DNA-Methylation-Mediated lncRNA *HOXB-AS4* Promotes Gastric Cancer Progression Through Regulating miR-130a-5p/PKP4

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Research

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Abstract

Background Gastric cancer (GC) is one of the most common cancer in the world, possessing the second leading cause of cancer-related mortality. Long noncoding RNAs (lncRNAs) have been shown to play important roles in tumorigenesis. However, the effect of lncRNA *HOXB-AS4* in GC progression and the underlying mechanisms remain unknown.

Methods Firstly, the expression of lncRNA *HOXB-AS4* in gastric cancer tissues and cancer cells was investigated according to GEPIA database and Real time fluorescence quantitative PCR(qRT-PCR). Then, MTT, clone formation, Transwell and Western blot were used to study the effects of overexpression or down-regulation of *HOXB-AS4* on the proliferation, invasion and epithelial mesenchymal transformation of cancer cells. We further studied the molecular mechanism of *HOXB-AS4* by fluorescence in situ hybridization, bioinformatics analysis, luciferase reporting, methylation specific PCR (MSP) and chromatin immunoprecipitation (chip).

Results: In the study, the GEPIA database and quantitative Real-Time PCR (qRT-PCR) assay showed that *HOXB-AS4* was upregulated in GC tissues and cells. Then, MTT, clone formation, transwell, and western blot assays suggested that overexpression of *HOXB-AS4* increased cell proliferation, migration, and invasion, and regulated epithelial-mesenchymal transition (EMT) markers expression, while knockdown of *HOXB-AS4* showed the opposite effect. Fluorescence in situ hybridization (FISH) assay found that *HOXB-AS4* localized in the cytoplasm of the GSE-1 and AGS cells. Further mechanism experiments, including bioinformatics, luciferase reporter, qRT-PCR, and western blot assays showed that *HOXB-AS4* sponged to miR-130a-5p to regulate the *PKP4* expression. Knockdown of miR-130a-5p obliterated the effect of *HOXB-AS4*, which was further abolished by knockdown of *PKP4 in vitro* and *in vivo*. Methylation-specific PCR (MSP) and chromatin immunoprecipitation (CHIP) assay showed that overexpression of *HOXB-AS4* in GC was mediated by *SP1*-dependent DNA methylation. Abnormal upregulation of lncRNA *HOXB-AS4* contributed to GC progression, which was mediated by DNA methylation. The study clarified that DNA-methylation-mediated *HOXB-AS4* played its role through miR-130a-5p/*PKP4* axis.

Conclusions: Our study provides new insights for the understanding of epigenetic regulation on lncRNA expression in GC, and indicates that *HOXB-AS4* could be a biomarker of GC prognosis. Moreover, targeting *HOXB-AS4* /miR-130a-5p/*PKP4* axis might be a promising strategy to treat GC.

Background

Gastric cancer (GC) is one of the most common cancer in the world, possessing the second leading cause of cancer-related mortality[1]. Although impressive progresses have been made in the treatment of GC, the survival rates of GC remain unoptimistic[2]. The survival rates of gastric correlate most with the early diagnosis, that the surgical removal of the tumor in the early stage benefits the prognosis of GC the most[3, 4]. However, the symptom of GC in the early stage may be mild and untypical, and many people were diagnosed in an advanced stage, leading to poor prognosis even then were given proper

treatments[4]. The pathogenesis and molecular mechanism of GC are complicated, which resulted from the dysregulation of various signaling pathways[5]. So, it's of vital importance to clarify the molecular mechanism of GC progression and find new markers and targets for GC diagnosis and treatment.

Long noncoding RNAs (LncRNAs) are a group of non-coding RNAs with the length longer than 200 nucleotides[6]. LncRNA has been proved to be involved in various biological processes, including cell proliferation, differentiation, and apoptosis[7, 8]. LncRNA plays its role by regulating protein-coding genes, mRNA processing, and maintenance of genomic integrity[9]. LncRNA functioned as the competitive endogenous RNA (ceRNA) which compete with the mRNA to bind to the microRNA (miRNA), leading to the change of gene expression[10]. Although ceRNA mechanism has been reported to play roles in GC progression[11, 12], the specific molecular mechanism on the occurrence of ceRNA mechanism remains largely unknown.

Homeobox (*HOX*) genes are a highly conserved subgroup of the superfamily, which contain 39 transcription factors[13]. It is reported that *HOX* family is associated with the development and progression of numerous cancers, including GC[14]. For instance, the transcription factor *HOXD9* was up-regulated in GC, and accelerated the GC progression[15]. Moreover, lncRNAs generated in *HOX* family genes have been proved to be a vital biomarker when evaluating the prognosis of multiple cancers [16, 17]. For instance, lncRNA *HOXA11-AS* served as an oncogene to participate in GC [18, 19]. As is known, abnormal DNA methylation is related to many cancers, and this epigenetic disruption may lead to abnormal lncRNA expression in tumor tissues [20]. Evidence has shown that lncRNAs generated in *HOX* family genes, as *HOTAIR*, *HOTTIP*, *HOXA11-AS*, *HOXB-AS4*, and *HOXC-AS3*, were regulated by aberrant DNA methylation in GC [21]. However, the roles and mechanism of lncRNA *HOXB-AS4* in GC remain unknown.

In the present study, we designed experiments to investigate the role of lncRNA *HOXB-AS4* in the GC progression and the effect of epigenetic regulation on its abnormal expression (Additional fle 1: Fig.S1). Our results showed that *HOXB-AS4* is epigenetically activated by DNA methylation at the CpG islands within its promoter region. *HOXB-AS4* regulates the cell proliferation, migration, invasion, and EMT markers expression by competitively binding to miR-130a-5p, leading to the upregulation of plakophilin 4 (*PKP4*), which promotes the GC progression.

Materials And Methods

Patients and specimens

A total of 16 pairs of GC tissues and the pair-matched non-tumoral gastric tissues were acquired from the first people's hospital of suqian. The written consent agreement was signed and approved by the Ethics Committee of the Affiliated Suqian first People's Hospital of Nanjing Medical University. All patients involved in the present study didn't receive chemotherapy or radiotherapy before the surgery. 16 tissues were stored at -80℃ immediately after the surgery.

Cell culture

The human gastric epithelial cell line GES-1, and human gastric cancer cell lines MKN-45, MKN-28 were purchased from the American Type Culture Collection (MD, USA). The human gastric cancer cell lines NCI-N87, AGS, HGC-27 were purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). All cells were cultured in RPMI-1640 culture medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) at 37 °C with 5% CO_2.

Plasmids construction and transfection

The full-length lncRNA *HOXB-AS4* was cloned into pcDNA3.1 (Invitrogen, USA), and the lncRNA *HOXB-AS4* plasmid and corresponding empty vector were transfected into GES-1 or AGS cells using Lipofectamine 3000 reagent (Invitrogen, USA). The lncRNA *HOXB-AS4* siRNAs, *SP1* siRNAs, *PKP4* siRNAs were synthesized by Genscript Biotech (Nanjing, China). miR-130-5p mimics, miR-130-5p inhibitors and relative control were purchased from Ribo Bio (Guangzhou, China). All siRNAs, miRNA mimics, inhibitors, or *HOXB-AS4* plasmid were transiently transfected to cells by using Lipofectamine 3000 for 24 h. The sequences of si-*HOXB-AS4*: AAGAGGACCACTCAGTTTAGG; si-*SP1*: AAGTGCAGCAGGATGGTTCTG; si-*PKP4*: GAGACCACAGCCACCACTATT.

RNA Extraction and quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using either TRIzol Reagent (Invitrogen, USA) following the manufacturer's instructions. Then mRNAs were reverse transcribed using the PrimeScript RT kit (Takara, Japan). The cDNAs were then analyzed by qRT-PCR using a QuantiTect SYBR Green PCR Kit (Qiagen, Germany), gene-specific primers and a Rotor gene Q real-time PCR cycler (Qiagen, Germany). The reaction condition was as follows: 95 °C for 30 s, at 60 °C for 30 s, 45 cycles at 60 °C for 30 s. RNA was quantified by 2−ΔΔCT method. After qRT-PCR reactions, the cycle threshold (CT) data were normalized to the internal control (U6 snRNA or GAPDH). Primers used were as follows: *HOXB-AS4*-F: CTT GTG CTT TAC AAG GCG GC, *HOXB-AS4*-R: GAC CGG CCC CTA GGT TTA TC; miR-130a-5p-F: GCG CGG ATC CAG GCG GCA AAA GGA AGA GTG GTG, miR-130a-5p-R: CGG CGA ATT CCA CAA GCA CTG CAT ACA GAA GTA G; miR-140a-5p-F: CGG GAT CCG GTC CTC TGT TCG GTG GTG GCG; miR-140-5p-R: CGG AAT TCA ACC AGC AAG GGG ATG TCC CAA G; U6-F: GCT TCG GCA GCA CAT ATA CTA A, U6-R: AAC GCT TCA CGA ATT TGC GT; GAPDH-F: TGT GTC CGT CGT GGA TCT GA; GAPDH-R: CCT GCT TCA CCA CCT TCT TGA.

Western blot

Western blot assay was used to assess epithelial-mesenchymal transition (EMT)-related makers Vimentin, N-Cadherin, and E-Cadherin and *PKP4* protein expression. Total protein from tumor tissues or cells was extracted by using RIPA lysis buffer plus PMSF (Beyotime, China). BCA assay kit (Santa Cruz, USA) was used to detect total protein concentration. Prepared protein samples were separated by using SDS-PAGE electrophoresis, and transferred into PVDF membranes and incubated with prepared antibodies. Finally, enhanced chemiluminescence (ECL, ThermoFisher, USA) was used to visualize the

membrane. The protein band analysis was conducted with ImageJ software. Antibodies against Vimentin, N-Cadherin, E-Cadherin, and GAPDH were purchased from CST (MA, USA). Antibody against *PKP4* was purchased from Abcam (Cambridge, USA).

Cell viability assay

The viability of GSE-1 and AGS cells was assessed using MTT. Briefly, cells were plated in the 96-well microplates at a density of 1×10^4 cells per well. After transfection with indicated time, the cells were incubated with MTT (0.5 mg/ml) at 37 °C for 3 h. Then the medium was removed, and 100 mM DMSO solution was added to dissolve the formazan crystals. The absorbance at 570 nm wavelength was detected using a microplate reader (Molecular Devices, Sunnyvale, USA).

Clone formation assay

The GSE-1 or AGS cells were digested and plated in a 6 cm culture dish at a density of 5×10^3/well after indicated treatments. The cells were subjected to the normal culture condition at 37℃, 5% CO_2 for 14 days. Then the cells in the dish were washed with PBS, and fixed with 3 mL methanol for 10 minutes. The cells were subjected to Giemsa dyeing for another 15 minutes. Then the cells were observed under a light microscope (Olympus, Japan), and the number of colonies was counted.

Cell migration and invasion assay

Cell migration and invasion were determined by transwell assay. GSE-1 and AGS cells were harvested and seeded to the upper transwell chamber at a density of 5×10^4 cells per well into an 8-mm pore transwell plate (Costar, USA) pre-treated with Matrigel (BD biosciences, USA) or not. Serum-free medium was added onto the upper chamber, and the culture medium containing 20% FBS was added into the lower chamber. Following 24 h incubation, the unmigrated or uninvaded cells were cleaned using a cotton swab and then fixed in 4% paraformaldehyde for 20 min and stained with hematoxylin. Images were taken, and the cell number was calculated.

Methylation-specific PCR (MSP)

Genomic DNA was extracted from the GC tissues and adjacent normal tissues by using the DNA extraction Kit (Qiagen, Germany) according to the manufacturer's instruction. The purified DNA was then exposed to bisulfite treatment by using the EpiTect Bisulfite Kit (Qiagen, Germany). Then the MSP of bisulfite-transformed DNA was performed with a nested, two-stage PCR method followed with the detection of the PCR products by using agarose gel electrophoresis.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with an EZ-ChIP Kit (Millipore, USA) according to the manufacturer's instructions. Briefly, GES-1 and AGS cells were cross-linked with 1% formaldehyde for 10 min followed with glycine treatment. Cell lysates were then sonicated to generate chromatin fragments and then

immunoprecipitated with *SP1* antibody (CST, USA). IgG antibody (CST, USA) was used as the negative control. The DNA fragment was amplified by PCR. The *HOXB-AS4* primer sequences are as follows: F: CTGAGTTTTCAGCCCTCCTG, R: AAGCTCCAATGAAGGGGTCT. The product was then analyzed by using agarose gel electrophoresis.

Fluorescence in situ hybridization (FISH)

To detect *HOXB-AS4* expression, GSE-1 and AGS cells were fixed in 4% formaldehyde for 15 min at room temperature and then permeabilized with 70% ethanol. After rehydrated for 5 min at room temperature, the cells were incubated by using biotin-labeled *HOXB-AS4* probe (Genepharma, Shanghai, China) at 37 °C for 8 h. Then the Alexa Fluor 647-conjugated secondary antibody (Abcam, USA) was used to detect the biotin-labeled *HOXB-AS4*.

Luciferase reporter assay

The wild-type or mutant 3'UTR of *PKP4* and the full length of *HOXB-AS4* were amplified and cloned into pGL3-basic vector (Promega, WI, USA) separately. The binding site of 3'UTR of *PKP4* was mutated from UAU UAU GUU UUU UAA AAU GUG AG to TTT ATA GTA TTA AAT TTT GAG TG for miR-130a-5p, and the potential binding sites of *HOXB-AS4* was mutated from AAAAGAGA to TTTTGTG for miR-130a-5p. Then, AGS cells were plated on a 24-well plate and co-transfected with wild-type or mutant luciferase plasmids and miR-23c mimic, miR-23c inhibitor or control miRNA. A Dual-Luciferase Reporter Assay System (Promega, WI, USA) was used to measure the luciferase activity. The relative luciferase activity of each sample was normalized to Renilla luciferase activity.

Tumor xenograft model

1×10^7 AGS cells were suspended in 200 μl PBS and subcutaneously injected into right flank of 4–6-week-old BALB/c nu/nu male mice (Charles River Lab, Beijing, China). The mice were recorded the tumor volume (volume = (length × width^2)/2) every 3 days. Knockdown of miR-130a-5p was performed by using miR-130a-5p antagomir (Ribo Bio, Guangzhou, China) which was administrated by tail injection at the dosage of 80 mg/kg. Knockdown of *HOXB-AS4* or *PKP4* was performed by using the related knockdown lentivirus (GenePharma, Shanghai, China) by intra tumoral injection of 50 μL virus (4×10^7 IU/mL) after the tumor cells injection. After 15 days, the mice were sacrificed. The tumor tissues were subjected to western blot analysis, hematoxylin-eosin (H&E) staining and immunohistochemical (IHC) analysis. The animal experiments were approved by the Animal Care and Use Committee of the first people's hospital of suqian.

H&E and IHC analysis

For H&E staining, the prepared tumor tissue slices were dewaxed and hydrated. After washed by water, tumor tissue slices were stained in hematoxylin solution for 5 min. Next, after treated by 1% hydrochloric alcohol for 15s, the slices were washed with water. And then the slices were stained by eosin solution for

1 min. Finally, tissue slices were dehydrated, transparentized and sealed by neutral gum, observed under an optical microscope (Olympus, Tokyo, Japan). For IHC staining, the paraffin-embedded tumor sections were dewaxed and treated with 3% H_2O_2 to deactivate endogenous peroxidase. After blocking non-specific antigen binding with 5% BSA at 37 °C for 1 h, the sections were incubated with a specific primary antibody against *PKP4* (1:100 dilution, Abcam, USA) at 4 °C overnight. After incubating with the corresponding secondary antibodies at 37 °C for 1 h, the sections were stained with diaminobenzidine and counterstained with hematoxylin. Representative images were taken using a light microscope (Olympus, Tokyo, Japan).

Kaplan-meier analysis

The overall survival data were derived from the online database Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/). Of the 110 GC samples, 108 samples showed high *HOXB-AS4* expression, whereas 82 samples showed low *HOXB-AS4* expression. The survival rates were analyzed by Kaplan-Meier curves. Then, the log-rank test was performed to evaluate the mean of the two groups.

Bioinformatics

The online database Starbase (http://starbase.sysu.edu.cn/) was used to predict possible miRNAs that may be sponged by *HOXB-AS4*. In addition, the online database miRCancer (http://mircancer.ecu.edu/) was used to predict the abnormal expression of miRNAs in gastric cancer. Then, the miRNAs that may be sponged by *HOXB-AS4* in gastric cancer were screened through the intersection of the Starbase and miRCancer. The online databases miRDB (http://mirdb.org/) and Targetscan (http://www.targetscan.org/) were used to screen the potential targets of miR-130a-5p.

Statistical analysis

All data were shown as mean ± s.e.m. and experiments were carried out at least three times. GraphPad 6.0 version was adopted for statistical analyses. Student's t-test was performed to evaluate the mean of the two groups and one-way ANOVA followed Tukey's poc host was used to analyze significant differences among multiple groups. $P < 0.05$ was regarded as statistically significant.

Results

LncRNA HOXB-AS4 is up-regulated in GC tissues and GC cells

To determine whether lncRNA *HOXB-AS4* plays a role in the pathogenesis of GC, we first investigated the expression of lncRNA *HOXB-AS4* in the tumor tissues. According to the GEPIA database (http://gepia.cancer-pku.cn/), we found that lncRNA *HOXB-AS4* was significantly up-regulated in various types of tumors, especially in GC (Fig. 1A). The *HOXB-AS4* expression in the GC tissues was further verified by using qRT-PCR. The results showed that *HOXB-AS4* was significantly up-regulated in the 16 pairs of GC tissues compared to the pair-matched non-tumoral gastric tissues (Fig. 1B). Moreover,

Kaplan-meier analysis showed that *HOXB-AS4* expression was associated with the prognosis of 110 of GC patients (Fig. 1C). 108 of GC patients with higher *HOXB-AS4* expression showed a poorer prognosis than 82 of GC patients with lower *HOXB-AS4* expression (Fig. 1C), indicating that *HOXB-AS4* expression may contribute to the GC progression. We further investigated the expression level of *HOXB-AS4* in various GC cells and in normal human gastric epithelial cell line GES-1. As shown in Fig. 1D, *HOXB-AS4* exhibited higher expression level in the cancer cells compared to the normal gastric cells, among which AGS cells showed the highest expression.

HOXB-AS4 modulates GC cells proliferation, migration, invasion and EMT markers expression

To investigate the effect of *HOXB-AS4* on tumor progression, we knocked down *HOXB-AS4* in AGS cells and overexpressed *HOXB-AS4* in GES-1 cells. The transfection efficacy was verified by using qRT-PCR (Fig. 2A). MTT assay showed that overexpression of *HOXB-AS4* increased the GES-1 cell viability, while knockdown of *HOXB-AS4* reduced the AGS cell viability (Fig. 2B). Overexpression of *HOXB-AS4* promoted the GES-1 cell clone formation, while knockdown of *HOXB-AS4* inhibited the AGS cell clone formation (Fig. 2C). GES-1 cells showed enhanced migration and invasion ability after overexpressed with *HOXB-AS4* (Fig. 2D). Knockdown of *HOXB-AS4* in AGS cells restrained the cell migration and invasion ability (Fig. 2D).

As activation of EMT process provides tumor cells with invasive and migratory ability[22], we then investigated the effect of *HOXB-AS4* on EMT markers expression. In GES-1 cells, overexpression of *HOXB-AS4* reduced Vimentin expression and increased the N-Cadherin and E-Cadherin expression (Fig. 2E). In AGS cells, knockdown of *HOXB-AS4* increased the Vimentin expression and inhibited the N-Cadherin and E-Cadherin expression (Fig. 2E).

HOXB-AS4 acts as a sponge for miR-130a-5p

We then explored the mechanism of the regulatory effect of *HOXB-AS4*. We examined the intracellular localization of *HOXB-AS4*. Results of FISH showed that *HOXB-AS4* localized in the cytoplasm of the GSE-1 and AGS cells (Fig. 3A). It is reported that cytoplasmic lncRNA exerts its regulatory function through several mechanisms, including mRNA turnover, translation, protein stability, sponging of cytosolic factors, and modulation of signaling pathways[23]. It's worth noting that ceRNA mechanism is one of the major functions of cytoplasmic lncRNA. Therefore, we then hypothesized that *HOXB-AS4* may play its role through the ceRNA mechanism. By using the Starbase and miRCancer database, we identified two miRNAs that may be sponged by *HOXB-AS4* (Fig. 3B). qRT-PCR showed that overexpression of *HOXB-AS4* in AGS led to lower miR-130a-5p level in cells, and miR-130a-5p showed an increased level in *HOXB-AS4*-knockdown cells (Fig. 3C). We then predicted the potential binding sites between *HOXB-AS4* and miR-130a-5p (Fig. 3D). Luciferase reporter showed that miR-130a-5p significantly decreased the luciferase activity of the cells transfected with WT rather than the mutant *HOXB-AS4*, indicating that miR-130a-5p specifically bound to the *HOXB-AS4* (Fig. 3E). Moreover, miR-130a-5p showed significant downregulation in 16 pairs of GC tissues compared to the pair-matched non-tumoral gastric tissues (Fig. 3F), which negatively correlated with the *HOXB-AS4* expression (Fig. 3G).

PKP4 is a direct target of miR-130a-5p

To determine the targets of miR-130a-5p which might contribute to the progression of GC, we screened the potential targets of miR-130a-5p by using TargetScan and miRDB, which identified the *PKP4* may be the potential targets of miR-130a-5p (Fig. 4A). We then predicted the potential binding sites (Fig. 4B), and performed luciferase reporter assays to verify whether *PKP4* was a target of miR-130a-5p. miR-130a-5p significantly decreased the luciferase activity of the cells transfected with WT rather than the mutant *PKP4*, indicating that miR-130a-5p specifically bound to the 3'UTR of *PKP4* (Fig. 4C). Furthermore, western blot showed that miR-130a-5p overexpression markedly reduced the *PKP4* protein expression, and miR-130a-5p knockdown increased the *PKP4* expression (Fig. 4D). Then, 16 pairs of GC tissues and the pair-matched non-tumoral gastric tissues were collected for western blot assay. *PKP4* showed an obvious upregulation in the GC tissues compared to the pair-matched non-tumoral gastric tissues (Fig. 4E). *PKP4* showed significant positive correlation with *HOXB-AS4* expression, and negative correlation with miR-130a-5p expression (Fig. 4F). All these data suggested that *HOXB-AS4*/miR-130a-5p/*PKP4* may be involved in the GC progression.

HOXB-AS4 modulates GC cells proliferation, migration, invasion and EMT markers expression via miR-130a/PKP4 axis.

We then explored the effect of *HOXB-AS4*/miR-130a-5p/*PKP4* axis in GC cells. As shown in Fig. 5A, *HOXB-AS4* knockdown decreased the cell viability. MiR-130a-5p knockdown eliminated the effect of *HOXB-AS4* knockdown, while *PKP4* knockdown further abolished the effect of miR-130a-5p inhibitor (Fig. 5A). *HOXB-AS4* knockdown inhibited the cell clone formation, which was abolished by miR-130a-5p knockdown, and *PKP4* knockdown further inhibited the effect of miR-130a-5p inhibitor (Fig. 5B). AGS cells knocked down of *HOXB-AS4* showed a reduced migration and invasion ability, which were abrogated by miR-130a-5p knockdown, and *PKP4* knockdown further reduced the cell migration and invasion ability, which were restored by miR-130a-5p inhibitor (Fig. 5C). In AGS cells, knockdown of *HOXB-AS4* increased E-Cadherin expression and decreased N-Cadherin and Vimentin expression (Fig. 5D). Knockdown of miR-130a-5p inhibited the effect of *HOXB-AS4* knockdown, and knockdown of *PKP4* knockdown further repressed the effect of miR-130a-5p. All these results indicated that inhibition of *HOXB-AS4*/miR-130a-5p/*PKP4* axis suppressed the proliferation and modulated EMT markers in GC cells.

Knockdown of HOXB-AS4 inhibits GC progression and modulates EMT markers in vivo

We further verified the effect and underlying mechanism of *HOXB-AS4* in vivo. Mice were divided into four groups, with 10 mice in each group. Mice in *HOXB-AS4* knockdown group showed an obvious smaller tumor size compared to the control group, while treatment of miR-130a-5p antagomir inhibited the tumor-suppressive effect of *HOXB-AS4* knockdown (Fig. 6A, B). Knockdown of *PKP4* further inhibited the tumor growth promoted by miR-130a-5p knockdown, showing smaller tumor size compared to the knockdown of *HOXB-AS4* and miR-130-5p group (Fig. 6A, B). Western blot showed that knockdown of *HOXB-AS4* increased E-Cadherin expression and decreased N-Cadherin and Vimentin expression (Fig. 6C). Knockdown of miR-130a-5p inhibited the effect of *HOXB-AS4* knockdown on, and knockdown of *PKP4*

knockdown further repressed the effect of miR-130a-5p (Fig. 6C). IHC staining revealed that the *PKP4* expression showed the consistency with the tumor progression (Fig. 6D). *HOXB-AS4* knockdown induced lower *PKP4* expression in the tumors, while knockdown of miR-130-5p recovered the *PKP4* expression inhibited by *HOXB-AS4* knockdown (Fig. 6D). All these data suggested that inhibition of *HOXB-AS4*/miR-130a-5p/*PKP4* axis restrained the tumor progression *in vivo*.

DNA methylation and SP1 **participate in the regulation of the expression of** HOXB-AS4

We then explored the mechanism underlying the high expression of *HOXB-AS4* in the GC tissues and cells. By using the UCSC Genome Bioinformatics Site(http://genome.ucsc.edu/), we identified CpG islands together with high enrichment and overlapping H3K427AC peaks within the promoter region of *HOXB-AS4* (Fig. 7A), indicating a potential relationship between DNA methylation and *HOXB-AS4* expression. We then performed MSP analysis to verify whether DNA methylation was involved in the upregulation of *HOXB-AS4* in GC. As shown in Fig. 7B, DNA methylation levels were significantly decreased in the promoter region of *HOXB-AS4* in the GC tissues. Accordingly, in the AGS cells, DNA methylation levels in the promoter region of *HOXB-AS4* were lower than that in the GES-1 cells (Fig. 7C). We then treated the GES-1 cells or AGS cells with the DNA demethylation agent azacytidine for different time. We found that *HOXB-AS4* expression increased with time in GSE-1 and AGS cells, indicating that the inhibition of DNA methylation promoted the *HOXB-AS4* expression (Fig. 7D). We further used PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3) to predict the potential transcription factors of *HOXB-AS4*, which showed that *SP1* may be a transcription factor of *HOXB-AS4*. Results of CHIP revealed the enrichment of *SP1* at the promoter of *HOXB-AS4* (Fig. 7E). We then changed the *SP1* expression in GES-1 and AGS cells, and the overexpression or knockdown efficiency was verified by western blot (Fig. 7G). Overexpression of *SP1* enhanced *HOXB-AS4* expression in GES-1 and AGS cells, and *SP1* knockdown restrained *HOXB-AS4* expression (Fig. 7F). All these data suggested that DNA methylation and transcription factor *SP1* were involved in the regulation of *SP1* expression.

Discussion

Increasing evidence showed that lncRNAs play important roles in the development of GC[24, 25]. In the study, we revealed a novel function of *HOXB-AS4* in GC progression. We reported that DNA-methylation-mediated *HOXB-AS4* promoted GC proliferation, migration, and invasion, and modulated EMT markers expression through regulating miR-130a-5p/*PKP4* (Fig. 8).

HOXB-AS4 was HOXB cluster antisense RNA 4, whose effect has barely been reported. In the present study, by using the data of GEPIA database, we found that *HOXB-AS4* was upregulated in various cancer, including GC. We further verified *HOXB-AS4* overexpression in GC tissues and cells, and found its upregulation correlated with the poor prognosis of GC patients, indicating it may play potential roles in GC progression. Further in-vitro experiment showed that *HOXB-AS4* overexpression promoted the AGS and GSE-1 cells proliferation, migration and invasion.

EMT encompasses complex and dynamic changes in phenotypes of cellular organization from epithelial characteristics to mesenchymal traits[26]. The epithelial and mesenchymal cell characteristics include EMT-inducing signals, EMT-Transcription factors and EMT markers[26]. One of the specific changes is the decrease of epithelial marker proteins, such as E-cadherin, and the up-regulation of mesenchymal marker proteins, such as N-cadherin and Vimentin[26]. It is reported that activation of EMT process confers tumor cells with invasive and migratory ability, and supports tumor metastasis[27]. Various studies have reported that lncRNA regulates the EMT process in cancer development[28, 29]. For example, lncRNA *SNHG16* promotes colorectal cancer cell proliferation, migration, and modulates EMT markers expression through miR-124-3p/*MCP-1*[30]. LncRNA *LINC00525* regulates the proliferation and epithelial to mesenchymal transition of human glioma cells by sponging miR-338-3p [31]. In the present study, overexpression of *HOXB-AS4* increased GSE-1 cell proliferation, migration and invasion, and modulated EMT markers expression. In AGS cells, knockdown of *HOXB-AS4* inhibited the cancer cell proliferation, migration and invasion, accompanied with the modulation of EMT markers expression, indicating that *HOXB-AS4* may function tumor-promoting effect through modulating EMT process.

LncRNA could act as miRNAs sponge to regulate gene expression, which were defined as “ceRNA” mechanism [32, 33]. CeRNA mechanism is the main regulatory model of cytoplasm lncRNA[34, 35]. In the present study, we identified the *HOXB-AS4* as a cytoplasm lncRNA by using FISH, indicating its regulatory role in GC may rely on the ceRNA mechanism. Screen of the Starbase and miRCancer database showed that miR-130a-5p and miR-140a-5p may interactwith *HOXB-AS4*. Luciferase reporter assay further showed that *HOXB-AS4* was a miR-130a-5p sponge. Further experiments showed that miR-130a-5p targeted the 3’UTR of *PKP4*. MiR-130a-5p was reported to inhibit tumor invasion and metastatic potential in non-small cell lung cancer[36]. MiR-130a-5p enhanced the sensitivity of cisplatin-resistant gastric cancer cells to chemotherapy [37]. *PKP4* was also called “p0071”, which were reported to interact with E-cadherin to promote the invasion and metastasis of cancer cells[38]. In the present *in-vitro* and *in-vivo* experiments, we verified that knockdown of miR-130a-5p inhibited the anti-tumor effect of *HOXB-AS4* knockdown, showing an increase in cell proliferation and modulation of EMT markers expression. Knockdown of *PKP4* further abolished the effect of miR-130a-5p knockdown on recovering the cell proliferation and EMT markers expression. All these results indicated that *HOXB-AS4*/miR-130a-5p/*PKP4* axis promoted GC development.

We further investigate the mechanism of *HOXB-AS4* upregulation in GC. LncRNA expression is regulated by multiple factors, including chromatin state, the utilization of transcription factors, and some post-transcriptional regulation [39]. DNA methylation is a stable and heritable epigenetic mark which could influence the chromatin state to change the transcriptional state of genes [40]. Many lncRNAs have been reported to be regulated by DNA methylation [41]. Although DNA methylation was studied much as a epigenetic modifications in GC, the DNA methylation-mediated dysregulation of lncRNAs was barely reported[42–44]. In the present study, we identified abnormally low methylation levels of the promoter region of *HOXB-AS4*, which resulted in enhanced lncRNA expression. *SP1* is a transcription factor which has been reported to mediate various lncRNA aberrant expression [45, 46]. *SP1*-dependent DNA methylation was an important pattern for gene overexpression[47]. Consistently, we found that *SP1* was

involved in the DNA-methylation-mediated upregulation of *HOXB-AS4*, indicating that *SP1*-dependent DNA methylation was the molecular mechanism of *HOXB-AS4* aberrant upregulation in GC. To data, there are some limitations in the study. For instance, the number of tissue samples seems inadequate. Only 16 pairs of GC tissues and the pair-matched non tumoral gastric tissues were acquired to detect *HOXB-AS4*, miR-130a-5p, and *PKP4* expression. Furthermore, the involvement of EMT in any process cannot rely solely on a few salient molecular markers, such as E-cadherin and vimentin. As is known, cytoplasmic lncRNA exerts its regulatory function through several mechanisms, including mRNA turnover, translation, protein stability, sponging of cytosolic factors, and modulation of signaling pathways[23]. In the current study, we could not excluded the other mode of action, and just clarified the role of *HOXB-AS4* /miR-130a-5p/ *PKP4* axis on GC progression. We will further explore the mechanism of *HOXB-AS4* in the future study.

In conclusion, we identified that abnormal upregulation of lncRNA *HOXB-AS4* contributed to GC progression. *HOXB-AS4* was found to be epigenetically activated by DNA methylation at the CpG islands within its promoter region. *HOXB-AS4* regulated the cell proliferation, migration, invasion, and EMT markers expression by competitively binding to miR-130a-5p, leading to the upregulation of *PKP4* in GC progression. Our study provides new insights for the understanding of epigenetic regulation on lncRNA expression in GC, and indicates that *HOXB-AS4* could be a biomarker of GC prognosis.

Abbreviation

GC, Gastric cancer; lncRNAs, Long noncoding RNAs; qRT-PCR, quantitative Real-Time PCR; EMT, epithelial-mesenchymal transition; FISH, fluorescence in situ hybridization; MSP, methylation-specific PCR; CeRNA, competitive endogenous RNA; miRNA, microRNA; *PKP4*, plakophilin 4; FBS, fetal bovine serum; CT, cycle threshold; ChIP, Chromatin immunoprecipitation; H&E, hematoxylin-eosin; IHC; immunohistochemical.

Declarations

Acknowledgments

Not applicable

Authors' contributions

Yu Fan and Rong He conceived and directed the study. Yu Fan, Xiao-yan Wang and Rong He contributed to the project design.Xiaoyan Wang, Rong He,Yan Wang, Yunyun Liu, Yuxin Wang and Dandan Chen performed the experiments. Rong He analyzed the bioinformatics data.Rong He and Xiao-yan Wang analyzed and interpreted the data.. All of them read and approved the final manuscript.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics approval and consent to participate

The written consent agreement was signed and approved by the Ethics Committee of The Affiliated Suqian first People's Hospital of Nanjing Medical University.

Consent for publication

The authors declared that there are no conflicts of interest.

Consent for publication

All the listed authors have participated in the study, and have seen and approved the submitted manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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Figures

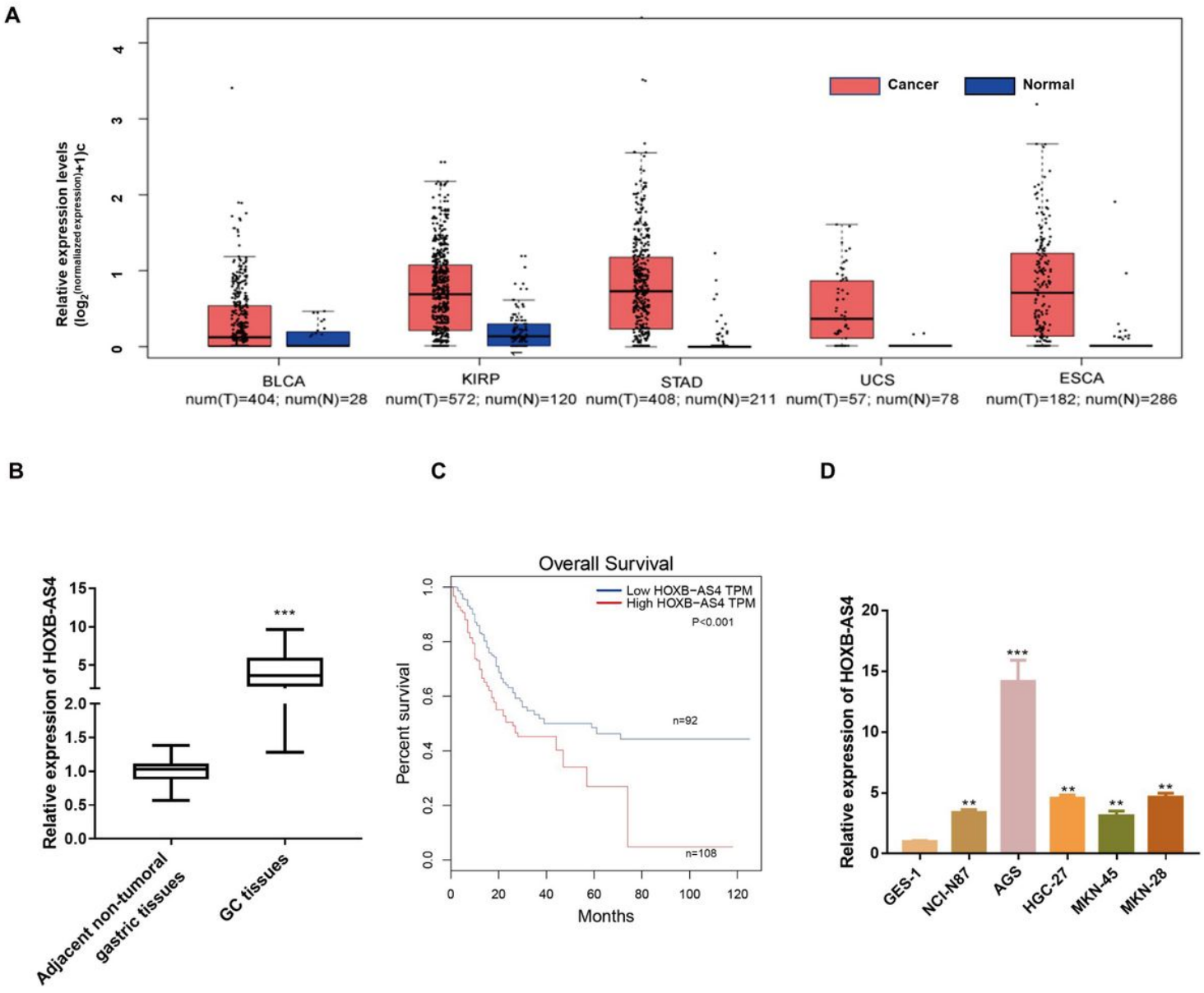

Figure 1

LncRNA *HOXB-AS4* is up-regulated in GC tissues and GC cells.

(A) LncRNA *HOXB-AS4* is up-regulated in the patients' cohort of bladder urothelial carcinoma (BLCA), kidney renal papillary cell carcinoma (KIRP), stomach adenocarcinoma (STAD), uterine carcinosarcoma (UCS), and esophageal carcinoma (ESCA). (B) qRT-PCR verified the upregulation of *HOXB-AS4* in GC tissues. (C) Kaplan-meier analysis showed overall survival in GC patients with *HOXB-AS4* expression. (D) Expression of *HOXB-AS4* in GES-1 cells and GC cancer cells was measured by qRT-PCR. Data are presented as mean ± s. e. m. **p<0.01,***p<0.001, compared to the control group.

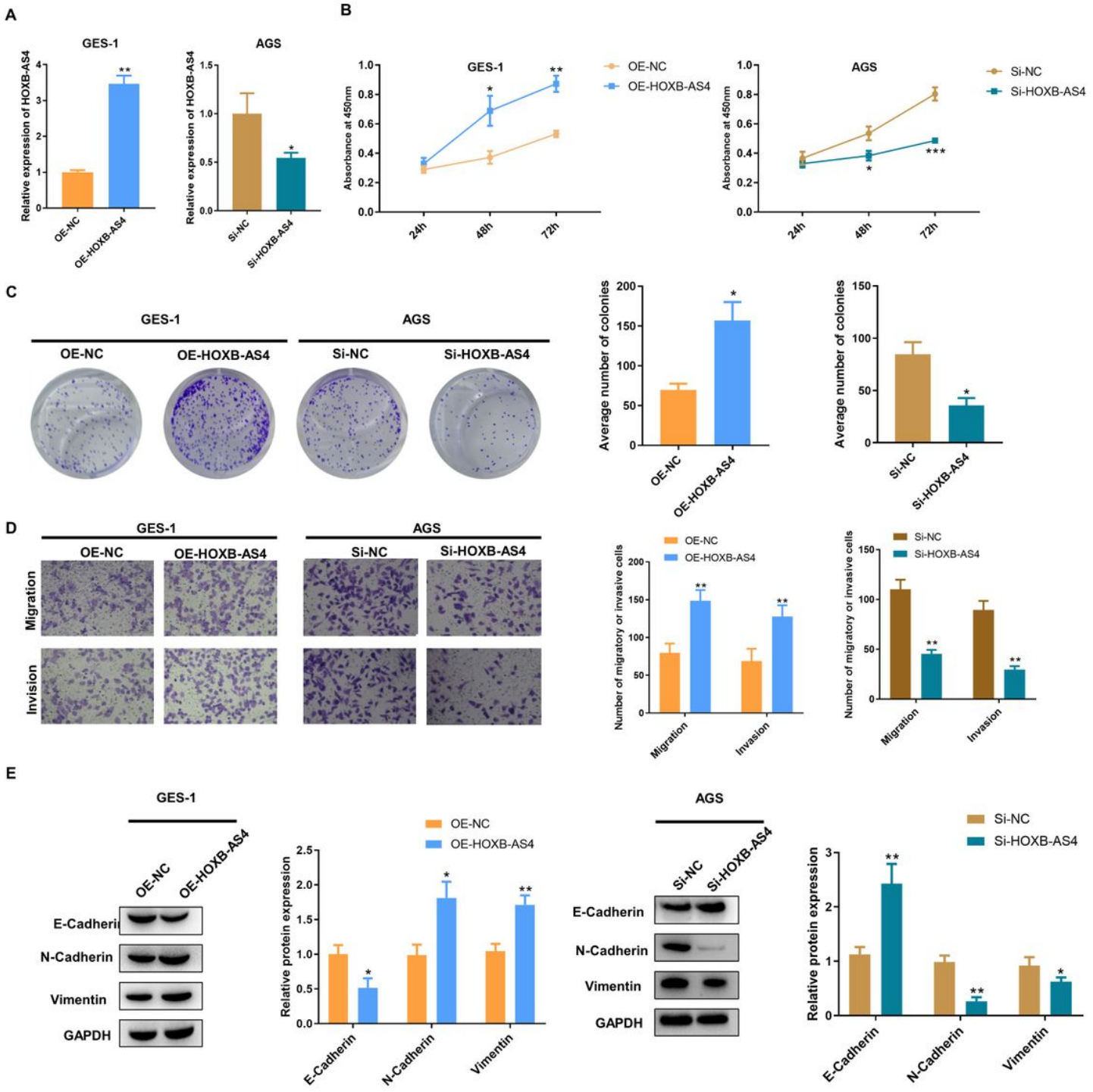

Figure 2

***HOXB-AS4* modulates GC cells proliferation, migration, invasion and EMT markers expression.**

(A) qRT-PCR showed the transfection efficiency of overexpression or knockdown of *HOXB-AS4* in GES-1 and AGS cells. (B) Cell viability of GES-1 overexpressed with *HOXB-AS4* and AGS knocked down of *HOXB-AS4* was detected by using MTT at 24, 48, and 72 hour. (C) Clone formation assay of GES-1 overexpressed with *HOXB-AS4* and AGS knocked down of *HOXB-AS4*. (D) Transwell assay showed the migration and invasion of GES-1 overexpressed with *HOXB-AS4* and AGS knocked down of *HOXB-AS4*. (E) Protein level of EMT markers in GES-1 cells overexpressed with *HOXB-AS4* and AGS cells knocked down of *HOXB-AS4*. n=3. Data are presented as mean ± s. e. m. *p<0.05, **p<0.01, compared to the control group.

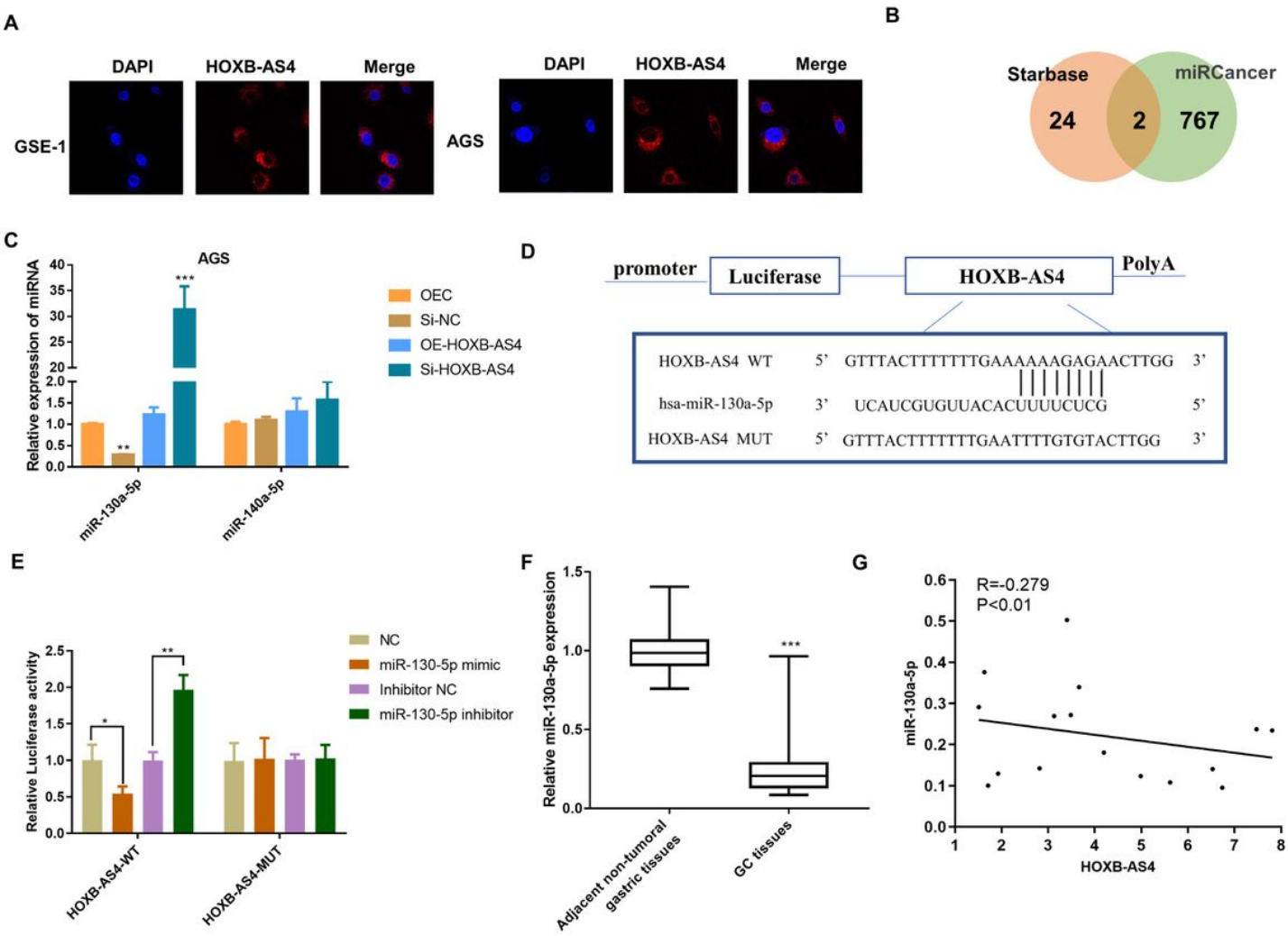

Figure 3

***HOXB-AS4* acts as a sponge for miR-130a-5p.**

(A) FISH analysis indicated subcellular location of *HOXB-AS4* (red) in GSE-1 cells and AGS cells. (B) Schematic drawing of the screening intersection of the candidate miRNAs. (C) qRT-PCR was used to determine the level of miRNAs responded to overexpression or knockdown of *HOXB-AS4*. (D) The

potential miRNA binding sites and the related mutant form were shown. (E) The luciferase reporter plasmid carrying wild type (WT) or mutant (MUT) *HOXB-AS4* was co-transfected with miR-130a-5p mimic or inhibitor. Relative luciferase activity of the cells was measured. (F) miR-130a-5p in GC tissues was measured by qRT-PCR. (G) The negative correlation of *HOXB-AS4* and miR-130a-5p expression in GC tissues (n=16) was shown. n=6. Data are presented as mean ± s. e. m. *p<0.05, **p<0.01, ***p<0.001, compared to the indicated control group.

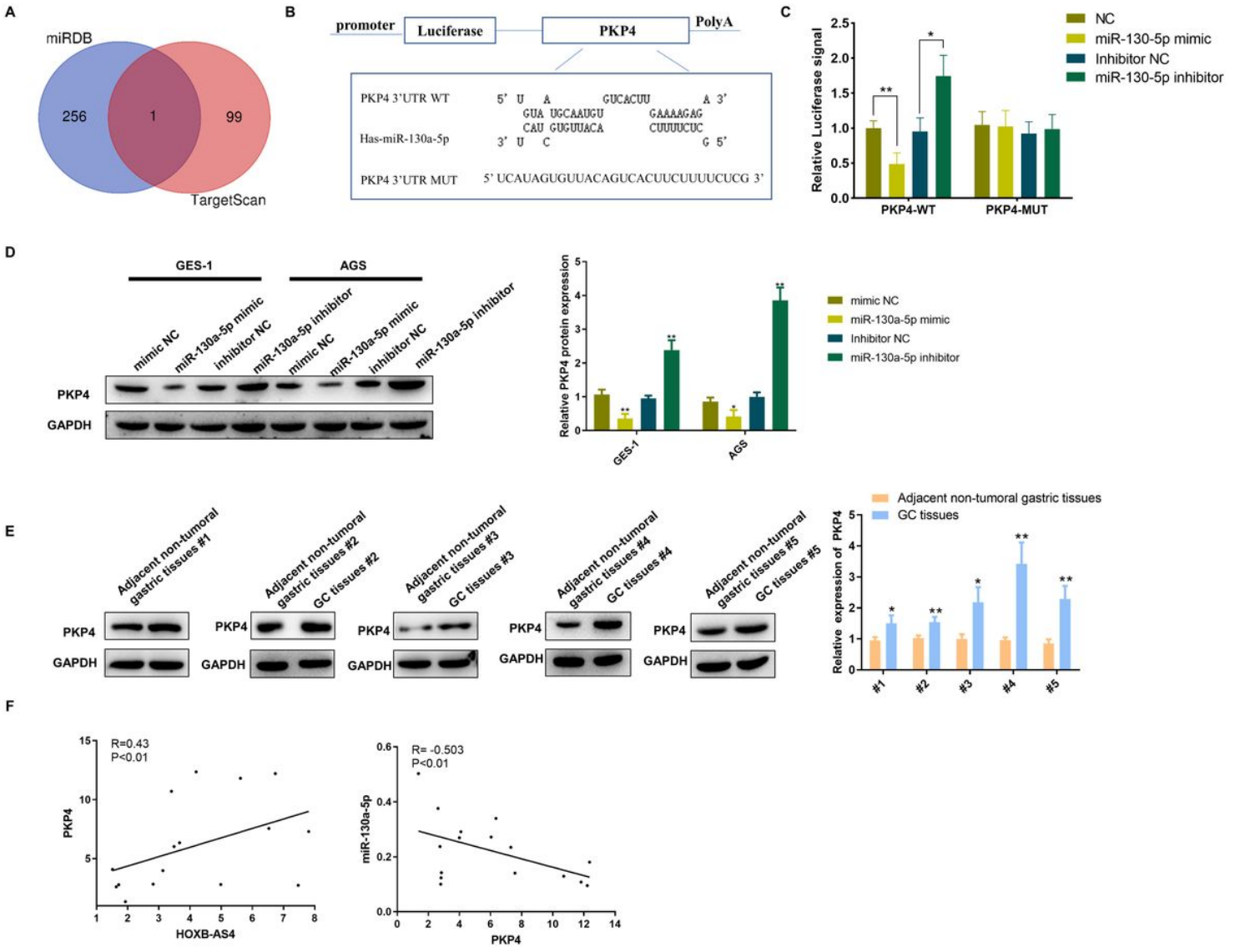

Figure 4

PKP4 is a direct target of miR-130a-5p.

(A) Schematic drawing of the screening intersection of the candidate target gene. (B) The potential miRNA binding sites and the related mutant form were shown. (C) The luciferase reporter plasmid carrying wild type (WT) or mutant (MUT) *PKP4* was co-transfected with miR-130a-5p mimic or inhibitor. Relative luciferase activity of the cells was measured. (D) *PKP4* expression in GES-1 and AGS-1 cells treated with miR-130a-5p mimic or inhibitor was measured by western blot. (E) Western blot was used to

determine the *PKP4* expression in GC tissues. (F) The positive correlation of *PKP4* and *HOXB-AS4* and the negative correlation of *PKP4* and miR-130a-5p expression in GC tissues (n=16) was shown. Data are presented as mean ± s. e. m. *p<0.05, **p<0.01, compared to the indicated control group.

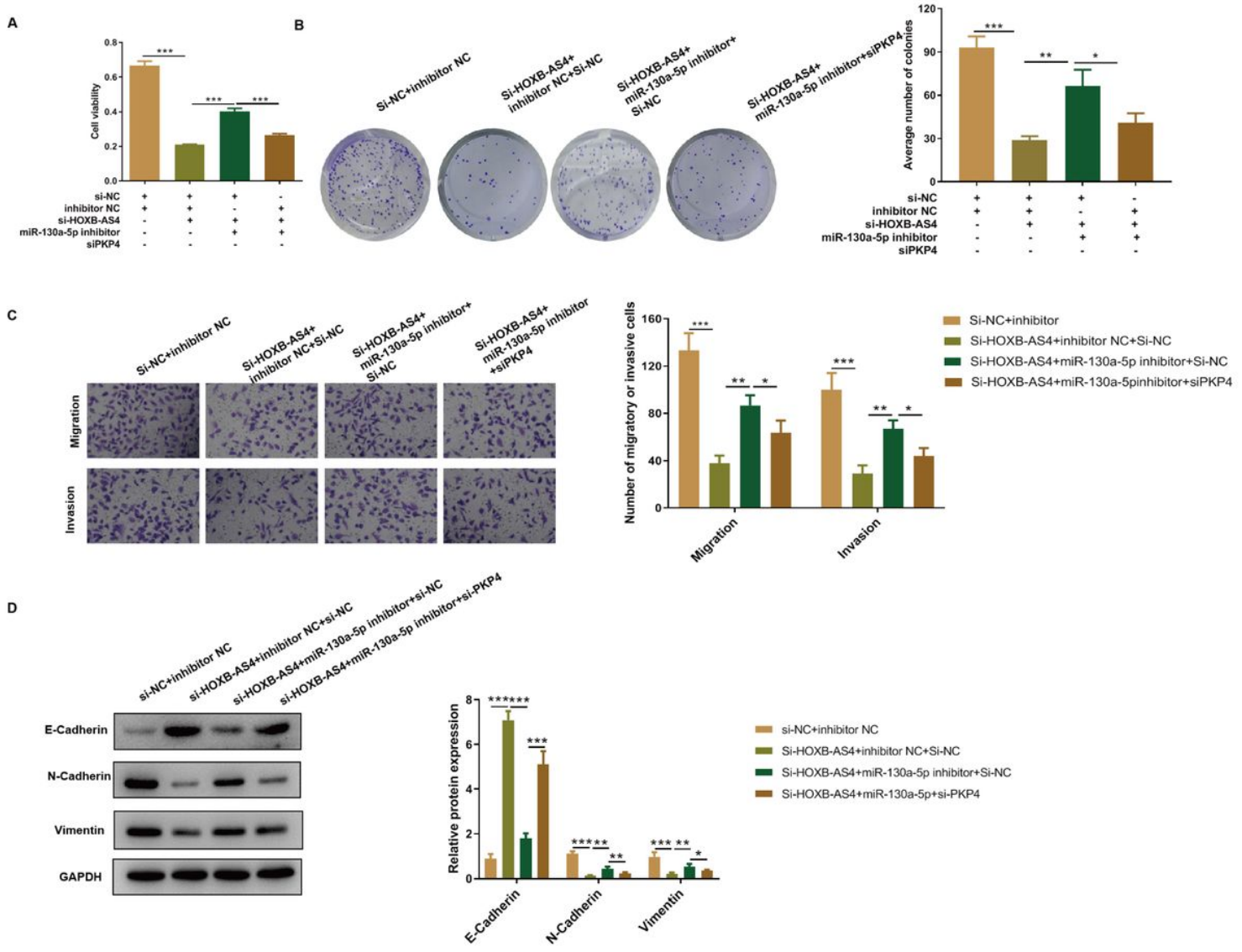

Figure 5

HOXB-AS4 modulates GC cells proliferation, migration, invasion and EMT markers expression via miR-130a/*PKP4* axis.

AGS cells were knocked down of *HOXB-AS4* singly, or with knockdown of miR-130a-5p or knockdown of *PKP4* collectively. (A) Cell viability of AGS cells was determined by MTT. (B) Clone formation assay of AGS cells in the indicated group. (C) Migration and invasion of cells in the indicated group were measured by transwell assay. (D) Protein markers of EMT in AGS cells were detected by western blot. n=6. Data are presented as mean ± s. e. m. *p<0.05, **p<0.01, ***p<0.001, compared to the indicated group.

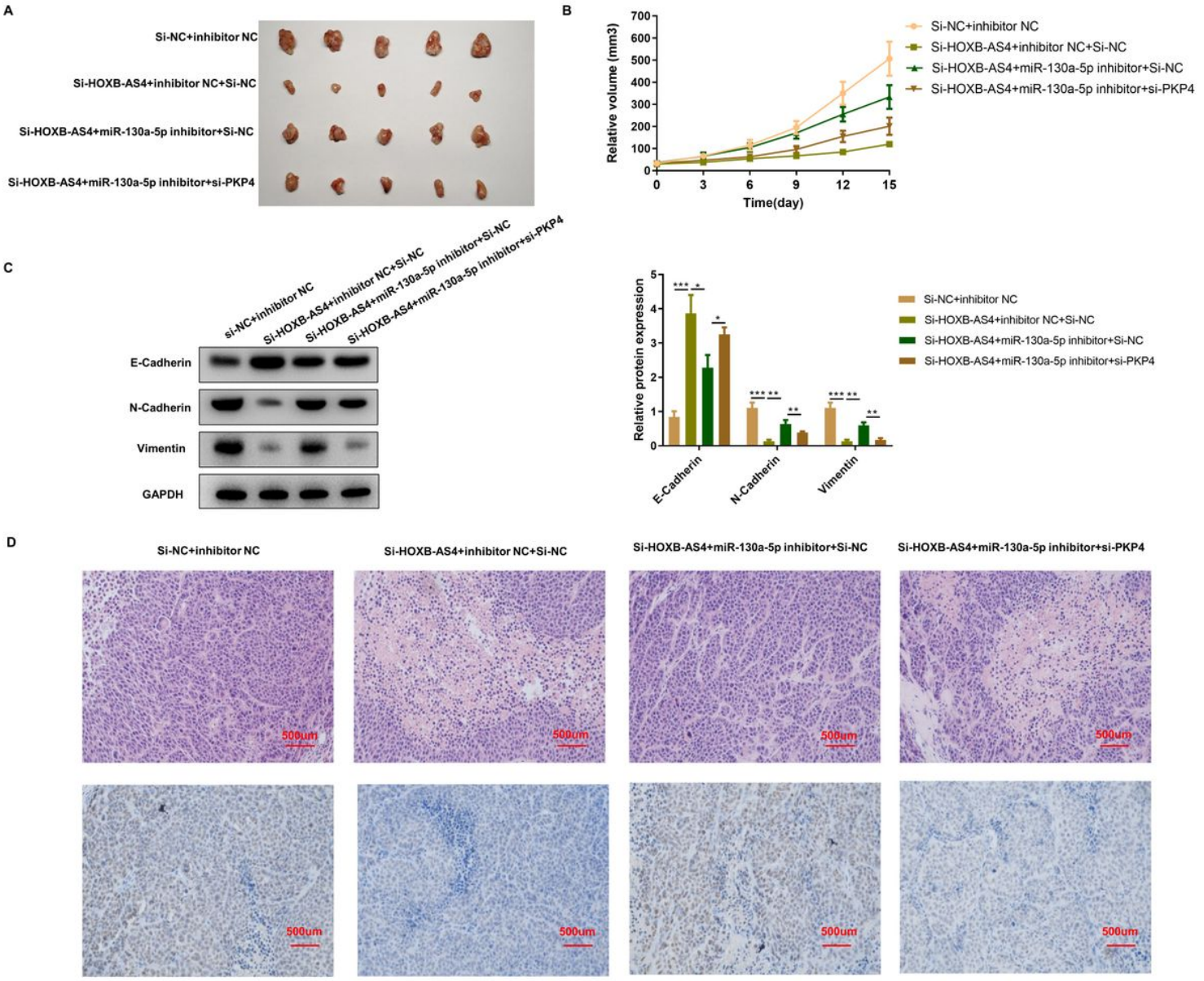

Figure 6

Knockdown of *HOXB-AS4* inhibits GC progression and modulates EMT markers expression *in vivo*.

Mice were divided into four groups, with 10 mice in each group. miR-130a-5p antagomir (80 mg/kg) was administrated by tail injection. Knockdown of *HOXB-AS4* or *PKP4* was performed by using the related knockdown lentivirus (4×10^7 IU/mL, 50 μL) by intra tumoral injection. After 15 days, the mice were sacrificed and subjected to following experiments. (A) Pictures of the isolated tumors of the indicated group. (B) Change of tumor volume was recorded every three days. (C) Protein markers of EMT in tumors were detected by western blot. (D) H&E staining of tumor tissues and IHC staining of *PKP4* expression in tumor tissues were shown. n=10. Data are presented as mean ± s. e. m. *p<0.05, **p<0.01, ***p<0.001, compared to the indicated group.

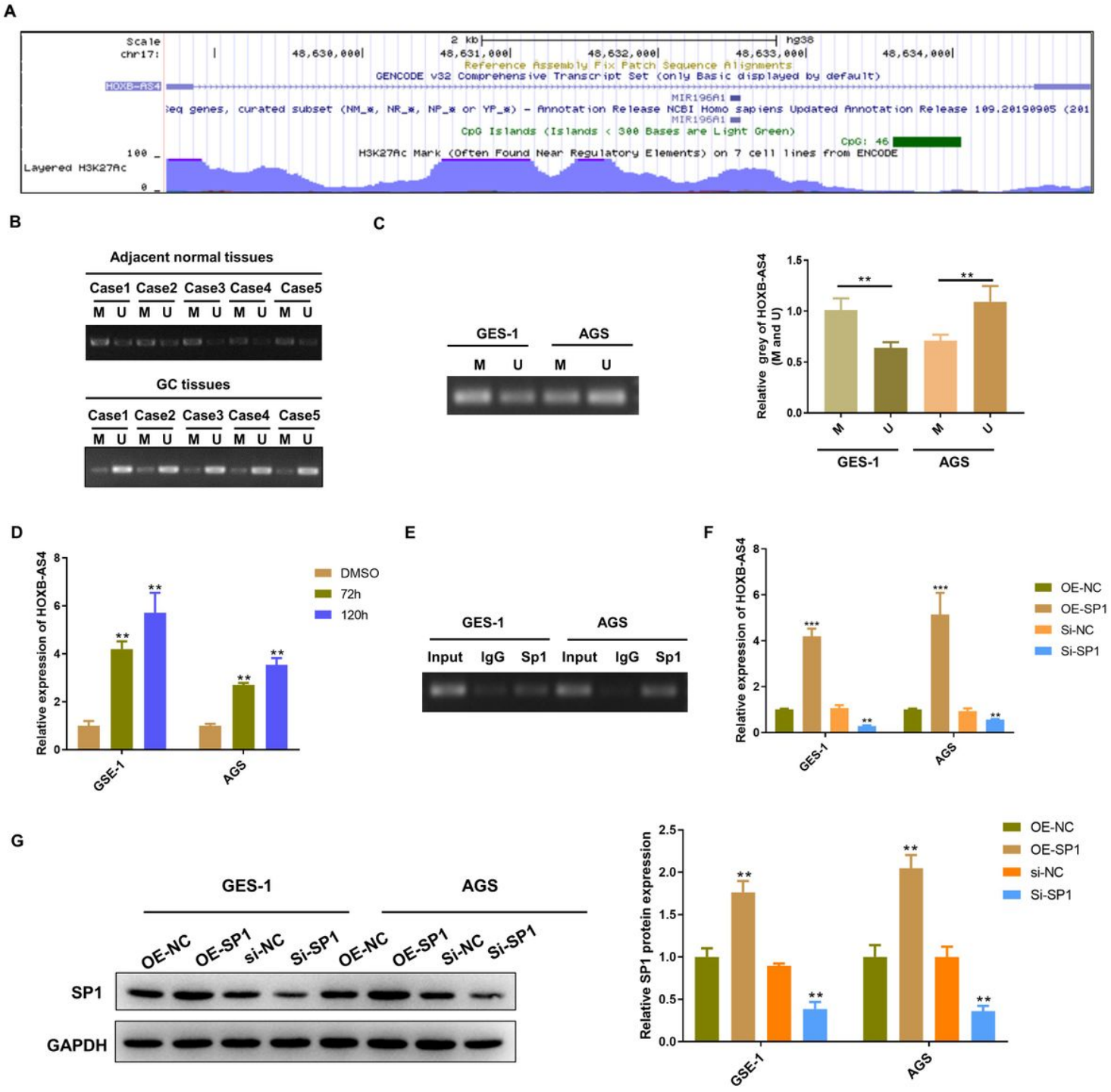

Figure 7

DNA methylation and *SP1* participate in the regulation of the expression of *HOXB-AS4*.

(A) The UCSC Genome Bioinformatics Site (http://genome.ucsc.edu/) showed high enrichment of H3K27ac at the promoter of *HOXB-AS4*. (B) MSP analysis was performed to examine methylation status of CpG island at the promoter region of *HOXB-AS4* in GC tissues and adjacent normal tissues. (B) MSP analysis was performed to examine methylation status of CpG island at the promoter region of *HOXB-*

AS4 in GES-1 cells and AGS cells. (D) qRT-PCR analysis was used to detect the *HOXB-AS4* level in GES-1 and AGS cells under the treatment with 5-azacytidine for 72 h and 120 h. (E) ChIP analysis was used to detect the *SP1* binding to the promoter region of *HOXB-AS4*. (F) *HOXB-AS4* level in GES-1 and AGS cells overexpressed or knocked down with *SP1* was determined by qRT-PCR. (E) The transfection efficiency of *SP1* was verified by western blot. n=6. Data are presented as mean ± s. e. m. **p<0.01, ***p<0.001, compared to the indicated control group.

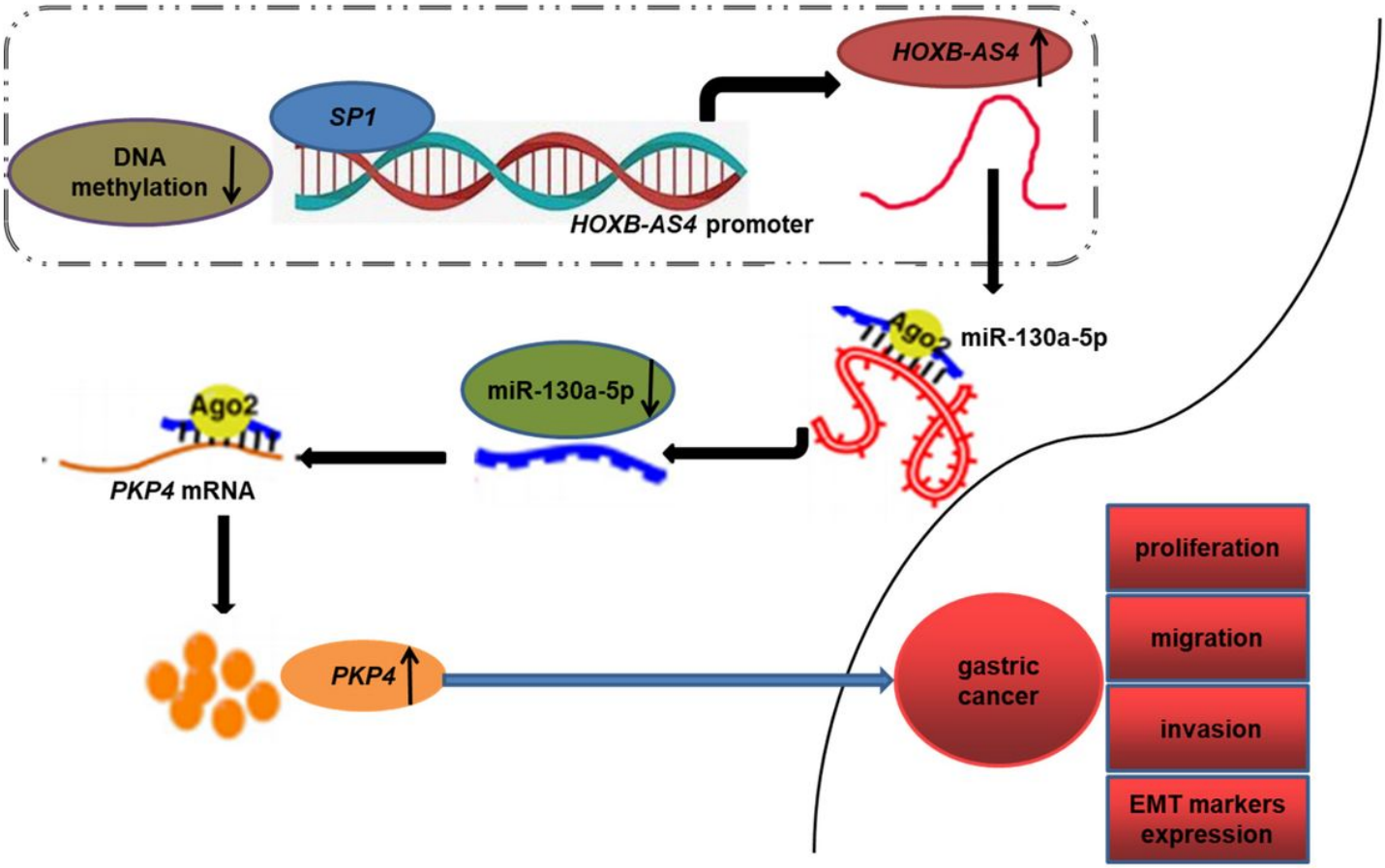

Figure 8

Proposed model in which *HOXB-AS4* mediates the progression of GC.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.jpg
- Additionalfile1.doc