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Supplemental Information

BTEB2-Activated lncRNA TSPEAR-AS2 Drives GC Progression through Suppressing GJA1 Expression and Upregulating CLDN4 Expression

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Figure S1

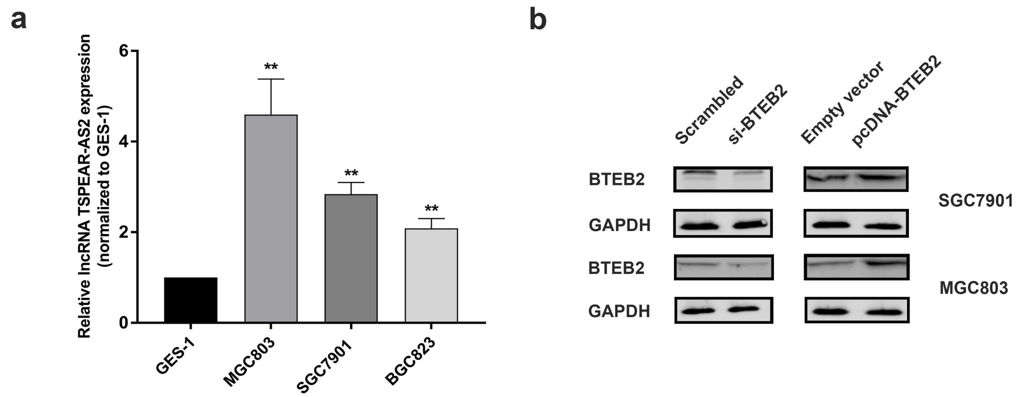

Figure S1 Relative expression level of lncRNA TSPEAR-AS2 in GC cells and the alteration of BTEB2 protein level in GC cell with BTEB2 knockdown or overexpression.

a Relative expression level of lncRNA TSPEAR-AS2 in GC at cellular level.

b BTEB2 level was detected in GC cells with BTEB2 knockdown or overexpression using western blot assays in SGC7901 and MGC803 cells.

Figure S2

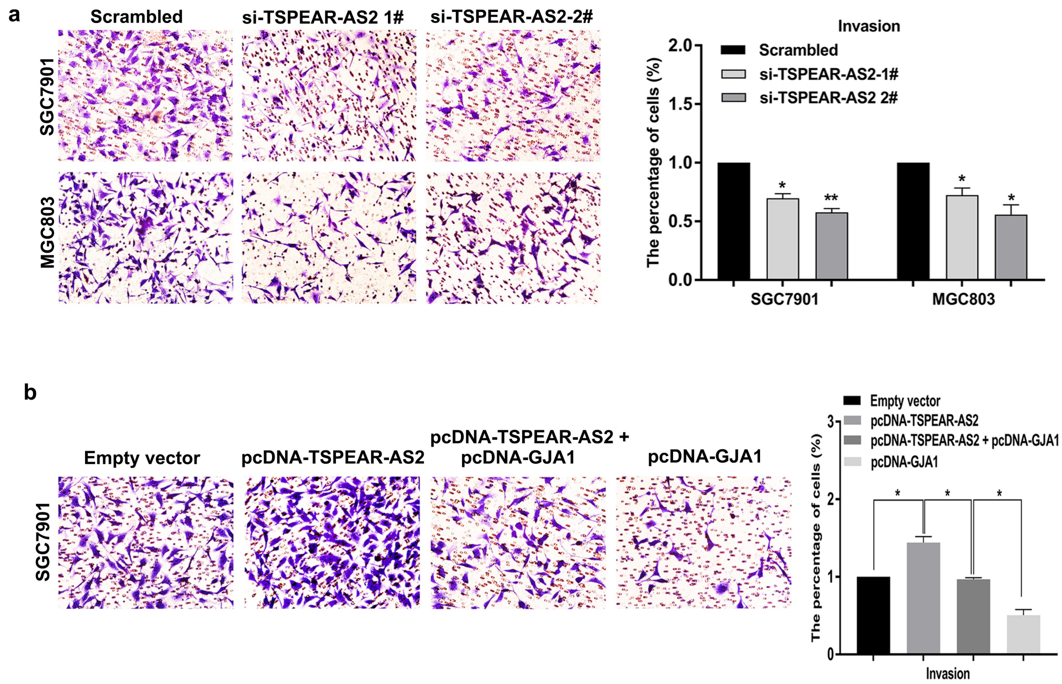

Figure S2 The involvement of GJA1 in TSPEAR-AS2-mediated GC cell invasion.

a The impact of TSPEAR-AS2 on GC cell invasion.

b GJA1 overexpression can partly reverse the promotion effects on GC cell invasion mediated by TSPEAR-AS2 overexpression. *$p < 0.05$, **$p < 0.01$.

Figure S3

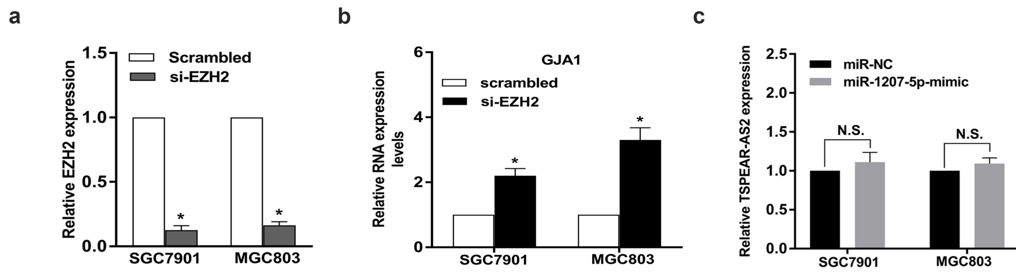

Figure S3 The impact of EZH2 depletion on GJA1 level in GC cells and the effects of miR-1207-5p on TSPEAR-AS2 regulation in GC cells.

a The knockdown efficacy of EZH2 siRNAs in GC cells.

b The expression of GJA1 in GC cells with EZH2 knockdown was analyzed by qRT-PCR assays.

c The effects of miR-1207-5p on the regulation of TSPEAR-AS2 in GC cells. *p < 0.05, **p < 0.01.

Table S1. Primers for qRT-PCR, siRNAs oligonucleotides and the company for antibody.

Primers used for qRT-PCR	
GAPDH-F	GCTCTCTGCTCCTCCTGTTC
GAPDH-R	ACGACCAAATCCGTTGACTC
CLDN4-F	AGTGGATGGACGGGTTTAGA
CLDN4-R	ACCCTCCCAGGCTCATTAGT
BTEB2-F	CTTCCACAACAGGCCACTTACTT
BTEB2-R	AGAAGCAATTGTAGCAGCATAGGA
EZH2-F	TGCACATCCTGACTTCTGTG
EZH2-R	AAGGGCATTCACCAACTCC
GJA1-F	TGTCCCTGGCCTTGAATATC
GJA1-R	GTGAGGAGCAGCCATTGAA
siRNAs oligonucleotides	
TSPEAR-AS2 1#	GGAUAAAGCCUCAAGUCCUGCAACU
TSPEAR-AS2 2#	GAGAGGAUGGCAUGGGUGACACGCA
TSPEAR-AS2 3#	GCGGCUCCUGUGUGCUUUGAAGUUU
si-EZH2	CGGCUUCCCAAUAACAGUATT
si-BTEB2	GCAGACUGCAGUGAAACAA
Antibody	**Company**
GAPDH	Cell Signaling Technology
GJA1	Abcam
IgG	Millipore
EZH2	Millipore
SUZ12	Millipore
AGO2	Millipore
CLDN4	Abcam
H3K27me3	Abcam

Supplementary Methods

Flow cytometric analysis

For apoptosis analysis, we purchased a FITC-Annexin V kit from BD Biosciences and stained GC cells with both FITC-Annexin V and PI. Then, a flow cytometer (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences) was used to analyze cells, which can be classified into viable cells, dead cells, early apoptotic cells, and late apoptotic cells.

RNA-expression data retrieval and analysis

Gastric cancer expression data were downloaded from the TCGA and GEO dataset. The datasets from TCGA, GSE66229, GSE65801 and GSE54397 were analyzed in this study. The BAM files and normalized probe-level intensity files were downloaded from TCGA and GEO databases, respectively. For multiple probes corresponding to a gene, the average signal generate lncRNAs. Download the probe sequences from GEO or the microarray manufacturer and reannotate probes by bowtie according to GENCODE Release 19 annotation.

RNA extraction and quantitative RT-PCR assays

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For quantification of RNA expression, cDNAs were synthesized using the PrimeScript RT Reagent Kit (TaKaRa). Real-time PCR assays were performed in triplicate with an Applied Biosystems Prism

7500 FAST Sequence Detection System using SYBR Premix Ex Taq (Takara). Human GAPDH was used as endogenous controls. RNA expression levels were investigated using the 7500 FAST System. The primers are summarized in **Table S1.**

Cell transfection

A DNA Midiprep kit (Qiagen, Hilden, Germany) was applied to manufacture the plasmid vectors for transfection. Three individual TSPEAR-AS2 siRNAs, miR-1207-5p mimics, miR-1207-5p inhibitor and scrambled negative control were obtained from Invitrogen. According to the manufacturer's instructions, GC cell lines and HEK-293T cells were transfected with siRNAs and plasmid vectors using Lipofectamine 3000 (Invitrogen). Stable knockdown of TSPEAR-AS2 level in GC cells was conducted by lenti-virus-mediated shRNA targeting TSPEAR-AS2. The sequences of these synthesized oligonucleotides for RNA interference (RNAi) are listed in **Table S1.**

Cell proliferation assays

A cell proliferation Reagent Kit I (MTT, Roche Applied Science) was used to test cell viability of GC cells with TSPEAR-AS2 knockdown or overexpression. The optical density 490 nm (OD490) was measured using a microplate reader. The other examinations of cell viability were conducted in GC cells using Cell Counting Kit-8 (CCK8; Beyotime Institute of Biotechnology, Shanghai, China) The optical density 450 nm (OD450) was measured using a microplate reader. For colony-formation

assays, cells were seeded in 6-well plates and grown for 10 days. We fixed cells with methanol and washed fixed cells with phosphate buffer saline (PBS). Then, 0.1% crystal violet (Sigma-Aldrich) was used to stain fixed cells, and the colony formation was determined by counting the number of stained colonies. The experiments were independently repeated three times.

Transwell assays

Cells were plated into the top chamber independent pathologists in migration assay, and cells were into the top chamber precoated with Matrigel in invasion assays. DMEM or RPMI1640 supplemented with 10% FBS were used to fill the bottom chamber. After incubation for 24-48 h, the cells were stained with 0.1% crystal violet for 30 min. Finally, microscope was used to calculate the number of migrated and invaded cells on the lower surface of the membrane.

Animal experiments

Four-week male BALB/c nude mice were kept in the specific pathogen-free condition. For the xenotransplantation mouse model, GC cells stably transfected sh-TSPEAR-AS2 or sh-Control were harvested and transplanted subcutaneously to either side of each mouse. The tumor volumes were calculated using the following equation: Volume = length × width × width × 0.5. Finally, the tumor weights were tested and recorded. All the mice were euthanized via CO2 asphyxiation. This study was conducted in strict accordance with the Guide for the care and Use of Laboratory

Animals of NIH. Animal protocols were approved by the Committee on the Ethics of Animal Experiments of the Nanjing Medical University. All institutional and national guidelines for the care and use of laboratory animals were followed.

RNA immunoprecipitation (RIP)

EZMagna RIP Kit (Millipore) was selected to perform RIP assays in SGC7901 and MGC803 cells. Cells lysates were prepared with complete RIP lysis buffer and followed by incubation with magnetic beads conjugated with antibodies at 4°C. Finally, the beads were washed using wash buffer, and then incubated with Proteinase K. The purified RNA was subjected to qRT-PCR assays.

Chromatin immunoprecipitation assays

EZ-ChIP Kit (Millipore, Billerica, MA, USA) was used to conduct ChIP assays in accordance with the instruction of manufacturer. SGC7901 and MGC803 cells were treated with formaldehyde and incubated for 10 min to generate DNA-protein cross-links. Anti-EZH2 and anti-H3K27me3 antibodies (Millipore) were used to immunoprecipitate chromatin fragments. Finally, qRT-PCR assays were performed to analyze the precipitated chromatin DNA.

Subcellular fractionation location and FISH assay

Based on the manufacturer's instructions, we used PAIRS Kit (Cat. AM1921, Invitrogen, CA, USA) to separate the nuclear and cytosolic portion in SGC7901 and

MGC803 cells. For FISH assay, cells were firstly fixed in 4% formaldehyde for 15 min. Fixed cells were washed with PBS and followed by treatment with pepsin (1% in 10 mmol/L HCl). Subsequently, 70%, 90%, and 100% ethanol was used for dehydration. The air-dried cells were 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 min. Hybridization was performed at 55°C for 2 h, and the slide was washed and dehydrated. RNA FISH probes were designed and synthesized by Bogu Co, Ltd. The images were collected by using Olympus Fluoview laser scanning confocal microscope.

Prognostic analysis

Firstly, launch the http://kmplot.com/analysis/ website. Then, select module titled Start KM plotter for gastric cancer. Subsequently, enter the gene symbol and select suitable methods to split patients. Finally, click the button of draw Kaplan-Meier plot.

5-Ethynyl-2-deoxyuridine (EdU) Staining Assay

The proliferative abilities of GC cells were assessed through EdU labeling/detection kit (Ribobio, Guangzhou, China) based on the instructions of the manufacturer. The cells were cultured in Edu labeling medium, with incubation for 2 h at 37 °C under 5 % CO2. 4 % paraformaldehyde was used to fix the treated cells for 30 minutes, which were subsequently processed with 0.5 % Triton X-100 for 20 min at room temperature. After washing in PBS, the fixed cells were treated with anti-Edu working

solution at 25 °C for 30 min and subsequently processed with 0.5 % Triton X-100 for 20 min at room temperature. Next, Hoechst 33342 was used to incubate the treated cells for 30 min. Finally, fluorescent microscope was implicated for cell observation and the ratio of EdU positive cells were calculated.

Transcriptome sequencing and Data analysis

Total RNA from the SGC7901 cells with TSPEAR-AS2 knockdown and control SGC7901 cells were isolated using RNeasy mini kit (Qiagen, Germany). Paired-end libraries were synthesized by using the TruSeq RNA Sample Preparation Kit (Illumina, USA) following TruSeq RNA Sample Preparation Guide. Briefly, The poly-A containing mRNA molecules were purified using poly-T oligo-attached magnetic beads. Purified libraries were quantified by Qubit 2.0Fluorometer (Life Technologies, USA) and validated by Agilent 2100 bioanalyzer (Agilent Technologies, USA) to confirm the insert size and calculate the mole concentration. Cluster was generated by cBot with the library diluted to 10 pM and then were sequenced on the Illumina HiSeq X-ten (Illumina, USA). The library construction and sequencing was performed at Shanghai Biotechnology Corporation. Hisat2 (version:2.0.4) was used to map the cleaned reads to the human GRCh38 reference genome with two mismatches[1]. Then ,we ran Stringti (version:1.3.0) with a reference annotation to generate FPKM values for known gene models[2, 3].

References:

1. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods 2015, 12(4):357-360.

2. Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 2015, 33(3):290-295.

3. Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nat Protoc 2016, 11(9):1650-1667.