Cancer Research

LncRNA HOXA11-AS Promotes Proliferation and Invasion of Gastric Cancer by Scaffolding the Chromatin Modification Factors PRC2, LSD1, and DNMT1

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Abstract

Long noncoding RNAs (lncRNA) have been implicated in human cancer but their mechanisms of action are mainly undocumented. In this study, we investigated lncRNA alterations that contribute to gastric cancer through an analysis of The Cancer Genome Atlas RNA sequencing data and other publicly available microarray data. Here we report the gastric cancer–associated lncRNA HOXA11-AS as a key regulator of gastric cancer development and progression. Patients with high HOXA11-AS expression had a shorter survival and poorer prognosis. *In vitro* and *in vivo* assays of HOXA11-AS alterations revealed a complex integrated phenotype affecting cell growth, migration, invasion, and apoptosis. Strikingly, high-throughput sequencing analysis after HOXA11-AS silencing highlighted alterations in cell proliferation and cell–cell adhesion pathways. Mechanistically, EZH2 along with the histone demethylase LSD1 or DNMT1 were recruited by HOXA11-AS, which functioned as a scaffold. HOXA11-AS also functioned as a molecular sponge for miR-1297, antagonizing its ability to repress EZH2 protein translation. In addition, we found that E2F1 was involved in HOXA11-AS activation in gastric cancer cells. Taken together, our findings support a model in which the EZH2/HOXA11-AS/LSD1 complex and HOXA11-AS/miR-1297/EZH2 cross-talk serve as critical effectors in gastric cancer tumorigenesis and progression, suggesting new therapeutic directions in gastric cancer. *Cancer Res; 76(21); 6299–310. ©2016 AACR.*

Introduction

Gastric cancer is one of the leading causes of cancer-related death worldwide and the most common gastrointestinal malignancy in East Asia (1, 2). Despite improvements in surgical techniques and targeted drug chemotherapy, the 5-year overall survival rate remains unsatisfactory owing to diagnosis of most patients at an advanced stage accompanied by lymphatic metastasis that limits successful therapeutic strategies (3). Furthermore, the molecular mechanisms underlying gastric cancer progression and tumor metastasis remain poorly understood. Therefore, better understanding of tumorigenesis and progression is essential for the development of diagnostic markers and novel effective therapies for gastric cancer patients.

Noncoding RNAs have recently emerged as critical regulators of gene expression (4). Long noncoding RNAs (lncRNA) are a class of ncRNAs longer than 200 nucleotides and lack of protein-coding ability. LncRNAs play essential roles in normal development and human diseases. LncRNAs participate in several biological processes, such as embryonic stem cell (5), Th-cell differentiation (6), autophagy and myocardial infarction (7), cell senescence (8), cancer cell apoptosis and metastasis (9), and chemotherapy drug resistance (10). Several studies have shown that lncRNA expression is tissue specific and frequently dysregulated in various types of cancers and some lncRNAs are correlated with cancer recurrence and poor prognosis (11). These lncRNAs involve in tumorigenesis and cancer metastasis through silencing tumor suppressors or activation of oncogenes via different mechanisms including epigenetic modification, alternative splicing, RNA decay, and post-translational modification regulation (12). Thus, lncRNAs have been highlighted as new players in tumorigenesis by functioning as tumor suppressors, oncogenes or both, depending on the circumstances.

Several lncRNAs have been well characterized in gastric cancer to date, particularly HOTAIR, ANRIL, H19, GAPLINC, PVT1, and LINC00152 (13). GAPLINC is highly expressed in gastric cancer and promotes cell proliferation and migration via regulating

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CD44 expression as a molecular decoy for miR211-3p (14). Overexpression of H19 promotes gastric cancer cell proliferation and invasion via actively binding to ISM1 (15). We previously found that HOTAIR promotes cell proliferation by completely sponging miR331-3p (16), whereas ANRIL promotes cell growth via epigenetically suppressing miR99a/449a in gastric cancer (17). GAS5 and MEG3 function as tumor suppressors in gastric cancer (18, 19). However, investigation of further gastric cancer–associated lncRNAs is warranted.

We speculated that there is still a large number of previously unexplored lncRNA alterations in gastric cancer, especially in the expression patterns of gastric cancer–specific lncRNAs. To conduct a comprehensive characterization of aberrantly expressed lncRNAs in gastric cancer, we analyzed The Cancer Genome Atlas (TCGA) stomach cancer and normal tissue RNA sequencing data (33 normal and 222 cancer samples) and four independent microarray datasets from Gene Expression Omnibus (GEO; 71 pairs of cancer and normal tissue samples). In addition to identifying several gastric cancer–associated lncRNAs, we discovered a gastric cancer–specific upregulated lncRNA termed HOXA11-AS, which only displayed remarkable increasing expression levels from normal gastric to carcinoma tissues. Its functional association and underlying molecular mechanism and the effector involved in its overexpression was determined.

Materials and Methods

RNA sequencing and microarray data analysis

Gastric cancer and other cancer gene expression data were downloaded from the TCGA and GEO dataset. The independent datasets from GSE50710 (14), GSE58828, GSE65801 (20), GSE51575 (21), GSE29431, GSE54002 (22), GSE20842 (23), GSE21510 (24), GSE56140 (25), GSE57957 (26), GSE53624 (27), GSE18842 (28), GSE19188 (29), GSE19804 (30), GSE21933 (31), GSE30219 (32), GSE31210 (33), GSE32863 (34), and GSE43458 (35) were analyzed in this study. The BAM files and normalized probe-level intensity files were downloaded from TCGA and GEO databases, respectively. The probe sequences were downloaded from GEO or microarray manufacturers, and bowtie was used to reannotate probes according to GENCODE Release 19 annotation for lncRNAs. For multiple probes corresponding to one gene, the probe with the maximum signal was selected to generate expression of lncRNAs.

Study subjects

We obtained 85 paired gastric cancer and adjacent nontumor tissues from patients who underwent surgery at Jiangsu Province Hospital between 2010 and 2011 and who were diagnosed with gastric cancer based on histopathologic evaluation. No local or systemic treatment was conducted in these patients before surgery. All collected tissue samples were immediately snap frozen in liquid nitrogen and stored at −80°C until required. The clinical characteristics of all patients are listed in Supplementary Table S1. This study was approved by the Nanjing Medical University.

5′ and 3′ rapid amplification of cDNA ends analysis

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Rapid amplification of cDNA ends (RACE) experiments were performed using the SMARTer RACE 5′/3′ Kit (Clontech Laboratories, Inc.), according to the manufacturer's instructions.

qRT-PCR

qRT-PCR was used to detect expression levels of HOXA11-AS and other genes in gastric cancer tissues and cells, following the manufacturer's instructions (TAKARA). GAPDH was used as the control. Primers are listed in Supplementary Table S1.

Plasmid construction and cell transfection

The full-length complementary cDNA of human HOXA11-AS was synthesized by Invitrogen and cloned into the expression vector pCDNA3.1 (Clontech Laboratories, Inc.). The small hairpin RNA (shRNA) of the HOXA11-AS was provided by Invitrogen. The final construct was verified by sequencing. Plasmid vectors for transfection were prepared using DNA Midiprep Kits (Qiagen), and transfected into gastric cancer cells using Lipofectamine 2000 (Invitrogen). The siRNAs were transfected into gastric cancer cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. All siRNA and shRNA sequences were listed in Supplementary Table S1.

Luciferase reporter assays

The E2F1-binding motif in the promoter region of HOXA11-AS was identified by JASPAR (http://jaspar.genereg.net/). The different fragment sequences were synthesized and then inserted into the pGL3-basic vector (Promega). All vectors were verified by sequencing and luciferase activities were assessed using the Dual Luciferase Assay Kit (Promega), according to the manufacturer's instructions.

Cell proliferation and apoptosis assays

Cell proliferation ability was examined using a Cell Proliferation Reagent Kit I (MTT; Roche Applied Science). Colony formation assays were performed to monitor gastric cancer cells' cloning capability. Gastric cancer cells transfected with si-HOXA11-AS or si-NC were harvested 48 hours after transfection by trypsinization. After double staining with FITC-Annexin V and propidium iodide (PI), the cells were analyzed with a flow cytometer (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences).

In vivo tumor growth assays

Four-week-old female athymic BALB/c nude mice were maintained under specific pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. sh-HOXA11-AS, HOXA11-AS overexpression vector, or empty vector stably transfected BGC823 or AGS cells were harvested. For tumor formation assay, 10^7 cells were subcutaneously injected into a single side of each mouse. Tumor growth was examined every 3 days, and tumor volumes were calculated using the equation $V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH (Bethesda, MD). The protocol was approved by the Committee on the Ethics of Animal Experiments of the Nanjing Medical University.

FISH and subcellular fractionation

Gastric cancer cells were fixed in 4% formaldehyde for 15 minutes and washed with PBS. Fixed cells were treated with pepsin

(1% in 10 mmol/L HCl) and dehydrated through 70%, 90%, and 100% ethanol. The air-dried cells were further incubated with 40 nmol/L FISH probe in hybridization buffer (100 mg/mL dextran sulfate, 10% formamide in 2× SSC) at 80°C for 2 minutes. Hybridization was performed at 55°C for 2 hours and the slide was washed and dehydrated. Finally, the air-dried slide was mounted with Prolong Gold Antifade Reagent with DAPI for detection. RNA FISH probes were designed and synthesized by Bogu Co., Ltd. Separation of nuclear and cytosolic fractions was performed using the PARIS Kit (Life Technologies) according to the manufacturer's instructions.

RNA immunoprecipitation

The EZMagna RNA immunoprecipitation (RIP) Kit (Millipore) was used following the manufacturer's protocol. BGC-823 and SGC-7901 cells were lysed in complete RIP lysis buffer, and the cell extract was incubated with magnetic beads conjugated with specific antibodies or control IgG (Millipore) for 6 hours at 4°C. Beads were washed and incubated with Proteinase K to remove proteins. Finally, purified RNA was subjected to qRT-PCR analysis. All antibody information is listed in Supplementary Table S1.

RNA pull-down assays

RNAs (HOXA11-ASF, HOXA11-ASD1, HOXA11-ASD2) were *in vitro* transcribed using T7 RNA polymerase (Ambio Life), purified using the RNeasy Plus Mini Kit (Qiagen), and treated with RNase-free DNase I (Qiagen). Transcribed RNAs were biotin labeled with the Biotin RNA Labeling Mix (Ambio Life). Positive, negative, and biotinylated RNAs were mixed and incubated with BGC823 cell lysates. Magnetic beads were added to each binding reaction, followed by incubation at room temperature. Beads were washed with washing buffer and eluted proteins were examined by Western blot analysis.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) experiments were performed using the MagnaChIP Kit (Millipore) according to the manufacturer's instructions as described previously (36).

Western blotting, immunocytochemistry, and immunofluorescence analysis

Western blot, immunocytochemistry, and immunofluorescence analyses were performed according to standard protocols as described previously (37).

Statistical analysis

Differences between groups were assessed by a paired, two-tailed Student *t* test. The Mann–Whitney *U* test was used to analyze the pathologic feature of HOXA11-AS expression in gastric cancer. The survival curves are drawn using Kaplan–Meier survival plot and tested using log-rank tests. The univariate and multivariate Cox proportional hazards modeling was used to determine the effects of variables on survival. Correlations between HOXA11-AS and *KLF2* or *PRSS8* were analyzed by Spearman rank correlation ($P < 0.05$). All statistical analyses were performed using SPSS17 software (Abbott Laboratories).

Results

The antisense-transcribed lncRNA HOXA11-AS is specifically upregulated in gastric cancer

To identify the lncRNAs that are involved in gastric tumorigenesis, we performed an integrative analysis of TCGA stomach cancer and normal tissue RNA sequencing data and gastric cancer microarray profile comprising GSE58828, GES50710, GSE51575, and GSE65801 from GEO datasets. We identified 137 lncRNAs misregulated in the TCGA data, 184 in GSE58828/GES50710 datasets, and 118 in GSE51575/GSE65801 datasets (fold change > 4.0, $P < 0.05$; Fig. 1A; Supplementary Table S1). However, the GSE51575 and GSE65801 datasets that used the Agilent-028004 SurePrint G3 Human GE 8 × 60 K Microarray platform only mapped 2966 lncRNAs, which led to only six lncRNAs consistently upregulated and four downregulated in all datasets (Fig. 1B). Then, we focused on overexpressed lncRNAs because these lncRNAs may be more readily used as early diagnosis markers or therapeutic targets.

To validate these findings, we analyzed the expression levels of six upregulated lncRNAs in 12 pairs of GC and nontumor tissues using qPCR. Among the six lncRNAs, HOXA11-AS is the most upregulated lncRNA (Supplementary Fig. S1A). Interestingly, further analysis of nine other cancers (lung squamous cell carcinoma, lung adenocarcinoma, kidney chromophobe, kidney renal clear cell carcinoma, kidney renal papillary cell carcinoma, liver hepatocellular carcinoma, prostate adenocarcinoma, thyroid carcinoma, and uterine corpus endometrioid carcinoma) using TCGA sequencing data or microarray datasets showed that HOXA11-AS is specifically upregulated in gastric cancer tissues (Fig. 1C; Supplementary Fig. S1B). Similar results were also found in an independent cohort of 12 paired esophageal squamous cell carcinoma, colorectal cancer, hepatocellular carcinoma, and non–small cell lung cancer and nontumor tissues using qPCR (Supplementary Fig. S1C). Therefore, we focused on HOXA11-AS and investigated its expression pattern, clinical significance, and biologic function in gastric cancer.

In situ hybridization assays showed that HOXA11-AS is expressed in tumor tissues, but not in adjacent normal tissue (Fig. 1D). We next analyzed an independent cohort of 85 paired gastric cancer and nontumor tissues and confirmed that HOXA11-AS was highly upregulated (fold change >1.5, $P < 0.01$) in 80% (68/85) of cancerous tissues (Fig. 1E). Similar results were found in gastric cancer cell lines (Supplementary Fig. S3A).

HOXA11-AS expression is correlated with gastric cancer progression and poor prognosis

To investigate the correlation between HOXA11-AS expression and gastric cancer patient clinical features, all patients were divided into high and low HOXA11-AS expression level groups according to the median value (Supplementary Fig. S1D). Higher HOXA11-AS expression levels were significantly correlated with larger tumor size ($P = 0.03$), poor differentiation ($P = 0.03$), advanced pathologic stage ($P = 0.003$), and lymph node metastasis ($P = 0.009$) in gastric cancer patients, but not associated with other factors including sex and age (Supplementary Table S2). Kaplan–Meier survival analysis showed that the overall survival and progression-free survival rates over 3 years for the high HOXA11-AS group were lower than those in the low HOXA11-AS group (Fig. 1F).

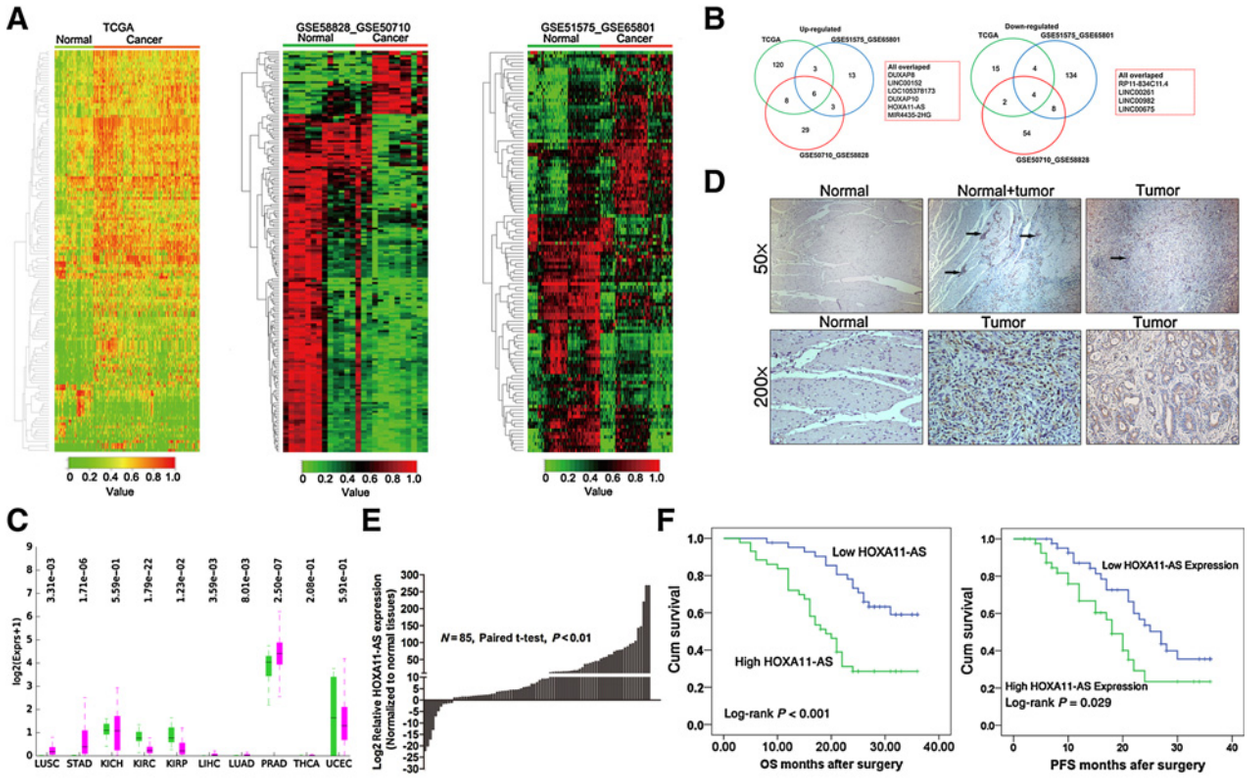

Figure 1.

lncRNA HOXA11-AS is overexpressed in gastric cancer tissues. **A,** hierarchical clustering analysis of lncRNAs that were differentially expressed (fold change > 2; $P < 0.05$) in gastric cancer and normal tissues. **B,** overlap of misregulated lncRNAs in TCGA data and GEO datasets. **C,** analyses of HOXA11-AS expression levels in lung squamous cell carcinoma (LUSC), stomach adenocarcinoma (STAD), kidney chromophobe (KICH), kidney renal clear cell carcinoma (KIRC), kidney renal papillary cell carcinoma (KIRP), liver hepatocellular carcinoma (LIHC), lung adenocarcinoma (LUAD), prostate adenocarcinoma (PRAD), thyroid carcinoma (THCA), and uterine corpus endometrioid carcinoma (UCEC) using TCGA sequencing data. **D,** *in situ* hybridization was used to detect HOXA11-AS expression in gastric cancer tissues and adjacent nontumor tissues. **E,** HOXA11-AS expression was analyzed by qRT-PCR in gastric cancer and adjacent nontumor tissues ($n = 85$). **F,** Kaplan–Meier survival analysis of overall survival and disease-free survival in gastric cancer patients ($P < 0.001$ for both overall survival and disease-free survival) based on HOXA11-AS expression. **, $P < 0.01$.

Univariate Cox regression analyses identified three prognostic factors: lymph node metastasis, TNM stage, and HOXA11-AS expression level. Other clinicopathologic features such as sex and age were not statistically significant prognosis factors. Multivariate Cox regression analyses showed that TNM stage ($P = 0.024$) was an independent prognostic factor for gastric cancer patients (Supplementary Table S2).

E2F1 activated HOXA11-AS transcription in gastric cancer cells

Our previous study and other reports demonstrated that transcription of lncRNAs is regulated by transcription factors such as p53, SP1, and E2F1. To investigate the potential regulators involved in HOXA11-AS overexpression, we first scanned for potential transcription factor–binding sites in the HOXA11-AS promoter and E2F1- and SP1-binding sites were found (Fig. 2A and Supplementary Fig. S2A). To assess the correlation between HOXA11-AS expression and E2F1 target gene signature, we performed the gene-set enrichment analysis (GSEA) of the dataset GSE15459 using a signature of E2F1-target genes V$E2F1_Q4_01 from the Molecular Signature Database. The positive enrichment score obtained from the analysis is indicative of a high enrichment in the expression of E2F1 target gene signature in the tumor subset with high HOXA11-AS expression. Meanwhile, correlation analysis revealed that HOXA11-AS has a significantly positive correlation with in the GSE15459 dataset (Fig. 2B). Moreover, E2F1 overexpression promoted HOXA11-AS expression and knockdown of E2F1 decreased HOXA11-AS expression, whereas upregulation or downregulation of SP1 had no effect on HOXA11-AS expression (Fig. 2C and Supplementary Fig. S2B and S2C). ChIP assays showed that E2F1 directly bound to the HOXA11-AS promoter region (Fig. 2D). In addition, luciferase report assays indicated that E2F1 binds to the E2 (-1612 bp) binding site, but not the other two sites (Fig. 2E).

HOXA11-AS promotes gastric cancer cell proliferation, migration, and invasion and inhibits apoptosis

The biologic function of HOXA11-AS in regulating cancer cell phenotype remains unclear. Therefore, we next performed loss-of-function and gain-of-function studies in gastric cancer cells. First, 5′ and 3′ RACE analysis were performed to determine the full-length HOXA11-AS (Supplementary Fig. S2D). After detecting the expression levels of HOXA11-AS in seven gastric cancer cell lines and normal gastric mucosa GES1 cells, we performed knockdown of HOXA11-AS in BGC823, SGC7901, and MGC803 cells that

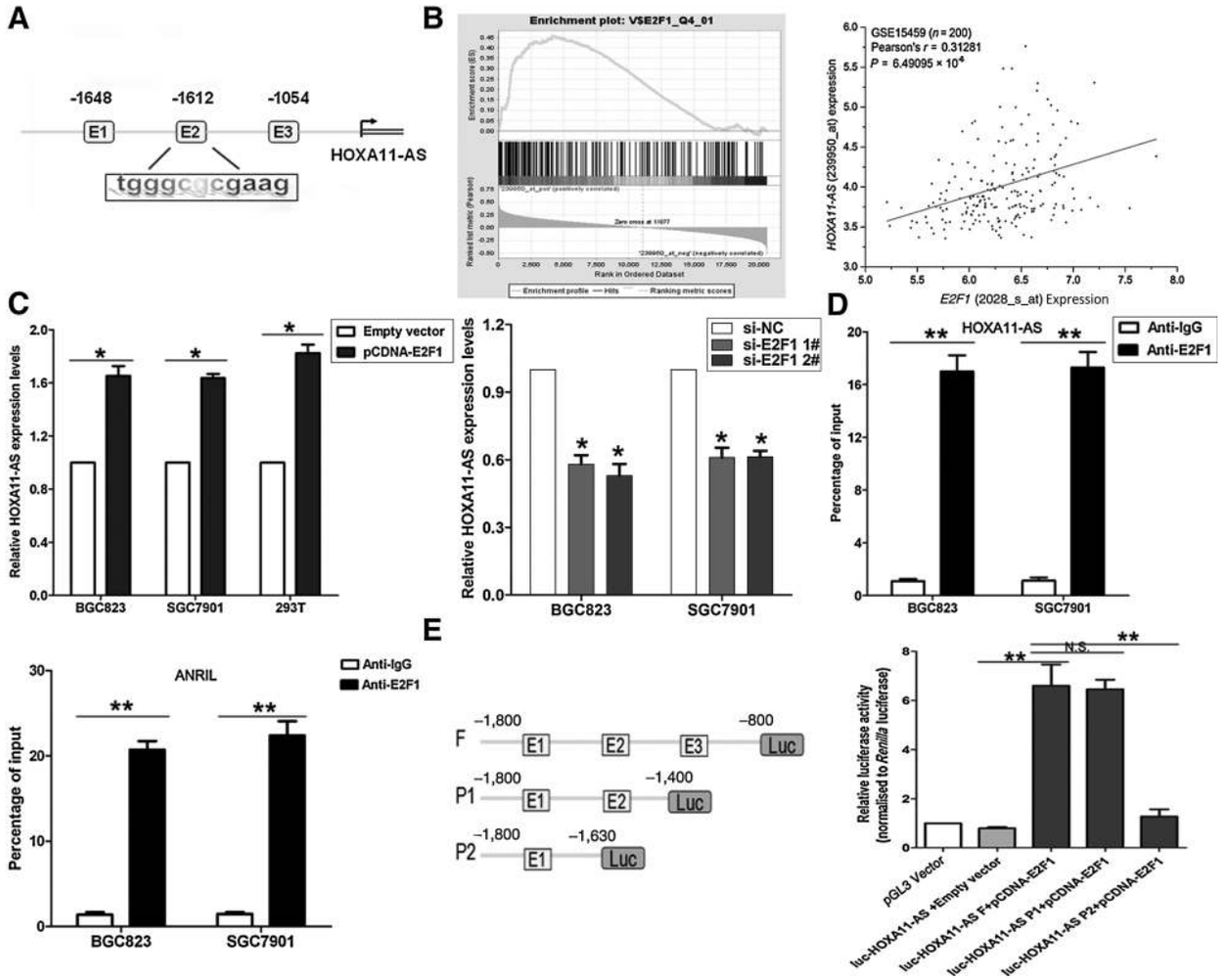

Figure 2.

E2F1 promotes HOXA11-AS transcription in gastric cancer cells. **A,** prediction of E2F1 binding sites in the HOXA11-AS promoter region using JASPAR. **B,** the correlation between E2F1 and HOXA11-AS expression was detected by analyzing GSE15459 data. **C,** HOXA11-AS expression was detected by qRT-PCR in BGC823, SGC7901, and 293T cells transfected with E2F1 vector. **D,** ChIP assays were performed to detect E2F1 occupancy in the HOXA11-AS promoter region. ANRIL was used as a positive control and IgG as a negative control. **E,** dual luciferase reporter assays were used to determine the E2F1 binding on the HOXA11-AS promoter region. *, $P < 0.05$; **, $P < 0.01$.

with higher HOXA11-AS expression and upregulated its expression in AGS cells that with low HOXA11-AS expression (Supplementary Fig. S3B). MTT and colony formation assays showed that knockdown of HOXA11-AS impaired gastric cancer cell proliferation, whereas HOXA11-AS overexpression promoted cell proliferation *in vitro* (Fig. 3A and B and Supplementary Fig. S4C).

As our results showed that HOXA11-AS exerts an oncogenic effect in gastric cancer cells, we investigated whether HOXA11-AS was involved in the regulation of cell apoptosis by performing flow cytometry and TUNEL staining analysis. The results showed that knockdown of HOXA11-AS significantly induced gastric cancer cell apoptosis (Fig. 3C and Supplementary Fig. S4D). Furthermore, Transwell assays were performed to investigate the role of HOXA11-AS on gastric cancer cell migration and invasion ability. The results showed that knockdown of HOXA11-AS dramatically decreased cell migration and invasion, while HOXA11-AS overexpression promoted cell migration and invasion (Fig. 3D and E and Supplementary Fig. S4E).

HOXA11-AS promotes gastric cancer cell tumorigenesis *in vivo*

We next injected HOXA11-AS stable knockdown BGC823 cells, HOXA11-AS overexpressing AGS cells, or control cells into nude mice to determine whether HOXA11-AS would influence gastric cancer cells tumorigenesis *in vivo*. The results showed that tumors grown from HOXA11-AS stable knockdown cells were smaller than tumors grown from control cells (Fig. 4A and B). Conversely, tumors grown from HOXA11-AS–overexpressing cells were larger than tumors grown from control cells (Fig. 4C and D). Moreover, hematoxylin and eosin staining showed that tumor tissues collected from the HOXA11-AS knockdown group had fewer Ki67-positive cells, whereas the HOXA11-AS overexpression group had more Ki67-positive cells than the control group (Fig. 4E).

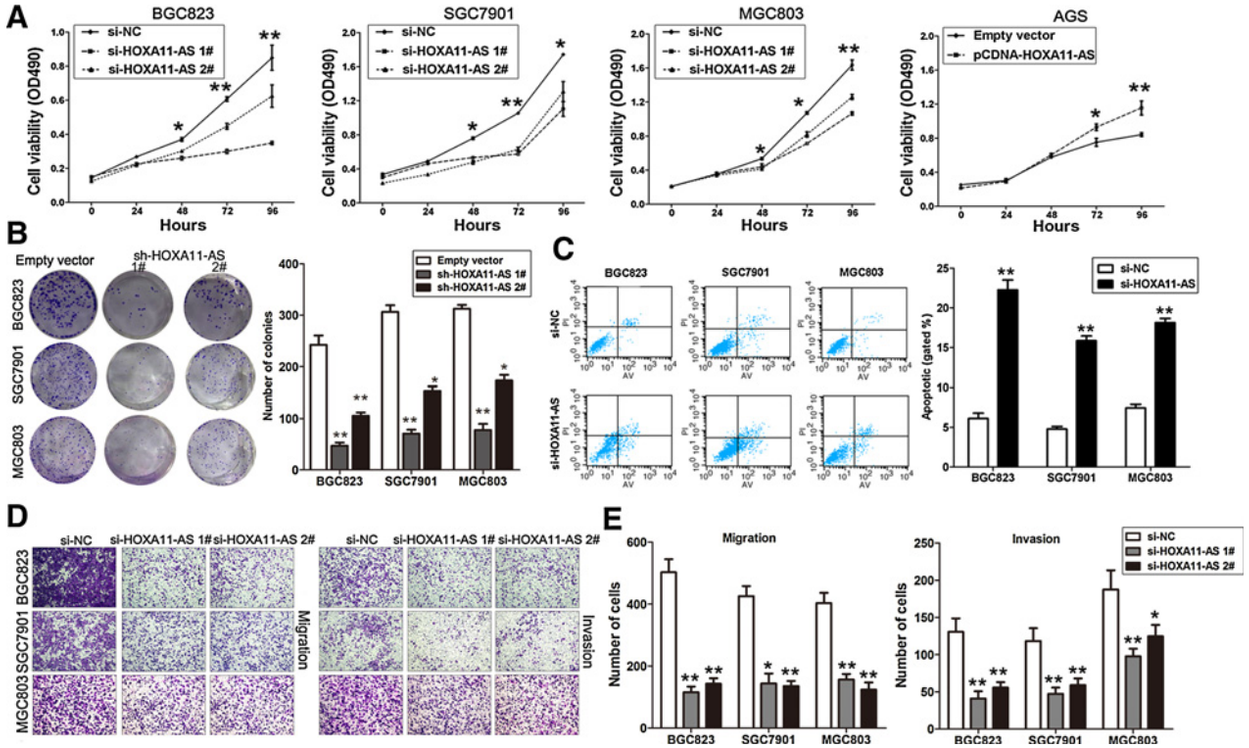

Figure 3.

HOA11-AS promotes cell proliferation, migration, and invasion in GC cells. **A,** MTT assays were used to determine the cell viability of si-HOXA11-AS or HOXA11-AS vector–transfected GC cells. **B,** colony formation assays were used to determine the cell colony formation ability of si-HOXA11-AS–transfected cells. **C,** FACS analysis of the effect of HOXA11-AS on cell apoptosis. **D** and **E,** Transwell assays showed that HOXA11-AS knockdown inhibits gastric cancer cell migration and invasion. *, $P < 0.05$; **, $P < 0.01$.

HOXA11-AS functions as a scaffold for EZH2 and LSD1

To investigate the potential mechanisms of HOXA11-AS in gastric cancer cells, we first examined its distribution in gastric cancer cells using FISH and subcellular fractionation. HOXA11-AS RNAs were more prevalent in the nucleus (Fig. 5A and Supplementary Fig. S4A). Then, we predicted the interaction probabilities of HOXA11-AS and RNA-binding proteins via RNA–protein interaction prediction (http://pridb.gdcb.iastate.edu/RPISeq/), and found that HOXA11-AS potentially binds EZH2, LSD1, DNMT1, AGO2, and STAU1 (as the RF or SVM score > 0.5; Supplementary Fig. S4B). We next performed RIP assays and confirmed that HOXA11-AS directly binds EZH2, LSD1, DNMT1, WDR5, STAU1, and AGO2 in gastric cancer cells (Fig. 5B). In addition, we performed RIP assays in AGS cells transfected with the HOXA11-AS overexpression vector and confirmed that HOXA11-AS directly binds with EZH2 and LSD1 (Supplementary Fig. S4C). Moreover, RNA pull-down assays further confirmed that HOXA11-AS indeed binds with EZH2, LSD1, and AGO2 in gastric cancer cells (Fig. 5C). Immunoprecipitation analysis showed that EZH2 could interact with LSD1 and DNMT1 in gastric cancer cells (Supplementary Fig. S4D).

Our results showed that HOXA11-AS could both bind EZH2 and LSD1, indicating that HOXA11-AS may function as a scaffold for EZH2 and LSD1 in gastric cancer cells. To verify this hypothesis, the HOXA11-AS was cut into HOXA11-AS-D1(1-856 nt) and -D2(857-1565 nt), and their overexpression vectors were constructed and transfected into AGS cells. RIP assays in AGS cells transfected with HOXA11-AS-D1 or -D2 vectors showed that HOXA11-AS-D1 mostly bound with EZH2, whereas HOXA11-AS-D2 bound with LSD1 (Fig. 5D and Supplementary Fig. S4E). RNA pull-down assays showed similar results (Fig. 5E). These data indicated that HOXA11-AS may function as a scaffold and bind with EZH2 at its 5′ region and LSD1 at its 3′ region in gastric cancer cells.

PRSS8 and KLF2 are key downstream targets of HOXA11-AS

There is evidence that antisense transcript lncRNAs can directly or indirectly regulate the expression of the sense genes; however, HOXA11-AS had no influence on HOXA11 expression (Supplementary Fig. S5A). To further investigate the potential genes involved in HOXA11-AS function, we performed RNA-sequencing assays. As shown in Fig. 5F, many genes involved in cell proliferation and cell–cell junction regulation were affected by HOXA11-AS (Supplementary Fig. S5B). Several genes that may contribute to gastric cancer were selected and confirmed by qPCR assays. Among these altered genes, PRSS8 and KLF2 have been identified as novel tumor suppressors that are involved in cancer cell proliferation, apoptosis, and invasion. Hence, we chose KLF2 and PRSS8 for further investigation. Western blot analysis showed that knockdown of HOXA11-AS upregulated PRSS8 and KLF2 in gastric cancer cells (Fig. 5G).

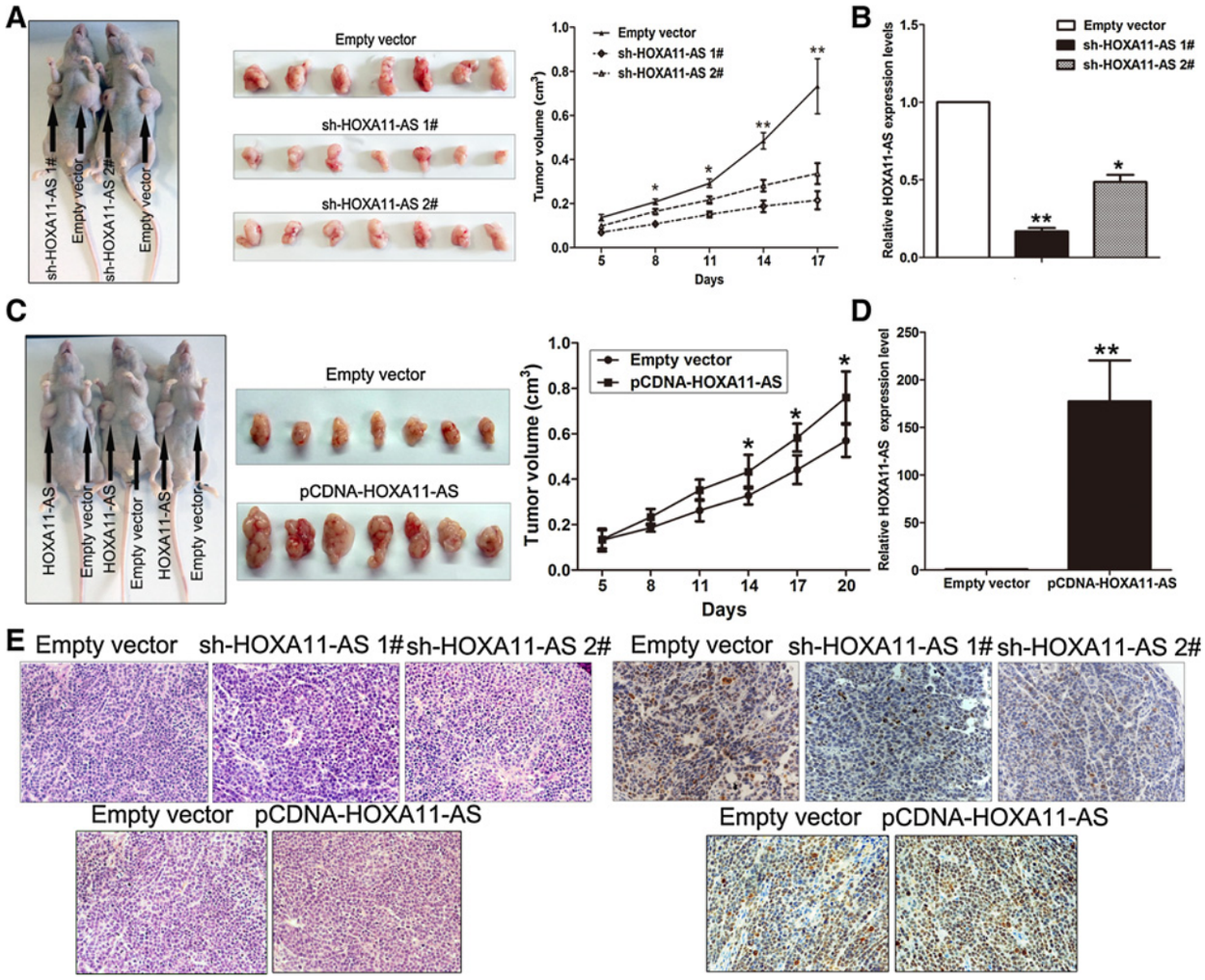

Figure 4.

HOXA11-AS promotes gastric cancer cell tumor growth *in vivo*. **A,** representative images of mice bearing tumors from empty vector, sh-HOXA11-AS 1# and sh-HOXA11-AS 2# groups, and the tumor volume growth curves after injections in different groups. **B,** HOXA11-AS levels were detected in tumors from mice by qRT-PCR. **C,** representative images of mice bearing tumors from empty vector and HOXA11-AS vector groups, and the tumor volume growth curves after injections in two groups. **D,** HOXA11-AS levels were detected in tumors from empty vector and HOXA11-AS vector groups mice by qRT-PCR. **E,** representative images for hematoxylin and eosin staining and Ki67 immunostaining of tumor samples from different groups. *, $P < 0.05$; **, $P < 0.01$.

To determine whether HOXA11-AS–interacting proteins are involved in the regulation of PRSS8 and KLF2, we first treated gastric cancer cells with DNMT1, EZH2, and LSD1 inhibitors and examined the impact on PRSS8 and KLF2 expression. The results showed that 5′aza, GSK343, or OGL002 treatment upregulated both *PRSS8* and *KLF2* expression (Supplementary Fig. S5C). However, knockdown of DNMT1 and EZH2 increased KLF2 expression, whereas knockdown of EZH2 and LSD1 upregulated PRSS8 expression in both BGC823 and SGC7901 cells (Fig. 5H and Supplementary Fig. S5D). Together, these findings indicate that DNMT1 and EZH2 may be involved in HOXA11-AS–mediated repression of *KLF2* expression, whereas EZH2 and LSD1 might contribute to HOXA11-AS–mediated repression of *PRSS8* expression in gastric cancer cells.

We next designed four paired primers across the promoter region (2,000 bp) of *KLF2* and *PRSS8*, and then performed ChIP assays to examine the regulatory mechanisms. The results showed that DNMT1 could directly bind to the *KLF2* promoter region (-956); EZH2 could directly bind to *PRSS8* (-1264) and *KLF2* promoter region (-956) and induce H3K27me3 modifications; LSD1 directly binds to the PRSS8 promoter region (-1264) mediated H3K4 demethylation, and HOXA11-AS knockdown decreased their binding ability and induced modification in gastric cancer cells (Fig. 5I and Supplementary Fig. S5E).

HOXA11-AS functions as ceRNA and sponges miR-1297 in gastric cancer cells

Besides regulation of targets epigenetically in the nucleus, several studies have revealed that some lncRNAs could also regulate the expression of target genes by functioning as competing endogenous RNAs (ceRNA) for specific miRNAs in cytoplasm. Our previous study found that HOTAIR could

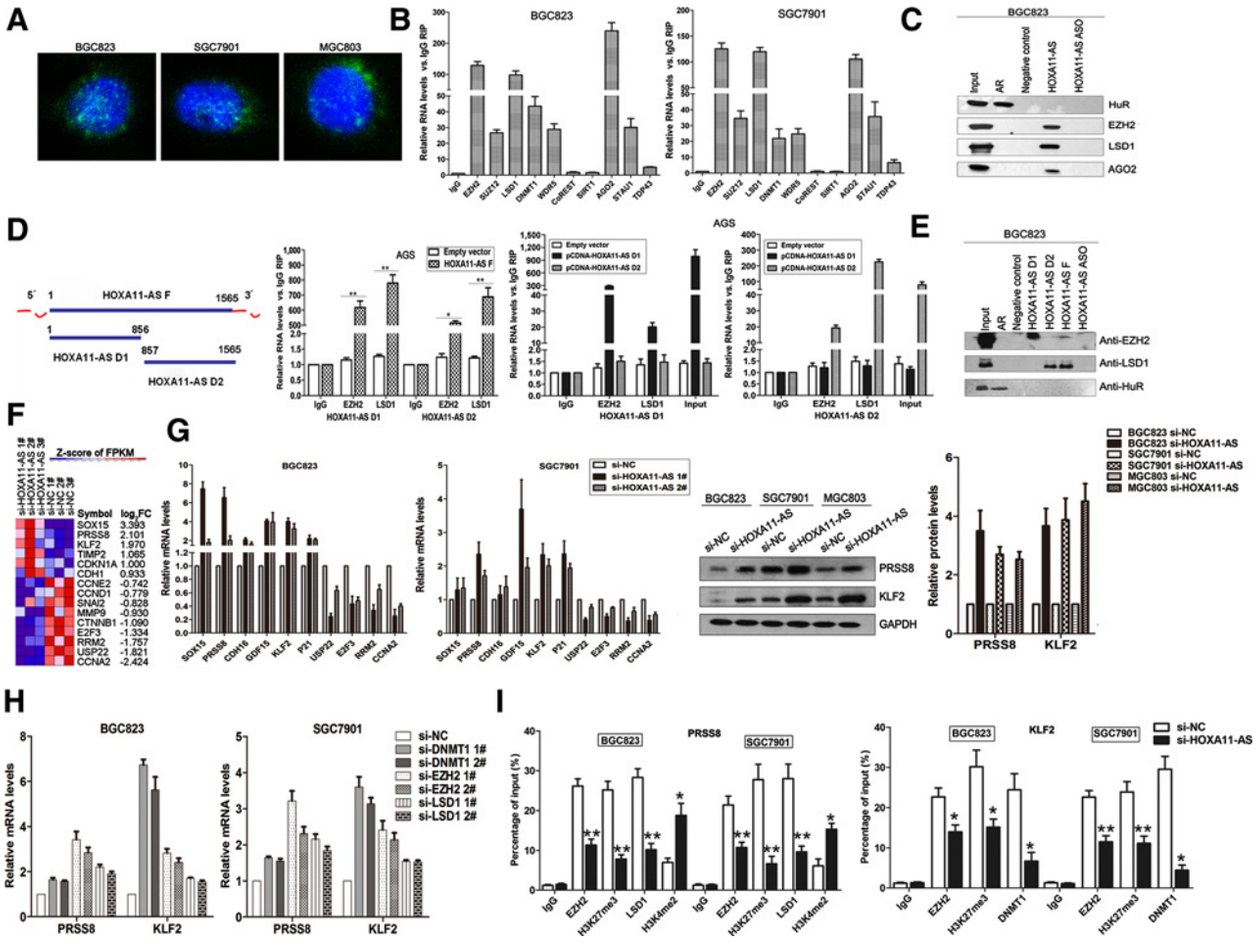

Figure 5.
HOXA11-AS functions as a scaffold for PRC2/LSD1/DNMT1 to regulate PRSS8 and KLF2 in gastric cancer cells. **A,** FISH was used to detect HOXA11-AS location in BGC823, SGC7901, and MGC803 cells. Green, HOXA11-AS; blue, DAPI. **B,** RIP assays of HOXA11-AS binding to indicated proteins in BGC823 and SGC7901 cell extracts. **C,** RNA pull-down was used to examine the association of HOXA11-AS and EZH2. AR binding to HuR was used as a positive control. **D,** the HOXA11-AS 5′ region bound with EZH2 and the 3′ region bound with LSD1 in RIP assays. **E,** biotinylated HOXA11-AS F, D1, and D2 RNAs were incubated with BGC823 cell lysates, and Western blot analysis was performed to evaluate the specific association between them and EZH2 or LSD1. **F,** heatmap of altered genes in HOXA11-AS knockdown BGC823 cells compared with control cells. **G,** qRT-PCR and Western blot analyses were performed to confirm the selected gene expression in HOXA11-AS knockdown cells. **H,** PRSS8 and KLF2 expression was examined in cells transfected with EZH2, LSD1, or DNMT1 siRNAs. **I,** ChIP shows EZH2, LSD1, or DNMT1 occupancy on the PRSS8 and KLF2 promoter regions, and knockdown of HOXA11-AS decreases their occupancy. *, $P < 0.05$; **, $P < 0.01$.

function as a ceRNA and sponge for miR331-3p (16), or repress miR34a transcription by binding with EZH2 in gastric cancer cells (38). Interestingly, our abovementioned findings showed that HOXA11-AS could bind AGO2 protein, suggesting that HOXA11-AS may also function as a ceRNA. To verify this hypothesis, we examined the HOXA11-AS sequence and found miR-1297, let-7f, miR148, and miR152-binding sites. Furthermore, dual luciferase reporter assays showed that only miR-1297 and let-7f directly bind the HOXA11-AS sequence, and the binding ability of miR-1297 is stronger (Fig. 6A). To further confirm the luciferase assay results, we mutated the miR-1297-binding site in HOXA11-AS by site-directed mutagenesis. As expected, the miR-1297-mediated suppression of luciferase activity was abolished in this mutated HOXA11-AS construct compared with the wild-type vector (Fig. 6B). Moreover, RIP experiments showed that HOXA11-AS and miR-1297 were preferentially enriched in Ago2-containing miRNPs relative to control IgG (Fig. 6C). Human FOS was used as a positive control.

Some studies showed that miR-1297 could inhibit HCC and colorectal cancer cell proliferation and invasion through targeting HMGA2 and EZH2 (39, 40). To investigate whether miR-1297 functions as a tumor suppressor in gastric cancer cells, we performed MTT and Transwell assays. As shown in Fig. 6D–F, overexpression of miR-1297 impaired gastric cancer cell proliferation and invasion. Importantly, Western blot assays also showed that knockdown of HOXA11-AS decreased EZH2 protein levels, which is consistent with miR-1297-induced downregulation of EZH2 protein (Fig. 6G). Finally, correlation analysis in gastric cancer tissues showed that HOXA11-AS expression is negatively associated with miR-1297 and positively associated with *EZH2* expression (Fig. 6H). Together, these data indicate that HOXA11-AS acts as a ceRNA for miR-1297, and thereby derepresses EZH2

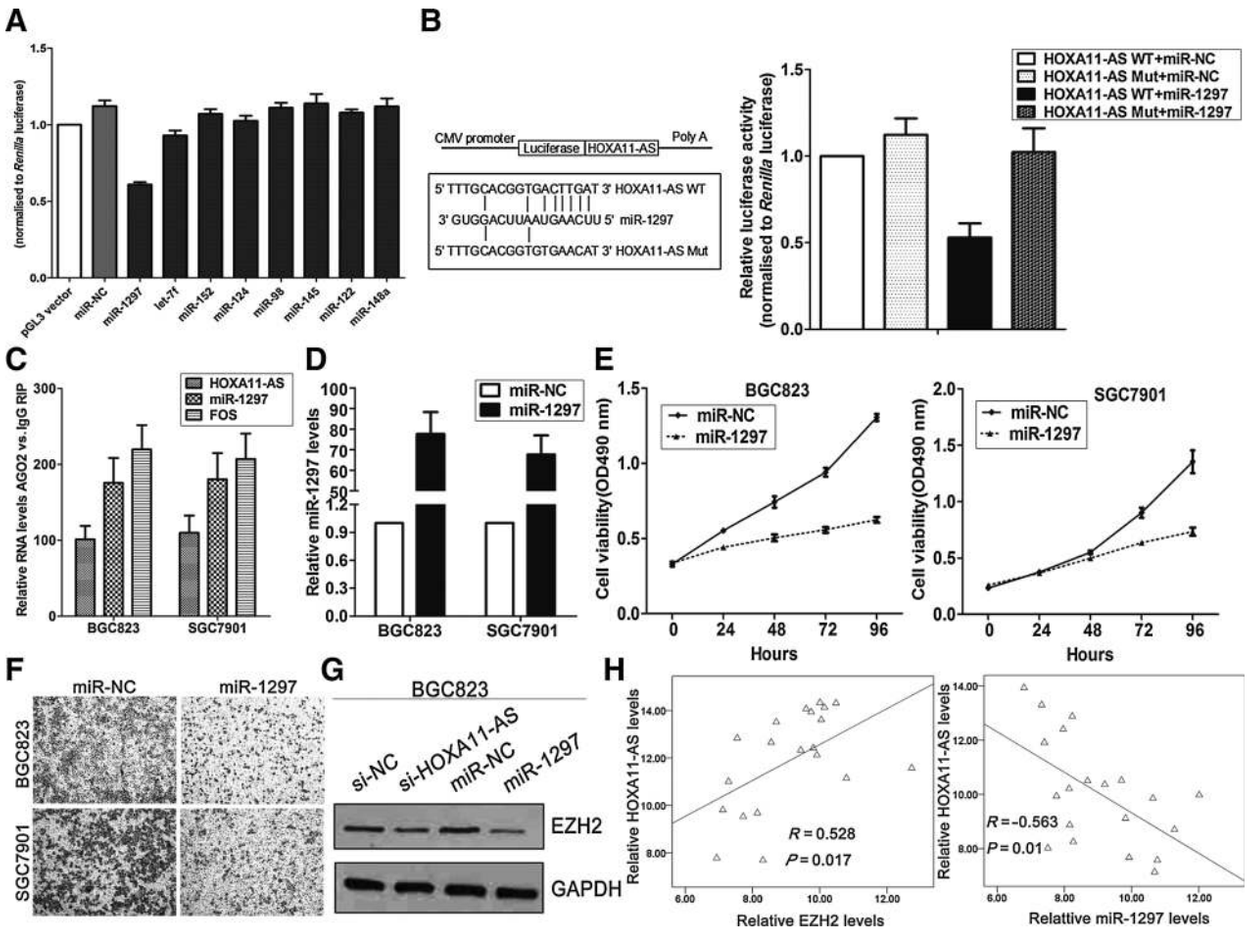

Figure 6.

HOXA11-AS acts as a ceRNA for miR-1297. **A** and **B,** luciferase reporter assays were used to determine the interacting activity between miRNAs and HOXA11-AS. Luciferase activity is shown as relative luciferase activity normalized to *Renilla* activity. **C,** RNA levels in immunoprecipitates are presented as fold enrichment in Ago2 relative to IgG immunoprecipitates. **D,** miR-1297 levels were examined in BGC823 and SGC7901 cells after transfection with mimics. **E** and **F,** MTT and Transwell assays showed that cell proliferation and invasion was suppressed by miR-1297 overexpression. **G,** Western blot analysis of EZH2 protein levels in BGC823 cells transfected with HOXA11-AS siRNA or miR-1297 mimics. **H,** the correlation between HOXA11-AS and EZH2, or miR-1297 expression was analyzed. *, $P < 0.05$; **, $P < 0.01$.

expression and imposes an additional level of post-transcriptional regulation.

Oncogenic function of HOXA11-AS by repressing PRSS8 and KLF2 expression

To further determine whether KLF2 and PRSS8 function as tumor suppressors in gastric cancer, we first analyzed their levels in normal and tumor tissues. The results showed that KLF2 and PRSS8 expression are both downregulated in tumor tissues (Fig. 7A and Supplementary Fig. S6A and S6B). Moreover, immunohistochemical staining assays showed that KLF2 and PRSS8 are upregulated in tumor tissues formed from HOXA11-AS knockdown cells (Fig. 7A). In addition, overexpression of both KLF2 and PRSS8 impaired BGC823 cell proliferation; however, overexpression of KLF2 was enough to inhibit SGC7901 cell proliferation (Fig. 7B). PRSS8 overexpression inhibited BGC823 and SGC7901 cell invasion (Fig. 7C). Previous studies have demonstrated that CDNN1A and β-catenin are KLF2 and PRSS8 underlying targets, and we also found that KLF2 overexpression increased CDKN1A expression whereas up-regulated PRSS8 expression decreased β-catenin protein expression (Fig. 7D). Kaplan–Meier survival analysis showed that downregulation of PRSS8 and KLF2 are associated with poor prognosis in gastric cancer patients (Supplementary Fig. S6C).

To determine whether PRSS8 and KLF2 are involved in the HOXA11-AS–induced increase of gastric cancer cell proliferation and invasion, we performed rescue experiments. AGS cells were cotransfected with HOXA11-AS, KLF2, or PRSS8 vector, and BGC823 cells were cotransfected with si-HOXA11-AS, si-PRSS8, or si-KLF2 (Fig. 7E). MTT assays showed that KLF2 partially rescued the HOXA11-AS–induced cell proliferation and PRSS8 partially reversed the HOXA11-AS–induced cell invasion, which may be owing to regulation of CDKN1A and β-catenin expression (Fig. 7F and G). Finally, we found a negative correlation between HOXA11-AS and *PRSS8* or *KLF2* expression in gastric cancer tissues (Fig. 7H). These data suggested that HOXA11-AS

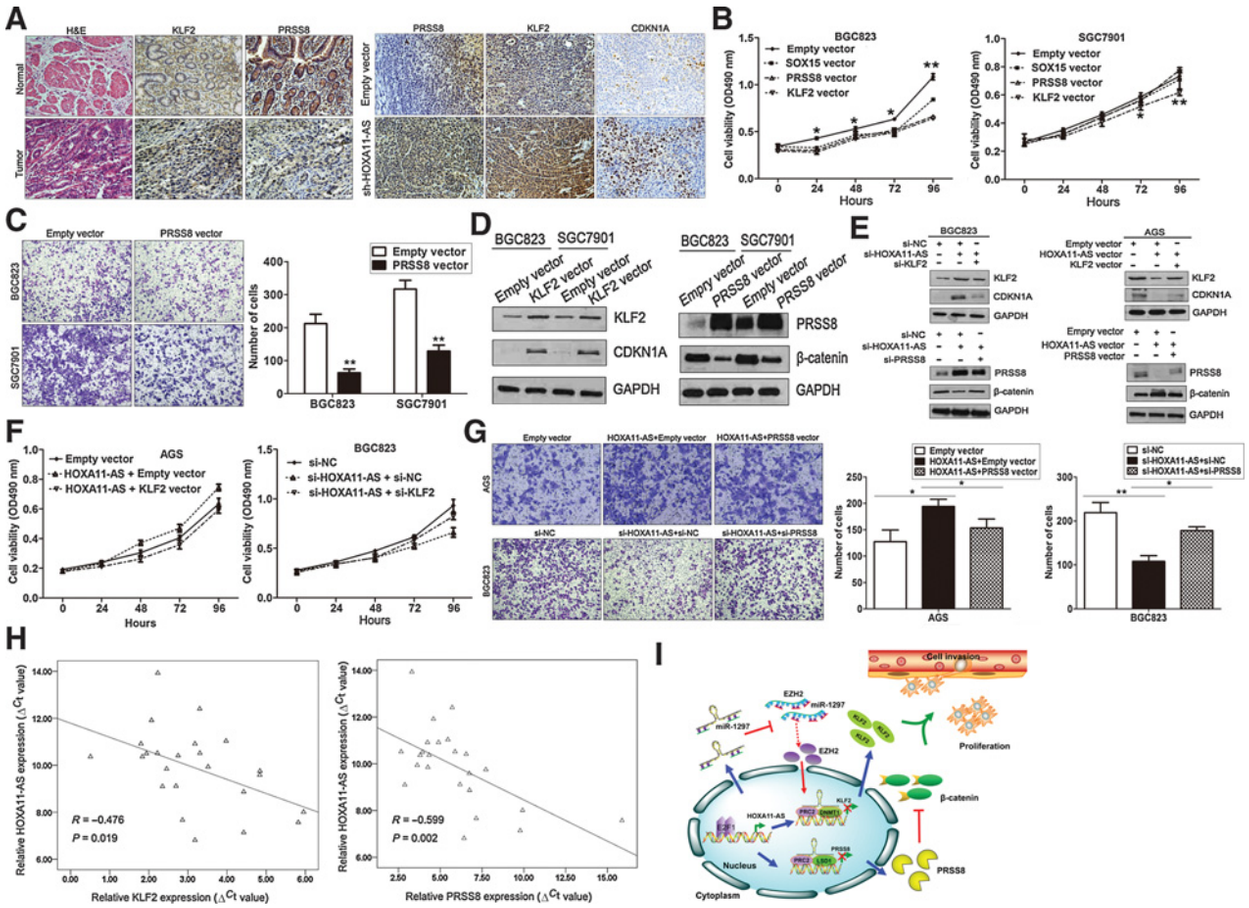

Figure 7.

HOXA11-AS promotes gastric cancer cell proliferation and invasion in part by regulating PRSS8 and KLF2. **A,** left, detection of PRSS8 and KLF2 protein levels in gastric cancer and normal tissues by IHC; right, immunohistochemical analysis of PRSS8, KLF2, and CDKN1A protein levels in tumor tissues formed from HOXA11-AS knockdown or control cells. **B,** MTT assays were used to determine cell viability of BGC823 and SGC7901 cells. **C,** Transwell assays were used to determine cell migration and invasion ability of BGC823 and SGC7901 cells. **D** and **E,** KLF2, CDKN1A, PRSS8, and β-catenin protein levels were detected by Western blot analysis in BGC823, SGC7901, or AGS cells. **F** and **G,** MTT and Transwell assays were used to determine the cell viability and invasion for HOXA11-AS, KLF2, or PRSS8 vectors cotransfected AGS cells or siRNAs cotransfected BGC823 cells. **H,** the correlation between HOXA11-AS and KLF2, or PRSS8 expression was detected by qPCR. **I,** summary of the mechanism of HOXA11-AS in gastric cancer cells. *, $P < 0.05$; **, $P < 0.01$.

promotion of gastric cancer cell proliferation and invasion may partially depend on regulation of KLF2 and PRSS8 expression.

Discussion

In recent years, thousands of lncRNAs have been discovered by RNA sequencing and annotated by the GENECODE project; however, little more than expression patterns of these lncRNAs has been established (41). As dozens of lncRNAs have been identified as critical players in cancer cell growth and metastasis, attention toward lncRNAs in cancer is continuing to increase. Although alterations of lncRNAs in gastric tumorigenesis are already a recognized phenomenon, the functional role and molecular mechanism of many GC-associated lncRNAs remain undetermined (42). In this study, we compared the lncRNA profiles of gastric cancer and normal gastric tissues using the sequencing data from TCGA project and microarray profile from GEO. Only lncRNA HOXA11-AS displayed specific overexpression in gastric cancer but not in other cancers. We further validated the HOXA11-AS expression pattern in gastric cancer clinical samples and found that HOXA11-AS expression was associated with poor prognosis.

It has been shown that lncRNAs play key roles in regulation of the malignant phenotypes of cancer cells. Our findings also showed that knockdown of HOXA11-AS inhibited gastric cancer cell proliferation and invasion, and induced cell apoptosis. Our findings suggest that HOXA11-AS may function as an oncogenic lncRNA in gastric cancer and potentially be considered as a novel prognostic indicator for this disease. Recent studies have demonstrated that many lncRNAs contribute to the development of multiple types of cancers (43). However, the regulators involved in lncRNA disorder expression in cancer cells are not well known. In this study, our data demonstrated that HOXA11-AS overexpression in gastric cancer cells could be activated by E2F1, which can also promote lncRNA ERIC (44) and ANRIL (45) expression.

LncRNAs can guide and recruit DNA, histone protein modification enzymes, or transcription factors to specific genomic loci, leading to inactivation of tumor suppressors or activation of oncogenes (46). In addition, a subset of lncRNAs is enriched in the cytoplasm, where the lncRNAs participate in cellular biological processes by functioning as ceRNAs or regulating mRNA stability, mRNA alternative splicing, and protein localization (47). Here, we found that HOXA11-AS could simultaneously bind several RNA-binding proteins (PRC2, LSD1, and DNMT1) and function as a scaffold to regulate PRSS8 and KLF2 at transcriptional level. The findings by Chang and colleagues, as well as ours, found that the 5′ domain of HOTAIR binds PRC2, whereas 3′ domain of HOTAIR binds the LSD1/CoREST/REST complex. The lncRNA-mediated assembly of PRC2 and LSD1 coordinates targeting of PRC2 and LSD1 for coupled H3K27 methylation and H3K4 demethylation (48). In addition, previous study has revealed that EZH2 interacts with DNA methyltransferases (DNMT) and associates with DNMT activity (49). Our findings suggest that DNMT1, EZH2, and LSD1 are directly binding target regions, and their interaction is mediated by lncRNA HOXA11-AS. Meanwhile, DNMT1, EZH2, and LSD1 are also likely to be recruited by other sequence-specific transcription factors. Moreover, HOXA11-AS also acts as a ceRNA for miR-1297 in the cytoplasm, and binding with miR-1297 releases its inhibition of EZH2 mRNA, resulting in elevated EZH2 protein levels. EZH2 protein could then enter into the nucleus, which could further be recruited by HOXA11-AS to repress PRSS8 and KLF2 transcription. The decreased PRSS8 protein could further stimulate β-catenin protein. This feedback loop resulted in loss of PRSS8 and KLF2 and continuous high β-catenin expression, which finally promotes gastric cancer cell proliferation and invasion (Fig. 7I). In addition, RNA sequencing in HOXA11-AS knockdown cells showed that many other genes involved in cell proliferation and migration, such as CDH1, TIPM2, CCNA2, and USP22, are also affected by HOXA11-AS in gastric cancer cells, suggesting that these genes may also be involved in the contributions of HOXA11-AS to gastric tumorigenesis.

PRSS8 is a member of the prostasin protein family, which is essential for terminal epithelial differentiation and is expressed abundantly in the normal epithelium (50). PRSS8 is downregulated in high-grade and hormone-refractory epithelial cancers including gastric cancer (51). PRSS8 was recently identified as an invasion suppressor through modulation of EGFR and EMT process, and epigenetic regulators might be involved in PRSS8 downregulation (52). Epigenetic alterations are also involved in the silencing of KLF2 expression in cancer cells (53). KLF2 is reduced in multiple cancers and functions as a tumor suppressor owing to its role in the inhibition of proliferation and induction of apoptosis, which is mediated by KRAS and partly through activating CDKN1A (54, 55). In this study, we found that PRSS8 and KLF2 also function as tumor suppressors in gastric cancer cells and their silencing is involved in the HOXA11-AS–exerted oncogenic function in gastric cancer cells.

Our results revealed that the gastric cancer–associated lncRNA HOXA11-AS is an oncogenic lncRNA that promotes tumorigenesis through serving as a scaffold and recruiting multiple chromosome modification enzymes to target specific genes or as ceRNA for specific miRNAs. Our findings support the idea that lncRNAs, such as HOXA11-AS, play crucial roles in gastric cancer progression and suggest that HOXA11-AS is potentially an effective target for gastric cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Sun, W. De, Z. Wang, J. Wang
Development of methodology: M. Sun, F. Nie, D. He
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Sun, F. Nie, Z. Zhang, M. Xie
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Sun, Y. Wang, J. Hou, D. He
Writing, review, and/or revision of the manuscript: M. Sun, L. Xu, W. De, Z. Wang, J. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Nie, W. De

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