected into five nude mice, respectively (total n = 10). After 25 days of tumor growth, the tumors, which were harvested from mice of the LINC00675-overexpressing

group, were evidently smaller in size and had lower weight than those harvested from the control group (Figures 2E–2G). These results together indicated that LINC00675 acted as a tumor-suppressor gene *in vivo*.

LINC00675 Binds to LSD1 in the Nucleus, Thus Enhancing the Binding of LSD1 and H3K4me2 and Epigenetically Silencing SPRY4

In order to unbiasedly probe the LINC00675-related pathways in GC, RNA sequencing (RNA-seq) in parallel with statistical analysis was performed using LINC00675-overexpressing and control SGC-7901 cells (Figure 3A). The results of the RNA-seq analysis showed that LINC00675 overexpression increased the abundance (more than 2-fold) of 744 genes, whereas 224 genes presented more than 2-fold decreased abundance (Table S1). Moreover, Gene Ontology (GO) analysis illustrated that multiple pathways of cell proliferation and migration were markedly involved in GC cell dysregulation upon LINC00675 overexpression (Figure 3B). The key genes that were significantly dysregulated in LINC00675-overexpressing GC cells

Table 1. Correlation between LINC00675 Expression and Clinicopathological Features of GC (n = 78)

Clinical Parameter	LINC00675		Chi-Square Test p Value
	Low-Expression Cases (n = 57)	High-Expression Cases (n = 21)	
Age (Years)			0.937
<50	25	9	
>50	32	12	
Gender			0.857
Male	34	13	
Female	23	8	
Location			0.321
Distal	26	9	
Middle	20	6	
Proximal	8	6	
Size			0.029[a]
>5 cm	34	6	
<5 cm	23	15	
Histologic Differentiation			0.096
Well	4	1	
Moderately	16	8	
Poorly	35	8	
Undifferentiated	2	4	
Invasion Depth			0.003[a]
T1	12	6	
T2	17	13	
T3	18	2	
T4	10	0	
TNM Stages			0.001[a]
I	13	14	
II	13	4	
III	24	3	
IV	7	0	
Lymphatic Metastasis			0.041[a]
Yes	24	15	
No	33	6	
Regional Lymph Nodes			0.005[a]
pN0	23	15	
pN1	5	4	
pN2	11	1	
pN3	18	1	
Distant Metastasis			0.180
Yes	7	0	
No	50	21	

[a]$p < 0.05$.

included DUSP6, ETV4, PSAT1, SERPINE2, SPRED1, SPRY4, etc., some of which were then verified by qRT-PCR (Figure 3C).

To elucidate the mechanism for LINC00675-mediated dysregulation, first, LINC00675 was found to have a much higher ratio in nuclei than in cytoplasm utilizing subcellular fractionation and RNA fluorescence in situ hybridization (FISH) analysis (Figures 3D and 3E), which suggested that LINC00675 probably functioned as a major regulator at the transcriptional level. Recent research has pointed out that lncRNAs could regulate gene expression by functioning in cooperation with chromatin-modifying enzymes.[30,37] Then, based on the prediction from the RNA-Protein Interaction Prediction (RPISeq) website, a panel of chromatin modifiers, including LSD1, EZH2, and SUZ12, etc., displayed positive possibility to bind with LINC00675 (Figure 4A). We performed RNA immunoprecipitation (RIP) assays with antibodies and confirmed that LINC00675 overexpression could establish more intense binding with LSD1 but not EZH2 (Figures 4B and S2).

Among the multiple potential target genes of LINC00675, SPRY4 was of particular interest to us since it had remarkable fold change in mRNA expression level, according to RNA-seq analysis and qRT-PCR identification. Of note, a strong decrease was caused in SPRY4 protein level upon LINC00675 overexpression, consistent with the decrease in SPRY4 mRNA level (Figure S3). Since LSD1 has been proven to be an oncogene in GC in many studies,[38,39] we hypothesized that the overexpression of LINC00675 suppressed GC proliferation and metastasis through its competitive binding with LSD1, which partially inhibited the function of LSD1. To confirm the hypothesis, we designed the small interfering (si)-LSD1 plasmid and found that when LSD1 was efficiently decreased (Figure S4), the expression of SPRY4 was also downregulated, which was consistent with the results upon LINC00675 overexpression (Figure 4C).

Evidence has shown that LSD1 mainly targeted histone methylation modification H3K4me2 and predominately induced transcriptional changes.[23] High levels of H3K4me2 at promoter regions may promote gene transcription, whereas low levels of H3K4me2 could inhibit transcription.[40–42] Therefore, we performed the ChIP assay for H3K4me2 to further explore the promoter modulation of SPRY4 upon LINC00675 overexpression. The ChIP assay results revealed that SPRY4 exhibited a decreased H3K4me2 level at its promoter region in LINC00675-overexpressing cells compared to control cells, thus suppressing the gene transcription of SPRY4 (Figure 4D). Consistently, after inhibition of the expression of LSD1 through si-LSD1 plasmid transfection, ChIP assay results showed that the binding capacity of H3K4me2 and the SPRY4 gene promoter was also reduced (Figure 4E).

Subsequently, the immunoprecipitation (IP) experiment was conducted and revealed that the binding capacity of LSD1 and H3K4me2 was increased when LINC00675 was overexpressed (Figure 4F). Based on these experimental results, we proposed a hypothetical mechanism, in which LINC00675 competitively bound with LSD1 and resulted in a stronger binding of LSD1 and its target H3K4me2, which eventually proved to attenuate the expression of
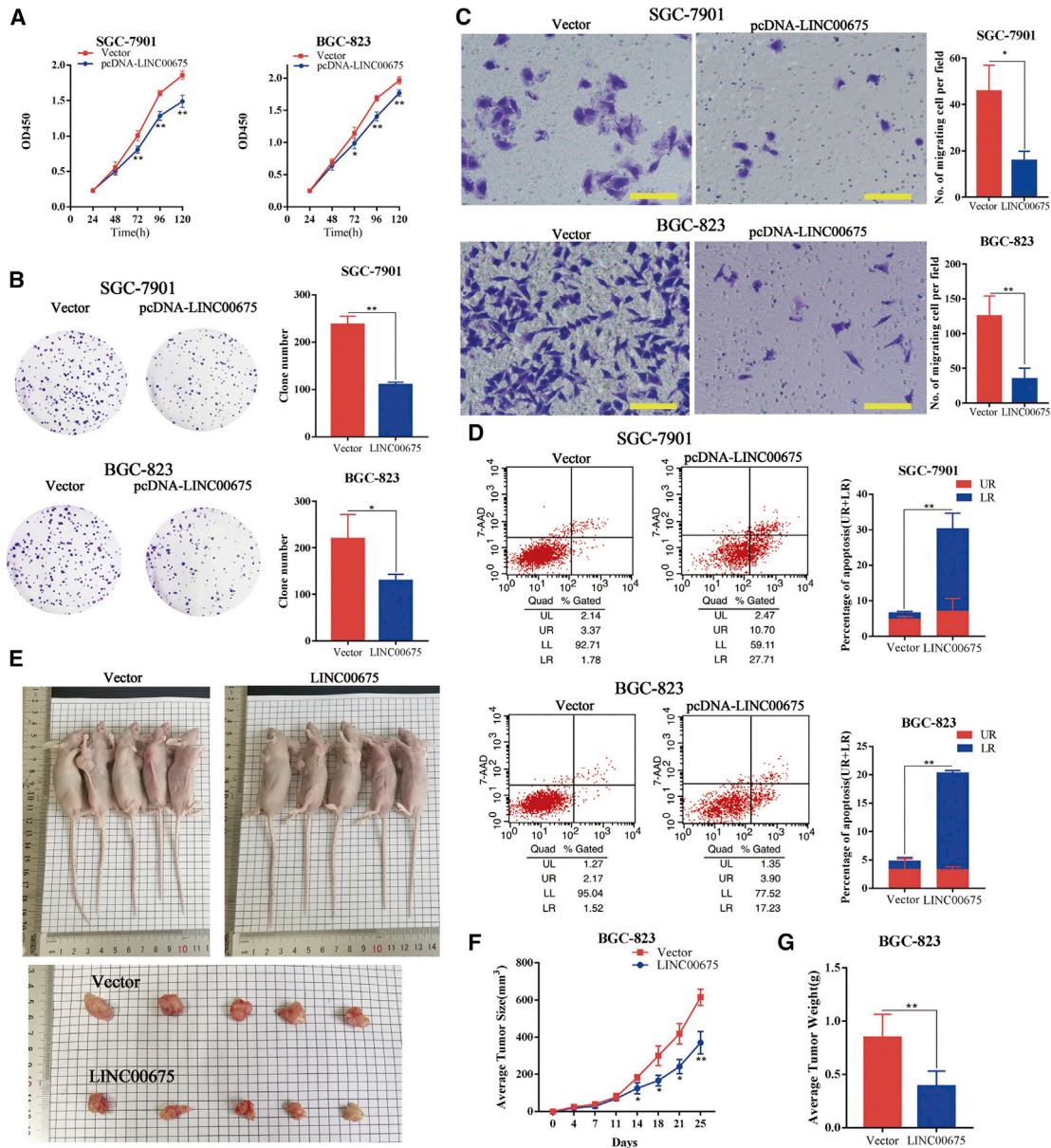
Figure 2. LINC00675 Suppresses GC Cell Proliferation and Migration *In Vitro* and *In Vivo*

(A) CCK-8 assays were conducted to determine cell proliferation of SGC-7901 and BGC-823 cells after transfection of LINC00675-overexpressing plasmids and control plasmids. (B) Colony-formation assays were performed to identify the effects of LINC00675 on GC cell proliferation. (C) Transwell assays showed that overexpressing LINC00675 could suppress GC cell migration (scale bars, 100 μm). (D) FACS analysis elaborated the effects of LINC00675 on GC cell apoptosis. (E–G) BGC-823 cells transfected with LINC00675-overexpressing lentivirus and empty vectors were injected into five nude mice, respectively (E; total n = 10). Tumor weights and sizes were represented as means of tumor weights (G)/sizes (F) ± SD (standard deviation). *p < 0.05, **p < 0.01.

H3K4me2 at the promoter region of SPRY4 and suppress SPRY4 gene transcription (Figure 7).

SPRY4 Acts as an Oncogene in GC Cell Proliferation and Migration

Finally, we examined the functional roles that SPRY4 played in GC progression. We initially explored the expression level of SPRY4 in GC from TCGA database, which indicted that SPRY4 was significantly upregulated in GC by analyzing 375 cancer tissues and 32 normal tissues of GC patients (Figure 5A). Then, taking 78 paired GC tissues and normal tissues into research, higher expression of SPRY4 was shown in tumor tissues compared with nontumor tissues (p < 0.001; Figure 5B). GC patients were then divided into SPRY4 high-expression (n = 52) and low-expression (n = 26) subgroups,
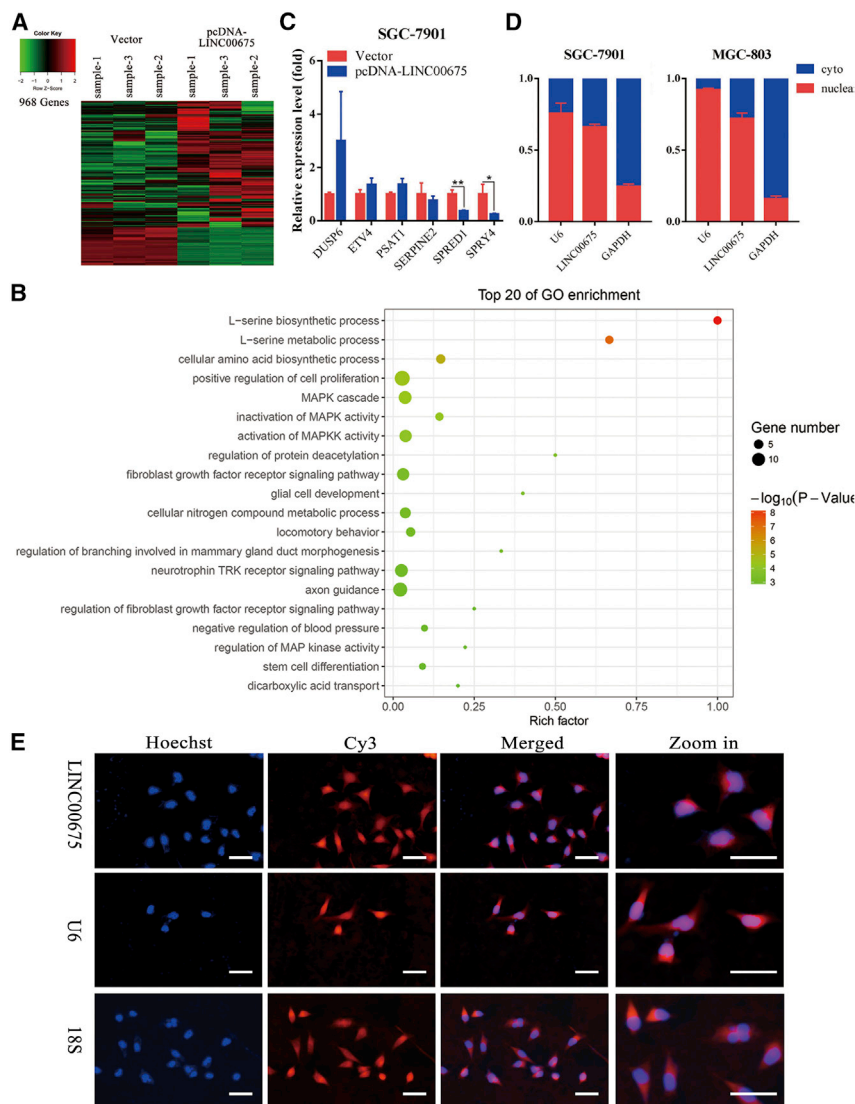
Figure 3. RNA-Seq Was Performed after LINC00675 Overexpression in SGC-7901 Cells, and LINC00675 Is Mainly Located in the GC Cell Nucleus

(A) RNA-seq was performed upon LINC00675 overexpression in SGC-7901 cells. Hierarchical clustering-illustrated 968 transcripts were altered ($\geq$2-fold change) in SGC-7901 cells treated with LINC00675-overexpressing plasmids and vectors with three repeats. (B) GO analysis identified multiple pathways involved in GC cell dysregulation upon LINC00675 overexpression. (C) qRT-PCR experiment was conducted to verify the significantly dysregulated genes in LINC00675-overexpressing GC cells. (D and E) Subcellular fractionation (D) and FISH analyses (E) suggested that LINC00675 had much higher ratio in nuclei than in cytoplasm (scale bars, 10 μm). *p < 0.05, **p < 0.01.

gain-of-function assays and identified the downregulation of SPRY4 mRNA and protein levels by qRT-PCR and western blotting (Figures S5 and S6). As shown in Figures 6A and 6B, low expression of SPRY4 significantly inhibited GC cell proliferation by CCK-8 assays and colony-formation assays. GC cell migration was also found to be attenuated upon SPRY4 downregulation by Transwell assays (Figure 6C), suggesting oncogenic capability of SPRY4 in GC.

To uncover the *in vivo* function of SPRY4 in GC proliferation, the xenograft tumor mouse model was constructed, in which the short hairpin (sh) SPRY4 group was found to have much lower mean tumor volume and weight than the control group (Figures 6D–6F). Overall, it could be noted that SPRY4 acted as an oncogene in the malignant progression of GC.

DISCUSSION

Accumulating evidence has demonstrated that epigenetic dysregulation plays a pivotal role in cancer development, and lncRNAs involved in epigenetics have been a hotspot for research.[43–45] lncRNAs could contribute to tumorigenesis and metastasis through numerous mechanisms.[7–9] For instance, lncRNAs could repress or activate gene transcription via recruiting histone modification enzymes (such as EZH2, LSD1, and SUZ12) to the promoter regions of downstream genes.[46,47] In cytoplasm, lncRNAs sponged with microRNAs (miRNAs) inhibited their activities by acting as ceRNAs.[48] lncRNAs could also modulate mRNA stability and process a small, active peptide via interacting with RNA-binding proteins (e.g., UPF1, STAU1, and hnRNPL).[49–52]

LINC00675 may exert its biological functional roles depending on the specific types of cancer. For example, LINC00675 exhibited a high-expression level in PDAC and predicted an unfavorable prognosis.[19] LINC00675 was also upregulated in cervical cancer and promoted

and clinicopathologic features were analyzed. High-expression level of SPRY4 in GC tissues suggested its association with invasion depth (p = 0.001), TNM stages (p < 0.001), lymphatic metastasis (p = 0.008), and regional lymph nodes (p = 0.002) (Table 2). Online Kaplan-Meier analysis was conducted to evaluate the association between SPRY4 expression and the prognosis of GC patients. The results indicated that the median OS was 36.4 months in the high SPRY4 expression group and 113.2 months in the low SPRY4 expression group, whereas the median FP in high and low SPRY4 expression groups was 24.5 months and 80.1 months separately (Figure 5C). Furthermore, we analyzed the correlation between LINC00675 and SPRY4, finding that SPRY4 expression in paired GC tissues and normal gastric tissues was negatively correlated with LINC00675 expression (Figure 5D).

To better understand the biological roles of SPRY4 in GC, we transfected SGC-7901 and BGC-823 cells with SPRY4 siRNAs to perform
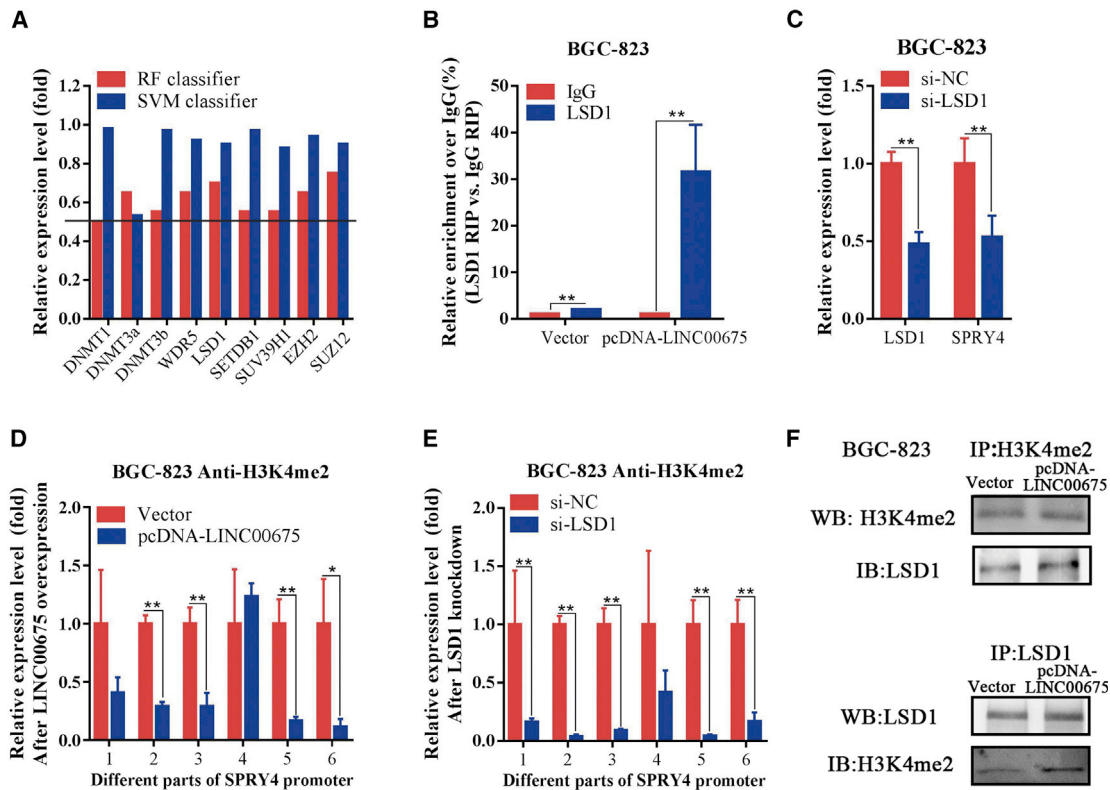
Figure 4. LINC00675 Binds to LSD1 in the Nucleus, Thus Enhancing the Combination of LSD1 and H3K4me2 and Epigenetically Silencing SPRY4

(A) A panel of chromatin modifiers, including LSD1, EZH2, and SUZ12, displayed a positive possibility to bind with LINC00675, according to the RPISeq website. (B) RIP assay revealed that LINC00675 could bind to LSD1 more intensely upon LINC00675 overexpression. (C) After treating GC cells with si-LSD1, the expression of SPRY4 was downregulated. (D) ChIP assay suggested that SPRY4 exhibited a decreased H3K4me2 level at its promoter region in LINC00675-overexpressing cells compared to control cells. (E) After treating GC cells with si-LSD1, ChIP assay results showed that the binding capacity of H3K4me2 and the SPRY4 gene promoter was reduced. (F) IP experiment was conducted and revealed that the binding capacity of LSD1 and H3K4me2 was increased upon LINC00675 overexpression. *p < 0.05, **p < 0.01.

cervical cancer progression via increasing the protein level of Bcl-2 and decreasing the expression of Bax.[15] In other cases, LINC00675 has been identified as tumor suppressors, in which it was downregulated in colorectal cancer and acted on miR-942 and Wnt/β-catenin signaling, thus inhibiting tumor development.[14] In GC, Zeng et al.[13] has demonstrated that LINC00675 had a lower expression level in cancer tissues and cells relative to normal tissues and cells and functioned as a tumor suppressor by interacting with vimentin protein, which enhanced the phosphorylation level of Ser83 and contributed to the collapse of vimentin filament.

In our study, we verified that LINC00675 acted as a tumor-suppressor gene in GC. H3K4me2 is a post-translational modification in the lysine residue at the promoter regions of genes, and a high methylation level of H3K4me2 promotes gene expression. LSD1 functions as a histone demethylase via binding to and interfering with the methylation of H3K4me2.[20,21] As shown in Figure 7, mechanically, LINC00675 bound with LSD1 and resulted in a stronger binding of LSD1 and its target H3K4me2, which eventually proved to reduce the expression level of H3K4me2 at the promoter region of SPRY4 and suppress the gene transcription of SPRY4 (Figure 7). Most previ-

ous studies concerning lncRNA-LSD1 binding have tended to focus on highly expressed lncRNAs that acted as oncogenes in cancer development.[53–56] lncRNAs were indicated to function as scaffolds to bind with LSD1 and/or some other functional proteins and recruit LSD1 to the promoter regions of target genes to induce demethylation and suppress gene transcriptions.[11,40–42] For example, HOTAIR, an oncogenic lncRNA, could recruit the polycomb repressive complex 2 (PRC2) and LSD1 to the promoter region of the PRB gene, thus enhancing the H3K27me3 level and decreasing the H3K4me2 level and epigenetically suppressing PRB expression.[40] LINC00460 could also enhance tumorigenesis in GC by directly interacting with EZH2 and LSD1, resulting in downregulation of cyclin G2 (CCNG2), which acted as a tumor-suppressor gene.[41]

However, our hypothetic mechanism may be different from the direct action of LSD1 shown in previous studies, since we focused on a tumor-suppressor gene LINC00675. Our research illustrated that LINC00675 bound to oncogene LSD1 upon overexpression, which was equivalent to partially inhibiting the functional role of LSD1. According to the direct action mechanism, if the demethylation function of LS D1 was weakened, the expression of H3K4me2 at the promoter
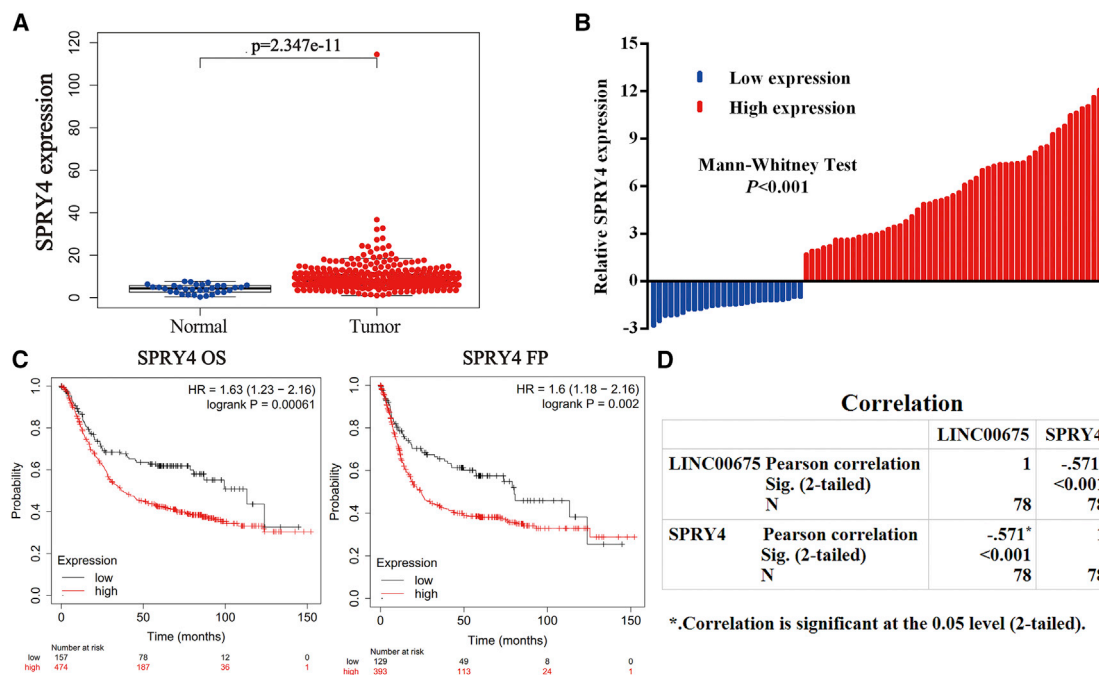
Figure 5. SPRY4 Is Upregulated in Human GC Tissues and Cells and Is Associated with Poor Prognosis

(A) The expression level of SPRY4 was analyzed in TCGA. SPRY4 was upregulated in GC tissues compared to nontumor tissues. (B) SPRY4 was detected in 78 pairs of GC tissues and nontumor tissues by qRT-PCR. The levels of SPRY4 in GC tissues were significantly higher than those in nontumor tissues. (C) Online Kaplan-Meier analysis showed that GC patients with higher expression of SPRY4 had shorter median OS and FP time, which predicted poor prognosis. (D) SPRY4 expression in paired GC tissues and normal gastric tissues was found negatively correlated with LINC00675 expression.

regions of downstream genes should be upregulated. This may explain the upregulation of certain genes that acted as tumor suppressors as LINC00675 did. However, we elected an oncogene SPRY4 as the target gene, whose expression level was significantly downregulated upon LINC00675 overexpression, thus suggesting that there must be other different underlying mechanisms affecting the expression of downstream oncogenes. After overexpressing LINC00675, we found that the binding capacity of LSD1 and H3K4me2 was largely enhanced by IP assay, whereas the binding capacity of the H3K4me2 and SPRY4 promoter was weakened by the ChIP assay, indicating that once LINC00675 bound with LSD1, LSD1 could bind with H3K4me2 more closely, thus weakening the binding of H3K4me2 and the promoter regions of target genes and suppressing gene expressions. In conclusion, we presented a novel mechanism through which tumor-suppressor gene LINC00675 inhibited GC progression via binding to LSD1. However, the specific conformational changes of protein domains and the spatial conformational changes between H3K4me2 histone and the promoter regions still need further study.

Spry proteins, which include four homologs, represent regulators of receptor tyrosine kinase (RTK)-driven signaling pathways.[57,58] Primarily, Spry proteins interfere with mitogen-activated protein kinase (MAPK)-extracellular-regulated protein kinase (ERK) activation specifically, as well as the phosphatidylinositol 3-kinase (PI3K) pathway.[26] Members of the Spry family have been proven to function as oncogenes or tu-

mor-suppressor genes in different kinds of cancers. For instance, tumor-promoting effects of SPRY2 and SPRY1 could be seen in colon carcinoma[59,60] and rhabdomyosarcoma,[61] respectively. Repression of SPRY2 was revealed to interfere with tumor progression of glioblastoma (GBM).[62,63] Das et al.[31,32] also found that SPRY4 was highly expressed in TGCT and miR-302s could induce SPRY4 expression and activate the MAPK-ERK pathway, thus promoting TGCT tumor development. In other circumstances, SPRY2 and SPRY4 could act as tumor suppressors in liver,[64] lung,[27] breast,[29] colon,[28] and esophagus[30] carcinoma. With the consideration of these results, the Spry family may exert different functions depending on different tumor types. In our research, we confirmed that SPRY4 could act as an oncogene in GC for the first time, and we would continue to work on its underlying functional mechanism in the future.

Moreover, new treatment perspectives have been offered for GC treatment via proposing several targeted approaches. Since LSD1 acted as a functional promoter in cancer cell proliferation and migration, it has been proven to be a good candidate for therapeutic molecular targets.[65–67] Our study demonstrated that LINC00675 could competitively bind to LSD1; thus, it may be utilized as a LSD1 inhibitor for cancer treatment in the future.

Conclusions

In summary, our research suggested that LINC00675 was downregulated in GC tissues and cells and functioned as a tumor-suppressor

Table 2. Correlation between SPRY4 Expression and Clinicopathological Features of GC (n = 78)

Clinical Parameter	SPRY4		Chi-Square Test p Value
	High-Expression Cases (n = 52)	Low-Expression Cases (n = 26)	
Age (Years)			0.572
<50	21	13	
>50	31	13	
Gender			0.935
Male	32	15	
Female	20	11	
Location			0.126
Distal	22	13	
Middle	23	6	
Proximal	7	7	
Size			0.173
>5 cm	30	10	
<5 cm	22	16	
Histologic Differentiation			0.128
Well	3	2	
Moderately	14	10	
Poorly	33	10	
Undifferentiated	2	4	
Invasion Depth			0.001[a]
T1	9	9	
T2	16	14	
T3	17	3	
T4	10	0	
TNM Stages			< 0.001[a]
I	11	16	
II	11	6	
III	23	4	
IV	7	0	
Lymphatic Metastasis			0.008[a]
Yes	32	7	
No	20	19	
Regional Lymph Nodes			0.002[a]
pN0	19	19	
pN1	5	4	
pN2	11	1	
pN3	17	2	
Distant Metastasis			0.088
Yes	7	0	
No	45	26	

[a]p < 0.05.

gene in GC development. Since LSD1 functioned as a histone demethylase via binding to and interfering with methylation of H3K4me2, we proposed a novel mechanism in which LINC00675 was demonstrated to lead LSD1 to bind with H3K4me2 more intensely, thus decreasing the H3K4me2 level at the SPRY4 promoter region and downregulating SPRY4 expression. Furthermore, SPRY4 was found to act as an oncogene in GC for the first time and could be regulated by LINC00675 through LSD1 binding.

MATERIALS AND METHODS

Tissue Collection and Ethics Statement

A total of 78 paired GC tissues and nontumor tissues were from patients with GC at the First Affiliated Hospital of Nanjing Medical University. The research was approved by the Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, People's Republic of China) and was conducted in compliance with the Declaration of Helsinki Principles. Written, informed consents were obtained from all patients.

Cell Culture

BGC-823, AGS, SGC-7901, MGC-803, and GES-1 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences. The cells were maintained in RPMI 1640 or DMEM (Gibco), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 37°C with 5% CO_2.

RNA Extraction and qRT-PCR Analyses

Total RNAs from cells and tissue samples were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then RNA (1 μg) was reversely transcribed to cDNA using PrimerScript RT Master Mix (Takara). cDNA was used for real-time PCR assays utilizing SYBR Premix Ex Taq (Takara). Results were normalized to the expression of β-actin. Primer sequences were listed in Table S2.

Plasmid Constructs

LINC00675 cDNA was synthesized and inserted into the expression vector pcDNA3.1 (Genepharma, Shanghai, People's Republic of China). LSD1 and SPRY4 siRNAs (si-LSD1, si-SPRY4-1, and si-SPRY4-2) and scrambled negative control siRNAs (si-NCs) were purchased from Invitrogen. SPRY4 shRNA was inserted into pLKO vectors. Plasmid vectors for transfection were prepared using DNA Miniprep or Midiprep kits (QIAGEN) before transfection.

Transfection of Cell Lines

The siRNAs and plasmid vectors were transfected into GC cells using Lipofectamine 3000 (Invitrogen), according to the instructions. The cells were collected for subsequent qRT-PCR and western blotting analyses after 48 h.

Cell Proliferation Analysis

Cell viability was tested using CCK8 (Beyotime Biotechnology), according to the manufacturer's instructions. For colony-formation assay, cells were inoculated into 6-well plates and maintained with 10% FBS-supplemented medium for 2 weeks. Cell colonies were fixed with 4% formaldehyde for 2 min, and colonies were stained with
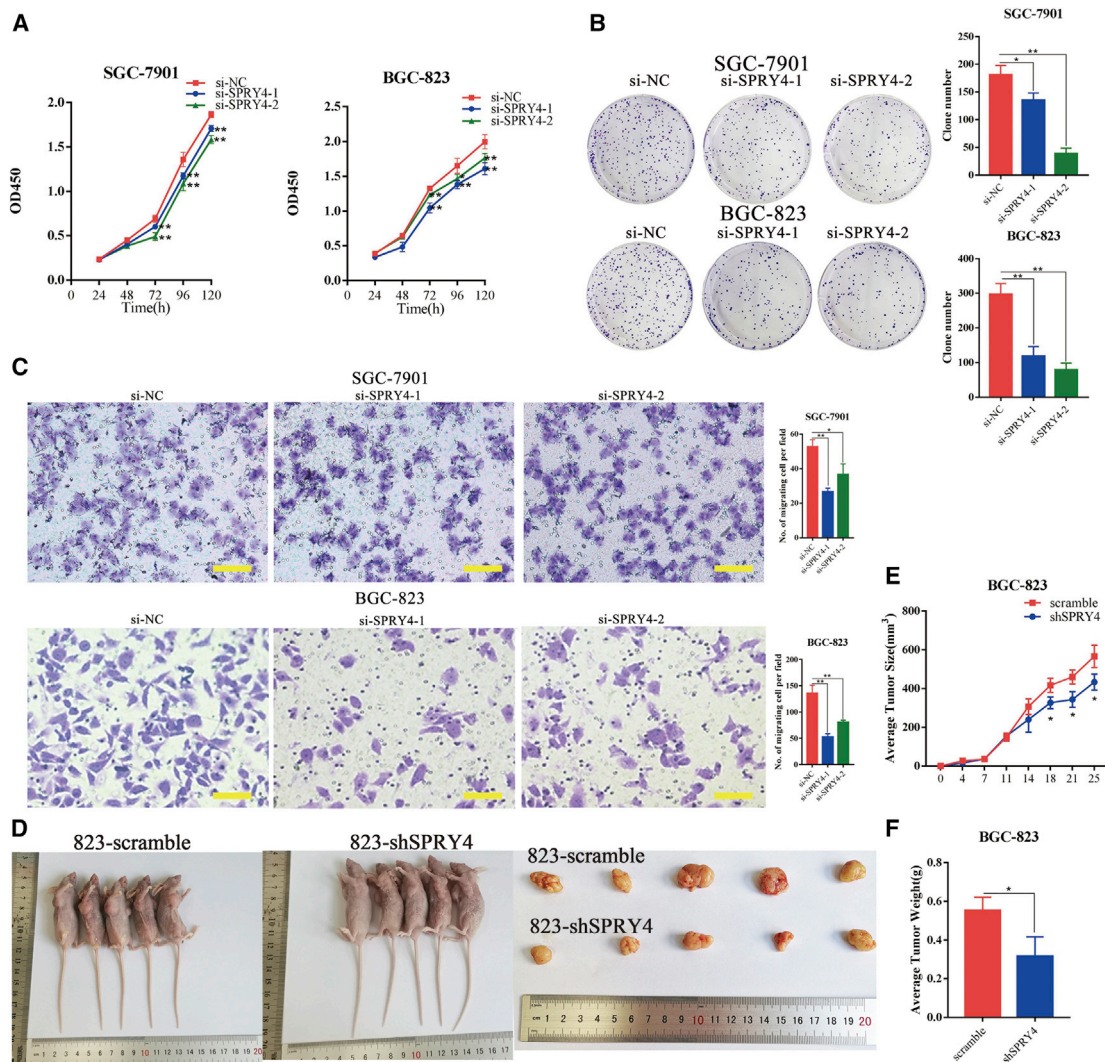
Figure 6. SPRY4 Acts as an Oncogene in GC Cell Proliferation and Migration

(A) CCK-8 assays were conducted to determine cell proliferation of SGC-7901 and BGC-823 cells after transfection of si-SPRY4 plasmids and control plasmids. (B) Colony-formation assays were performed to identify the effects of SPRY4 on GC cell proliferation. (C) Transwell assays showed that downregulated SPRY4 expression could suppress GC cell migration (scale bars, 100 μm). (D–F) BGC-823 cells transfected with scramble or shSPRY4 were injected into five nude mice, respectively (D; total n = 10). Tumor weights and sizes were represented as means of tumor weights (F)/sizes (E) ± SD. *p < 0.05, **p < 0.01.

crystal violet solution for 2 h. Colonies containing more than 50 cells were counted, and the mean colony numbers were calculated.

Flow Cytometry Analysis

For apoptosis analysis, a total of 1×10^6 cells were collected and mixed with 70% ethanol to fix at −20°C overnight. Then the cells were stained with propidium iodide (PI), according to the instruction of the CycleTEST PLUS DNA Reagent Kit (BD Biosciences) and analyzed in the flow cytometer.

Cell Migration Assays

Transwell assays were performed to detect cell migration. For the Transwell assay, 5×10^4 cells in serum-free media were seeded into

the upper Transwell chambers (Corning), and the medium with 10% FBS was added to the lower chamber. After 24 h incubation, cells that had migrated from the upper chamber into the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet and then counted with a microscope (Olympus). All of the experiments were repeated three times.

In Vivo Assay

4-week-old male athymic BALB/c nude mice were purchased from the Animal Center of Nanjing Medical University and raised under pathogen-free conditions. For cell-proliferation assay *in vivo*, BGC-823 cells stably transfected with LINC00675 and empty vector (or shSPRY4 and scrambled vector) were harvested. Then cells were
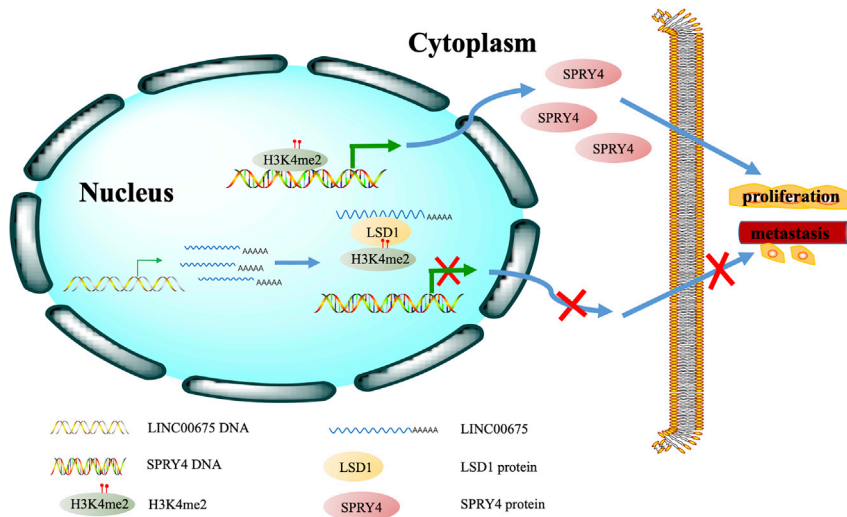
Figure 7. Proposed Model of the Functional Mechanism through Which LINC00675 Suppresses GC Development

H3K4me2 is a post-translational modification in the lysine residue at the promoter regions of genes and promotes gene expression. LINC00675 leads the histone demethylase LSD1 to bind with H3K4me2 more intensely, thus weakening the binding of H3K4me2 and the promoter region of oncogene SPRY4 and downregulating SPRY4 expression, which suppresses GC cell proliferation and migration.

injected subcutaneously into one side of each mouse. The tumor volumes and weights were determined every 4 days. 25 days after injection, mice were sacrificed, and tumors were removed to measure the weight and used for further analysis. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

Subcellular Fractionation Location and FISH Assay

The nuclear and cytosolic fractions were separated according to the instructions of the PARIS kit (Life Technologies). For the FISH assay, GC cell lines were fixed in 4% paraformaldehyde for 15 min and followed by washes with PBS. Fixed cells were treated with Triton X-100 and subsequently treated with a FISH kit (Ribo-Bio, People's Republic of China), following the manufacturer's instructions.

Western Blot Assay and Antibodies

Cells were treated with radioimmunoprecipitation assay (RIPA) (Beyotime), supplemented with a protease inhibitor cocktail (Roche). Cell protein lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride membranes (Millipore), and then incubated with H3K9me2, LSD1, H3K4me2, SPRY4, and β-actin antibodies (Cell Signaling Technology), followed by the incubation of secondary antibodies. Specific bands were exposed using the enhanced chemiluminescence (ECL) chromogenic substrate (Bio-Rad) in visualizer (Tanon, People's Republic of China).

RIP Assays

RIP experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA), according to the manufacturer's instructions. Antibody for RIP assays of LSD1 was from Abcam. A RT-PCR assay was conducted to measure the co-precipitated RNAs.

Coimmunoprecipitation (CoIP) and Western Blotting

coIP assays were performed using a coIP kit (Thermo Fisher Scientific), based on previously described procedures.[30] Precipitates were purified and analyzed by western blotting.

ChIP Assays

ChIP experiments were performed with a MagnaChIP kit (Millipore), according to the manufacturer's instructions. Cells were treated with formaldehyde and incubated for 10 min to trigger DNA-protein crosslinking. Cell lysates were sonicated to produce 200–300 bp chromatin fragments. Then the lysates were immunoprecipitated with H3K4me2 antibody and rabbit immunoglobulin G (IgG) used as a control. The precipitated chromatin DNA was analyzed via qRT-PCR. Primer sequences were listed in Table S2.

Bioinformatics Analyses

Prediction of the interaction between LINC00675 and LSD1 was performed according to a previously described method.[30]

Statistical Analysis

Statistical analysis was performed using SPSS software (SPSS, Chicago, IL, USA). The significance of differences between groups was estimated by Student's t test, χ^2 test, or Wilcoxon test, as appropriate. Data were expressed as the mean ± standard deviation (SD) from at least three independent experiments. Statistical significance was accepted for a p value <0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2020.09.038.

AUTHOR CONTRIBUTIONS

Y.P., Y.F., M.X., and Y.L. contributed to designing and organizing the experiments, carrying out the data analysis, and writing the manuscript. Y.P., T.Y., X.W., and T.X. contributed to laboratory

measurements and data analysis. P.M. and Y.S. contributed to conceiving the ideas, supervising the study, and writing the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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