European Review for Medical and Pharmacological Sciences 2019; 23: 8371-8376

The carcinogenic complex lncRNA FOXP4-AS1/EZH2/LSD1 accelerates proliferation, migration and invasion of gastric cancer

R.-Y. CHEN[1], Q. JU[1], L.-M. FENG[1], Q. YUAN[2], L. ZHANG[1]

[1]Department of Gastrointestinal Surgery, Qingdao Central Hospital, Qingda[illegible]ina
[2]Department of Chinese and Western Medicine, Qingdao Central Hospita[illegible]ngdao[illegible]ina

Abstract. – OBJECTIVE: To clarify the role of lncRNA FOX4-AS1 in the progression of gastric cancer (GC) via interacting with EZH2/LSD1.

PATIENTS AND METHODS: Relative level of FOXP4-AS1 in GC tissues and adjacent normal tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The potential influences of FOXP4-AS1 on cellular behaviors of GC cells were evaluated via a series of functional experiments. Bioinformatics prediction, RNA immunoprecipitation (RIP) assay, and Western blot were conducted to verify the potential of EZH2/LSD1 as a target of FOXP4-AS[illegible]

RESULTS: FOXP4-AS1 was upregulated[illegible] tissues relative to controls. Its level was [illegible]h-er in GC patients with stage III-IV than t[illegible] with stage I-II. The survival rate was lowe[illegible] GC patients presenting the hig[illegible]ression FOXP4-AS1 compared with [illegible]esenting low expression. Transfecti[illegible] sh-[illegible]P4-AS1 1# or sh-FOXP4-AS1 2#[illegible]enuate[illegible]oliferative, migratory, and invas[illegible]ilitie[illegible] BGC7901 cells. FOXP[illegible]AS[illegible]d to [illegible] and EZH2, and posi[illegible]y regu[illegible]their expression levels. Trans[illegible]on of sh-L[illegible]r sh-EZH2 reduced the pr[illegible]e ability of [illegible]lls.

CONCLUS[illegible]: F[illegible]-AS1 binds to EZH2/LSD1 to fo[illegible] a carcinc[illegible] complex, thus accelerati[illegible] cells to pro[illegible], migrate and invade.

Key[illegible]ds:
Ga[illegible] (GC), F[illegible]4-AS1, LSD1, EZH2.

[illegible]troduction

[illegible]cer (GC) is one of the most prevalent [illegible]alignancies, ranking second in the tumor-relat-[illegible]eath globally. The incidence of GC is on the ri[illegible]arly-stage symptoms of GC are not evident, and effective examinations lack. The pathogenesis of GC is diverse, including *H.pylori* infection, eating habits, environmental factors, etc.[1]. The diagnostic rate [illegible]rly-stage [illegible]vely low, which is a[illegible]ason for th[illegible]n mortality of GC[2,3]. [illegible]eat s[illegible] on surgical procedures, chemotherapy, targe[illegible] drugs, and biological treat[illegible]f GC have [illegible]made. However, the 5[illegible] survival of advanced GC is still low[4]. In re[illegible] years, releva[illegible] studies have been extensively [illegible]ucted to ex[illegible]e the molecular mechanisms o[illegible] etiology [illegible] pathogenesis of GC. It is of gre[illegible]ifica[illegible]to develop sensitive diagnostic hallma[illegible]herapeutic targets of GC.

Non-coding RNAs are hot topics in tumor [illegible]. They are divided into lncRNAs and [illegible]n-coding RNAs (microRNA, siRNA, and piRNA) based on the length[5-7]. LncRNA is 200-1000 Kb long, which lacks the open reading frame and protein-encoding functions. The production of lncRNAs is similar to that of mRNAs[8-11]. LncRNAs have been identified as regulators participating in cellular behaviors. They are also involved in regulating pathological progress through various mechanisms[9,10,12-14]. It is reported that lncRNA is widely involved in tumor progression. For example, lncRNA PCGEM1 locates on 2q32 and is upregulated in prostate cancer. It is capable of stimulating the proliferative rate and colony formation ability in prostate cancer cells. LncRNA HOTAIR enhances the migratory ability of glioma cells *via* silencing the WNT inhibitor WIF1[1,4,15]. LncRNA is crucial in the occurrence and progression of tumors[16].

LncRNA FOXP4-AS1 was firstly reported in 2016. It is upregulated in colorectal cancer (CRC) and closely related to cell cycle progression. FOXP4-AS1 remarkably influences the occurrence and progression of CRC[16-18]. The regulatory effect of FOXP4-AS1 is also reported in osteosarcoma[10,11,19]. It is believed that FOXP4-AS1 exerts a crucial role in tumor biology.

Corresponding Author: Lin Zhang, BM; e-mail: zhangne1975@126.com

This work mainly investigated the role of FOXP4-AS1 in the progression of GC and the underlying mechanism. Our study aims to provide novel directions for improving the prognosis of GC patients.

Patients and Methods

Baseline Characteristics

24 paired GC and adjacent normal tissues were surgically harvested from GC patients in Qingdao Central Hospital from March 2016 to October 2018. Tissue samples were immediately preserved in liquid nitrogen after removal. None of the enrolled patients had the preoperative treatment and family history. This investigation was approved by the Medical Ethics Committee of Qingdao Central Hospital and informed consent from each subject was obtained.

Cell Transfection

AGS and BGC7901 cells were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) cont[illegible] 10% fetal bovine serum (FBS; Gibco, Ro[illegible] MD, USA) in a 5% CO_2 incubator at 37°C.

Cell Transfection

Cells were subjected to trans[illegible]t 70-80[illegible] of confluence. 1.5 mL of [illegible] medium and 500 µL of Lipofecta[illegible]M 200 [illegible]ransfection solution (Invitroge[illegible]lsbad[illegible]) containing transfecti[illegible]vec[illegible] appli[illegible]n each well. A com[illegible] mediu[illegible]s replaced 6 h later. Sequenc[illegible] transfecti[illegible]tors were as follows: sh[illegible]S1 1 #: 5'-[illegible]GUAG-CUGCGGGA[illegible]GGUC[illegible]'; sh-FOXP4-AS1 2#: 5'-A[illegible]ACAGCUU[illegible]GGGCAAUU-3'; sh-FOX[illegible]AS1 3#: 5'-AA[illegible]ACUAAAUUA-CAG[illegible]GGC[illegible] sh-LDS1: 5'-GCCACCCA-GA[illegible]AUU[illegible]U-3'; sh-EZH2: 5'-GAGGUU-CAG[illegible]UGAU[illegible].

Extra[illegible]d Quantitative Re[illegible]Time-Po[illegible]erase Chain Reaction (q[illegible]CR)

[illegible]were lysed in 1 mL of TRIzol (In[illegible]trogen, Carlsbad, CA, USA) and incubated in [illegible]mL of chloroform. After maintenance at room te[illegible]rature for 5 min, the mixture was centrifuged at 4°C, 12,000 rpm for 10 min. The supernatant was transferred to a new EP tube (Eppendorf, Hamburg, Germany) and incubated with isodose isopropanol. The mixture was subjected to centrifugation under the same condition. The precipitant was washed with ethanol, air dried, and dissolved in Diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). [illegible] sample was quantified using the Na[illegible]rop 100[illegible] and preserved at –80°C. The extr[illegible]RNA was quantified and reversely transcribe[illegible] cDNA, followed by PCR using the S[illegible]R Gre[illegible]thod. The relative level was cal[illegible]ed using t[illegible] method. Primer sequen[illegible]were [illegible] follows: F: 5'-CTCGCTTCG[illegible]GCAC[illegible]CATATA-3', R: 5'-AATATGGA[illegible]C[illegible]TC[illegible]GA-3'; FOXP4-AS1 F: 5'-CCTC[illegible]TTC[illegible]GAA[illegible]-3', R: 5'-GCACTTT[illegible]AACAAT[illegible]C[illegible].

5-Ethyny[illegible]-De[illegible]ridine (EdU) Assay

Cells were seede[illegible]he 24-well plate with 4×10[illegible]per well. Ce[illegible]re labeled with 50 µ[illegible]L EdU at 37°C for [illegible] h and subjected to [illegible]min fixation [illegible] 4% paraformaldehyde and [illegible]min incubati[illegible]n phosphate-buffered saline ([illegible] containin[illegible]% Triton-100. After washing wit[illegible]co[illegible]ng 3% bovine serum albumin (BSA), [illegible]of the dying solution was applied [illegible]er well for 1 h incubation in the dark and cells [illegible]ounter-stained with 100 µL of 1×Hoechst [illegible]f 30 min. The ratio of EdU-positive cells was calculated.

Transwell Assay

Cell density was adjusted to 2×10^4/mL. 400 µL of suspension was applied in the upper side of the transwell chamber (Millipore, Billerica, MA, USA) pre-coated with Matrigel (BD Bioscience, Franklin Lakes, NJ, USA). In the bottom side, 700 µL of medium containing 10% FBS was applied. After 48 h of incubation, invasive cells were fixed in methanol for 30 min, dyed with 0.1% crystal violet for 10 min and counted using a microscope. Penetrating cells were counted in 6 randomly selected fields per sample. Transwell migration assay was similarly conducted without Matrigel pre-coating.

Cell Counting Kit (CCK-8) Assay

Cells were seeded in the 96-well plate with 1×10^4 cells per well. Absorbance (A) at 450 nm was recorded at the established time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for depicting the viability curve.

Colony Formation Assay

Cells were seeded in the 6-well plate with 2.5×10^3 cells per well and cultured for 2 weeks.

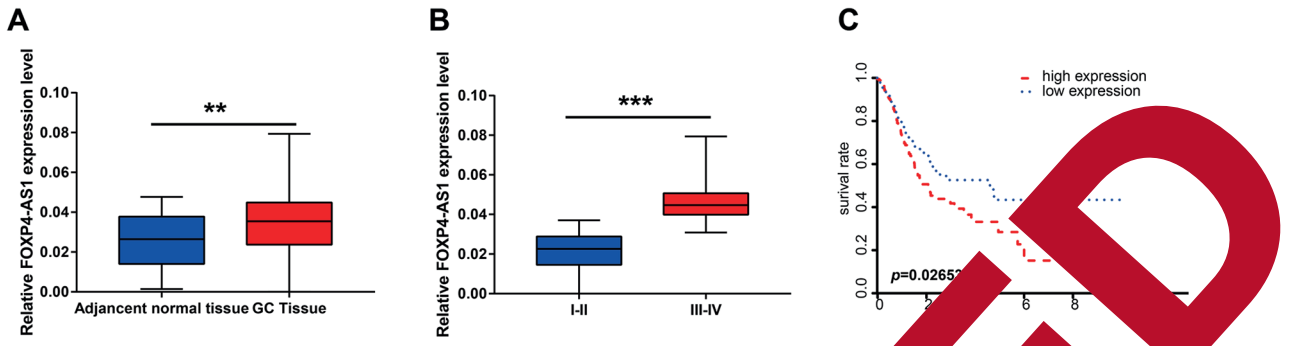

Figure 1. Upregulated FOXP4-AS1 in GC. **A,** Relative level of FOXP4-AS1 in GC tissue[illegible]djac[illegible]ormal ti[illegible]ues. **B,** Relative level of FOXP4-AS1 in GC patients with stage III-IV and stage I-II. **C,** Kaplan-[illegible]er c[illegible]uced fo[illegible]yzing the 5-year survival of GC patients with high expression and low expression of FOXP4[illegible].

Subsequently, cells were subjected to 15-min fixation in 4% paraformaldehyde and 10 min staining in Giemsa solution. After removing the staining solution, colonies were washed, air dried, and observed under a microscope.

RNA Immunoprecipitation (RIP)

Cells were treated according to the procedures of Millipore Magna RIPTM RNA-Binding[illegible] tein Immunoprecipitation Kit (Millipore, [illegible]ca, MA, USA). The cell lysate was incubate[illegible]th anti-EZH2, anti-LSD1 or IgG antibody at [illegible] for 6 h. A protein-RNA complex was captu[illegible] and digested with 0.5 mg/mL [illegible]e K co[illegible] taining 0.1% sodium dodecy[illegible]phi[illegible]SDS) to extract RNA. The magnet[illegible]eads w[illegible] repeatedly washed with RIP w[illegible]g buff[illegible] non-specific adsorptio[illegible]s [illegible]ossibl[illegible]nally, the extracted [illegible]A was s[illegible]ed to mRNA level determinati[illegible]ng qRT-PC[illegible]

Western Bl[illegible]

Total p[illegible]n was extra[illegible] from cells or tissues u[illegible] the radioimmun[illegible]cipitation assay (RIP[illegible] Beyoti[illegible] Shanghai, China) and loaded fo[illegible]roph[illegible]s. After transferring on a polyviny[illegible]oride [illegible]DF) membranes (Milli-[illegible] Bi[illegible] MA[illegible]A), it was blocked in 5% s[illegible] milk [illegible]ncubated with primary antibo[illegible]at 4°C o[illegible]ight and secondary antibodies fo[illegible] Bands were exposed by enhanced chemi-[illegible]nes[illegible](ECL; Pierce, Rockford, IL, USA) [illegible]d analyzed by Image Software.

S[illegible]tical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 (SPSS Inc., Chicago, IL, USA) software was used for data analyses. Data were expressed as mean ± st[illegible]ard d[illegible]ion. Intergroup differences were analyzed by the[illegible] Spearman correlation anal[illegible]s conducted [illegible]luate the expression re[illegible]n between the two ge[illegible]es. Kaplan-Meier was [illegible]duced to anal[illegible]e the survival rate. $p<0.05$ was [illegible]idered statis[illegible]ly significant.

Results

Upregulated FOXP4-AS1 in GC

[illegible]pared with adjacent normal tissues, [illegible]AS1 was upregulated in GC tissues (Figure 1A). Based on the tumor stage, it is found that FOXP4-AS1 level remained higher in GC patients with stage III-IV than those with stage I-II (Figure 1B). Kaplan-Meier curves revealed lower 5-year survival in GC patients presenting a high expression of FOXP4-AS1 compared with those presenting low expression (Figure 1C). It is speculated that FOXP4-AS1 may influence the progression of GC.

FOXP4-AS1 Influenced the Viability, Migration, and Invasion of GC Cells

To explore the biological function of FOXP4-AS1 in GC, we first constructed sh-FOXP4-AS1 vectors. Among the three vectors, transfection of sh-FOXP4-AS1 1# or sh-FOXP4-AS1 2# greatly downregulated FOXP4-AS1 level in AGS and BGC7901 cells, showing a pronounced transfection efficacy (Figure 2A). The CCK-8 assay showed that transfection of sh-FOXP4-AS1 1# or sh-FOXP4-AS1 2# inhibited the viability of GC cells at 48, 72, and 96 h (Figures 2B, 2C). Moreover, transwell assay demonstrated the attenuated invasive and migratory abilities in GC cells transfected with sh-FOXP4-AS1 1# or sh-FOXP4-AS1 2# (Figures 2D, 2E). Collectively, knockdown of FOXP4-AS1 suppressed the viability, migratory, and invasive abilities of GC.

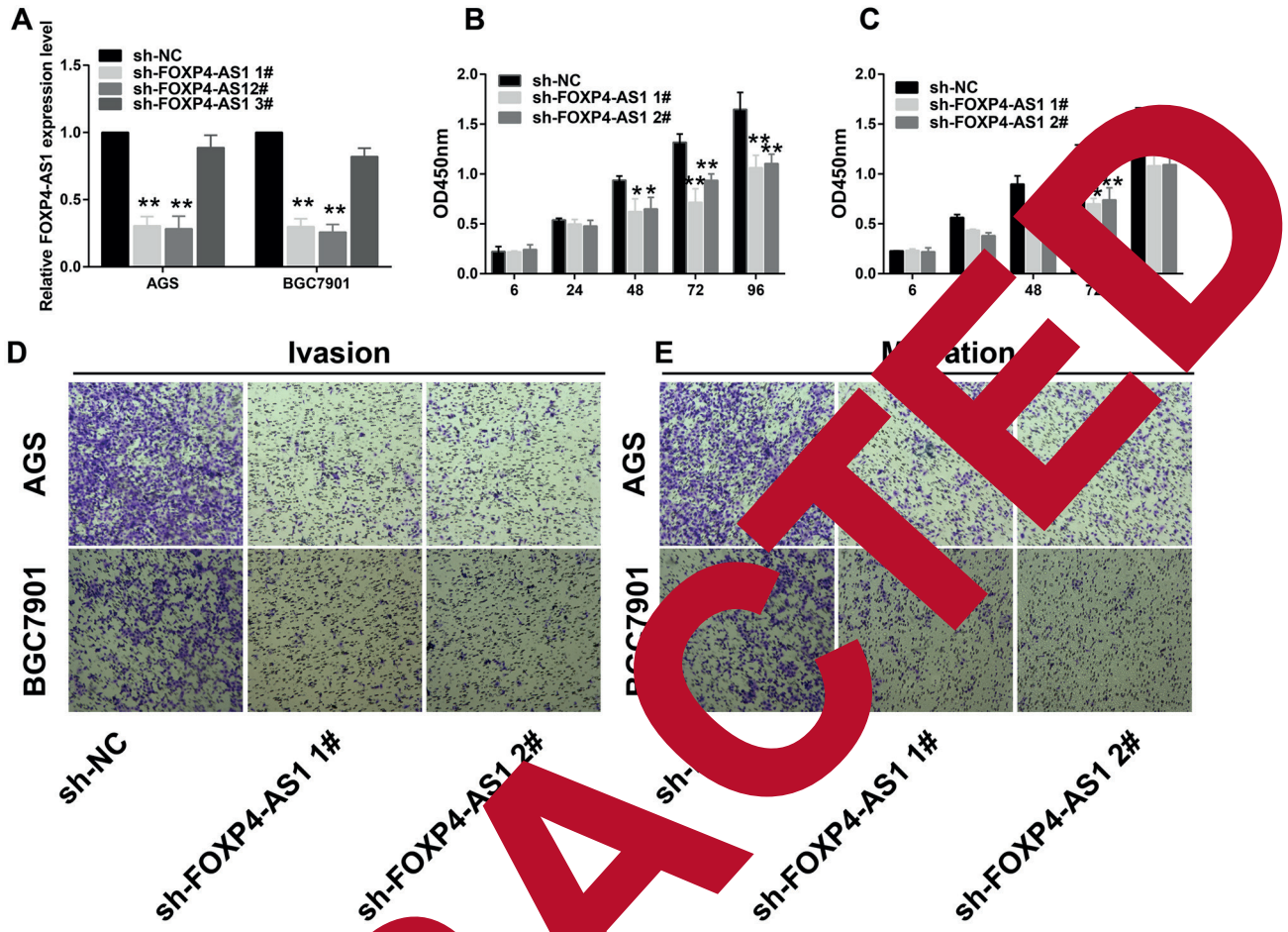

Figure 2. Knockdown of FOXP4 inhi the viabi igration and invasion of GC cells. **A,** Transfection efficacy of sh-FOXP4-AS1 1#, sh-FOXP 1 2# and FOXP4-AS 3# in AGS, and BGC7901 cells. **B,** CCK-8 assay showed the viability in AGS cells transfe h sh-N P4-AS1 1# or sh-FOXP4-AS1 2# at 6, 24, 48, 72, and 96 h, respectively. **C,** CCK-8 assay showed th iab sfected with sh-NC, sh-FOXP4-AS1 1# or sh-FOXP4-AS1 2# at 6, 24, 48, 72, and 96 h, resp ely. **D,** ll assay showed the invasion in AGS and BGC7901 cells transfected with sh-NC, sh-FOXP4-AS1 1# or s XP4-AS1 2 nification: 40×). **E,** Transwell assay showed the migration in AGS and BGC7901 cells transfected wi sh-FOXP4- or sh-FOXP4-AS1 2# (magnification: 40×).

FOXP4- Influence Proliferative Abilit GC Cells

Ed ssay w rther conducted to assess the inf e of P 4-AS1 on the proliferative ability o s. Tran ion of sh-FOXP4-AS1 r sh- P4-AS 1# markedly decreased the r of ed cells, suggesting the inhib proli e ability (Figure 3).

Were the Target Genes FOXP4-AS1

ncRNA-RNA binding protein complex has b eported to mediate biological processes. Through RV and SVm classifier analyses, EZH2 and LSD1 were predicted to interact with FOXP4-AS1 (Figure 4A). Furthermore, RIP assay verified that FOXP4-AS1 was abundantly enriched in anti-EZH2 and anti-LSD1 relative to control (Figure 4B). Hence, we believed that FOXP4-AS1 could bind to EZH2 and LSD1. Subsequently, sh-EZH2 and sh-LSD1 were constructed. Their transfection efficacy in AGS and BGC7901 cells was verified by Western blot (Figure 4C). The potential effects of EZH2 and LSD1 on the proliferative ability of GC cells were evaluated through the colony formation assay. Transfection of sh-FOXP4-AS1 1#, sh-EZH2 or sh-LSD1 in GC cells all could decrease the number of colonies (Figure 4D). Finally, FOXP4-AS1 level was identified to be positively correlated to LSD1and EZH2 levels in GC (Figures 4E, 4F).

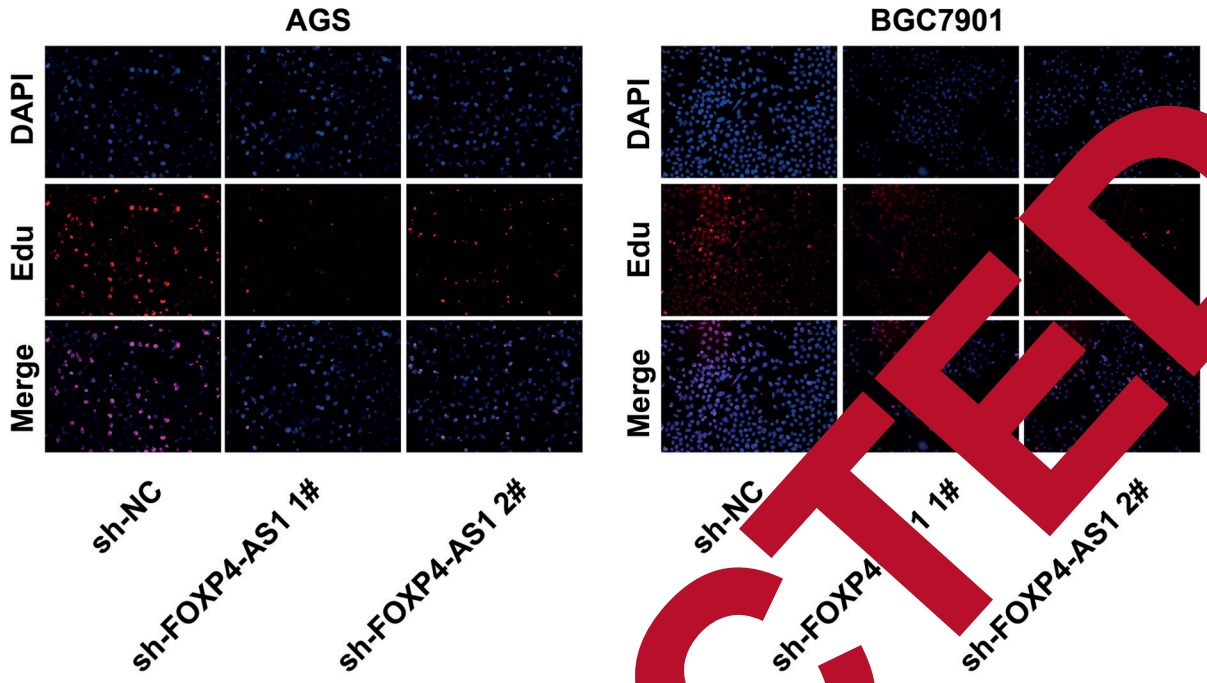

Figure 3. Knockdown of FOXP4-AS1 inhibited the proliferation of cells. EdU assay showed DAPI-labeled (blue), EdU-labeled (red), and merged images in AGS and BGC7901 cells transfected with sh-NC, sh-FOXP4-AS1 1# or sh-FOXP4-AS1 2# (magnification: 40×).

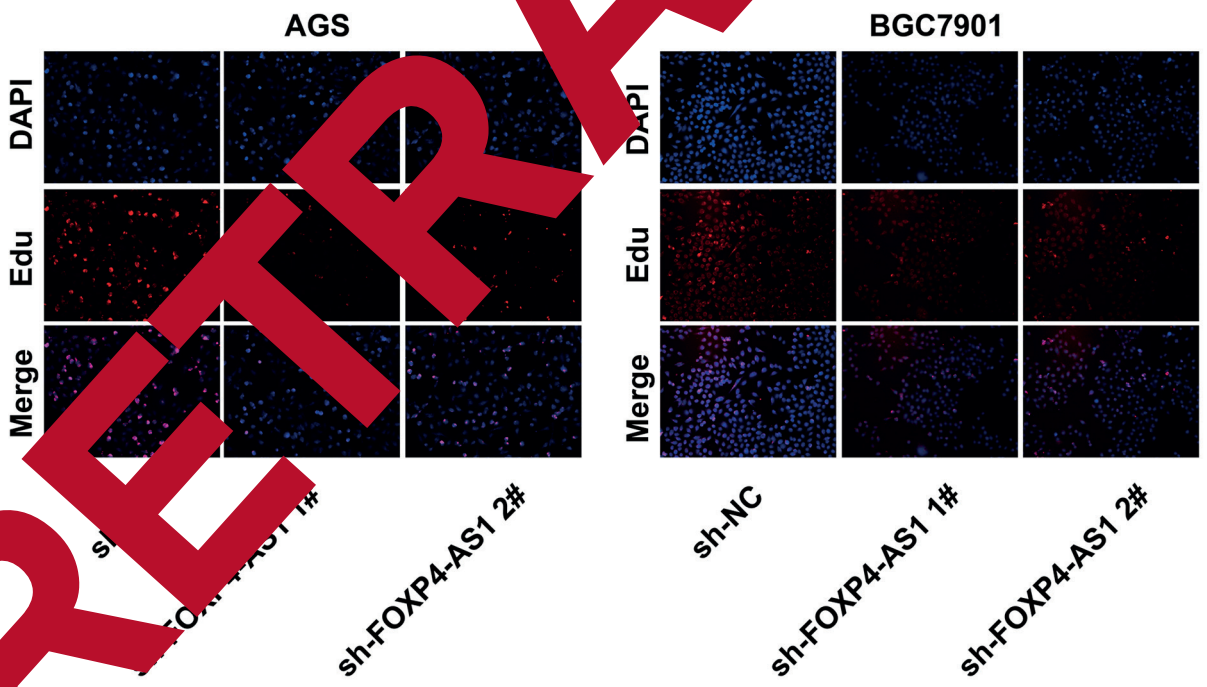

Figure 4. EZH2/LSD1 were the target genes of FOXP4-AS1. **A,** RF and SVM classifier showed the interaction probabilities of EZH2/LSD1 with FOXP4-AS1. **B,** RIP assay showed the enrichment of FOXP4-AS1 in the anti-EZH2, anti-LSD1, and anti-IgG. **C,** Transfection efficacy of sh-EZH2 and sh-LSD1 in AGS and BGC7901 cells. **D,** Colony formation assay showed the colonies in AGS and BGC7901 cells transfected with sh-NC, sh-FOXP4-AS1 1#, sh-EZH2 or sh-LSD1 (magnification: 10×). **E,** Correlation between LSD1 and FOXP4-AS1. **F,** Correlation between EZH2 and FOXP4-AS1.

Discussion

Here, we first examined the expression pattern of FOXP4-AS1 in GC by qRT-PCR. It was found that FOXP4-AS1 was upregulated in GC tissues relative to adjacent normal ones. In particular, FOXP4-AS1 level remained higher in stage III-IV GC than that of stage I-II. Survival analysis demonstrated a worse prognosis in GC patients presenting a high expression of FOXP4-AS1. *In vitro* experiments illustrated that knockdown of FOXP4-AS1 attenuated the proliferative, migratory, and invasive abilities of AGS and BGC7901 cells. Therefore, it is suggested that FOXP4-AS1 accelerated the progression of GC.

Recently, the ceRNA hypothesis proposed that lncRNA could sponge target genes to further mediate biological processes. Through bioinformatics methods, LSD1 and EZH2 were predicted to be the target genes of FOXP4-AS1. RIP assay further indicated the interaction between FOXP4-AS1 and EZH2/LSD1. Knockdown of EZH2/LSD1 was capable of inhibiting the proliferative ability of GC cells. Fi[illegible] correlation analyses revealed positive re[illegible] between FOXP4-AS1 and EZH2/LSD1. T[illegible]m up, our results indicated that FOXP4-[illegible] EZH2/LSD1 was a carcinogenic complex promote the progression of GC.

Conclu[illegible]ns

We demonstrated [illegible] bind[illegible] EZH2/LSD1 to fo[illegible] carci[illegible]ic complex, thus accelerating [illegible]ells to prol[illegible] migrate, and invade. FO[illegible] may be a pro[illegible]ing gene for the clinical [illegible]eatme[illegible]GC.

Conf[illegible] of int[illegible]st

Th[illegible]ors de[illegible] no conflicts of interest.

[illegible]erences

1) [illegible]moto H, Watanabe Y, Maehata T, Morita R, [illegible]ikawa R, Ishigooka S, Ozawa S, Matsuo Y, Hosoya K, Yamashita M, Taniguchi H, Nosho K, Suzuki H, Yasuda H, Shinomura Y, Itoh F. An updated review of gastric cancer in the next-gen-[illegible]ation sequencing era: insights from bench to bedside and vice versa. World J Gastroenterol 2014; 20: 3927-3937.
2) Bhan A, Soleimani M, Mandal SS. Long Noncoding RNA and cancer: a new paradigm. Cancer Res 2017; 77: 3965-3981.
3) Boon RA, Jaé N, Holdt L, Dimmeler S. Long noncoding RNAs: from clinical genetics to t[illegible] targets? J Am Coll Cardiol 2016; 67: [illegible]
4) Bolha L, Ravnik-Glavac M, Glavac D[illegible]g noncoding RNAs as biomarkers in canc[illegible]ease Markers 2017; 2017: 7243968.
5) Jalali S, Kapoor S, Sivadas [illegible]hartiya [illegible]ia V. Computational approac[illegible]owards unc[illegible]d ing human long non-[illegible]g RNA biology. formatics 2015; 31: [illegible]2251.
6) Dong Y, Yoshitomi T, [illegible] C[illegible]Long nc[illegible]oding RNAs coordin[illegible]fun[illegible]etween [illegible]chondria and th[illegible]ucleus. [illegible]netic [illegible]romatin 2017; 10: [illegible]
7) Kim TK[illegible] Gray JM. L[illegible]ncer RNAs: a class[illegible]ng nc[illegible]ing RNAs synthesized at enhancers. Cold Sp[illegible]arb Perspect Biol 2015; 7: [illegible]
8) [illegible]ng Y, Cheung BB, Atm[illegible]brata B, Marshall GM, Dinger ME, Liu PY, Liu T. The regulatory role of long noncoding F[illegible]s in cancer. Cancer Lett 2017; 391: 12-19.
9) [illegible]g L, Frob[illegible]E, Lee JT. Long noncoding RNAs: [illegible]ves into the RNA world. Trends Bi[illegible] 2014; 39: 35-43.
10) Yu B, Shan G. Functions of long noncoding RNAs [illegible]the nucleus. Nucleus 2016; 7: 155-166.
11) [illegible]g Y, Cao X. Long noncoding RNAs in innate immunity. Cell Mol Immunol 2016; 13: 138-147.
12) Schmitz SU, Grote P, Herrmann BG. Mechanisms of long noncoding RNA function in development and disease. Cell Mol Life Sci 2016; 73: 2491-2509.
13) Sun T. Long noncoding RNAs act as regulators of autophagy in cancer. Pharmacol Res 2018; 129: 151-155.
14) Sun W, Yang Y, Xu C, Guo J. Regulatory mechanisms of long noncoding RNAs on gene expression in cancers. Cancer Genet 2017; 216-217: 105-110.
15) Kung JT, Colognori D, Lee JT. Long noncoding RNAs: past, present, and future. Genetics 2013; 193: 651-669.
16) Hu X, Sood AK, Dang CV, Zhang L. The role of long noncoding RNAs in cancer: the dark matter matters. Curr Opin Genet Dev 2018; 48: 8-15.
17) Li J, Lian Y, Yan C, Cai Z, Ding J, Ma Z, Peng P, Wang K. Long non-coding RNA FOXP4-AS1 is an unfavourable prognostic factor and regulates proliferation and apoptosis in colorectal cancer. Cell Prolif 2017; 50. doi: 10.1111/cpr.12312.
18) Schmitt AM, Chang HY. Long noncoding RNAs in cancer pathways. Cancer Cell 2016; 29: 452-463.
19) Yang L, Ge D, Chen X, Qiu J, Yin Z, Zheng S, Jiang C. FOXP4-AS1 participates in the development and progression of osteosarcoma by downregulating LATS1 via binding to LSD1 and EZH2. Biochem Biophys Res Commun 2018; 502: 493-500.