 doi:10.20944/preprints202004.0264.v1

RBFOX3 Promotes Gastric Cancer Growth and Progression through Activating HTERT Signaling

Chen Luo[#1,2,3], Xiaojian Zhu[#1,2,3], Qilin Luo[1], Fanqin Bu[1,2,3], Chao Huang[1,2,3], Jingfeng Zhu[1,2,3], Jiefeng Zhao[1,2,3], Wenjun Zhao[1,2,3], Kang Lin[1,2,3], Cegui Hu[2,3], Zong Zeng[1,3], Hongliang Luo[1,3], Jun Huang[1], Zhengming Zhu[1]

[1]Department of General Surgery, The Second Affiliated Hospital of Nanchang University，Nanchang, China.

[2]Jiangxi Medical College of Nanchang University，Nanchang, China.

[3]Jiangxi Province Key Laboratory of Molecular Medicine, Nanchang, China.

[#]Chen Luo[#1,2,3] and Xiaojian Zhu[#1,2,3] contributed equally to this work.

Correspondence: *Zhengming Zhu, Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China. Email: zzm8654@163.com.

*Xiaojian Zhu, Department of General Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang 330006, China. Email: zhuxiaojian1203@163.com.

***Zhengming Zhu and Xiaojian Zhu are co-corresponding authors.**

Abstract

Tumor invasion, metastasis, and recrudesce remain a considerable challenge in the treatment of gastric cancer (GC). Herein, we first identified that RBFOX3 (RNA binding protein fox-1 homolog 3) was significantly up-regulated in GC tissues and negatively linked to the survival rate of GC patients. RBFOX3 promoted cell division and cell cycle progression *in vitro* as well as *in vivo*. Furthermore, RBFOX3 increased cell invasion and migration ability. Interestingly, both the suppression of GC cell multiplication and invasion moderated by the silencing of RBFOX3 was rescued by HTERT up-regulation. Additionally, RBFOX3 augmented the resistance of GC cells to 5-fluorouracil (5-Fu) by repressing RBFOX3. Mechanistically, exogenous up-regulation of RBFOX3 triggered promoter activity and HTERT expression thereby enhancing the division and development of GC cells. Importantly, our findings revealed that RBFOX3 interacted with AP-2β to modulate the HTERT expression as demonstrated by co-immunoprecipitation analysis. In conclusion, our study indicates

 doi:10.20944/preprints202004.0264.v1

that high expression of RBFOX3 promotes GC progression and development but predicts worse prognosis by stimulating HTERT signaling. Moreover, the results suggest that the RBFOX3/AP-2β/HTERT pathway is a novel target for the development of therapeutic agents for the prevention and treatment of GC reappearance and metastasis.

Keywords: RBFOX3; HTERT; gastric cancer; promoter-binding protein; cancer biomarker

Introduction

Gastric cancer (GC) is the 3rd leading cause of cancer deaths worldwide and the most common gastrointestinal malignancy in East Asia and Latin America[1]. China has recorded a growing rate of GC that has been attributed to lifestyle and dietary changes[2]. Despite recent advances in screening, diagnosis and treatment GC, prognosis remains poor, with a 5-year survival rate <50% as a result of disease recurrence and high metastasis rate[3]**.** GC pathogenesis is highly complex and poorly understood. Genetic factors, Helicobacter pylori (HP) infection, unhealthy eating habits, and smoking have all been implicated GC carcinogenesis[4]. Owing to the complexity of the abdominal microenvironment, GC recurrence is relatively high and further treatment does not effectively improve the patients' quality of life. Further investigations in to the molecular mechanisms of GC tumorigenesis and progression is urgently needed for more effective prevention and treatment options.

RNA binding protein fox-1 homolog 3 (RBFOX3) is encoded by the *Rbfox3* gene on chromosome 17 and comprises 15 exons and is a novel member of the Fox1 family of splicing factors[5]. In mammals, this family of splicing factors consists of 3 members, RBFOX1, RBFOX2, and RBFOX3. RBFOX1 is expressed in the heart, skeletal muscles, and neural tissues. RBFOX2 exhibits a broader expression pattern, including throughout the embryo, neurons, and muscles. RBFOX3 is primarily expressed in post-mitotic neurons in normal physiology[6, 7]. RBFOX3 is reported to regulate a variety of brain-specific alternative pre-mRNA splicing choices by binding to an RNA penta/hexa nucleotide UGCAUG motif[8]. It has been reported that RBFOX3 interacts with the polypyrimidine tract binding protein-associated splicing factor (PSF)[9], which

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

enhances RBFOX3 binding target UGCAUG motif[10]. However, recent studies suggest that RBFOX3 also modulates various physiological pathological processes. It has been reported that neuronal nuclei (NeuN) is a product of the Rbfox3 gene, revealing RBFOX3 as a marker exclusively expressed in post-mitotic neurons[11]. Another study showed that RBFOX3 could bind to DNA in vitro[12, 13]. RBFOX3 has also been reported to control biogenesis of some miRNAs including primary-miRNAs (pri-miRNAs) that lack a UGCAUG motif[14]. We hypothesized that in addition to its splicing functions, RBFOX3 regulates various biochemical processes that are poorly understood. Here, we find that RBFOX3 regulates HTERT expression to promote GC. We find that RBFOX3 is significantly upregulated in GC, and that it correlates with poor survival. Functional analyses revealed that that RBFOX3 enhances GC growth, metastasis, and chemoresistance. Mechanistic studies reveal that RBFOX3 overexpression elevates HTERT expression, promoting GC progression. Moreover, our data indicate that RBFOX3 interacts with AP-2β to regulate HTERT expression. Taken together, our findings highlight the RBFOX3/HTERT signaling axis as a potential novel therapeutic target against GC.

Materials and Methods samples

Clinical samples

178 paired human GC tissues and adjacent non-cancer tissue were obtained from the tissue biobank at the department of pathology at the Second affiliated hospital of Nanchang University. The samples had been biobanked between August 2017 and December 2019 and 92 were from males and 86 from females. Adjacent, matched non-cancer tissues were collected > 5 cm away from the edge of the cancerous foci. Ethical approval for this study was provided by the clinical research ethics committee of the second affiliated Hospital of Nanchang University.

Cell lines and antibodies

The human GC cell lines MKN45, AGS, SGC-7901, MGC-803 and BGC-823, as well as the normal gastric epithelial cell line, GES-1, were purchased from the American type culture collection (ATCC). All cells were authenticated via short tandem repeat profiling by the Cell Bank, and cultured in RPMI-1640 (Corning, 10-040-CVR)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

supplemented with 10% FBS (Sigma, 12103C) in a humidified incubator, at 37°C, 5% CO_2. Rabbit anti-RBFOX3 (Abcam, ab177487) was used at 1:1000 for western blot and 1:3000 for IHC. Rabbit anti-HTERT (Abcam, ab183105) was used at 1:1000 for western blot and 1:100 for IHC. Mouse anti-E-cadherin (Abcam, ab1416) was used at 1:50 for western blot, Rabbit anti-β-catenin (Abcam, Ab32572) was used at 1:5000 for western blot), Rabbit anti-IgG (Abcam, ab2410) and rabbit anti-GAPDH (Abcam, ab9485) were used at 1:2500 for western blot.

Streptavidin-agarose pull down assay

HTERT promoter binding proteins were analyzed by streptavidin-agarose pulldown assays. Briefly, nuclear proteins were extracted from GC cells and 1mg incubated with 10μg of biotinylated double-stranded DNA probes (Sigma-Aldrich) corresponding to nucleotide -351 to -149 of the HTERT promoter region and 100μl of streptavidin-agarose beads (Sigma-Aldrich) at 4°C overnight. The DNA-protein complex was then pulled down by centrifuging at 500 g for 10 minutes, at 4°C.

Identification of HTERT promoter-binding proteins

HTERT promoter-bound proteins from section streptavidin-agarose pulldown assay were analyzed by mass spectrometry. Briefly, bound proteins were separated by 10% SDS-PAGE and visualized by sliver staining (Beyotime, P0015A). After reduction and alkylation, the bands of interest were candidate protein bands were digested MS-grade trypsin solution (Promega, CAS9002-07-7). The digested peptides were then identified by mass spectrometry. The identities of the proteins of interest were verified on available databases and software.

Transient transfection

To overexpress RBFOX3 and AP-2β, we used lipofectamine 3000 (Invitrogen, L3000150) to transiently transfected pcDNA3.1-RBFOX3, pcDNA3.1-AP-2β or control vector plasmids into SGC-7901. To silence RBFOX3 and AP-2β expression, we used shRNA against RBFOX3 (5'-CAAATCGGGGGGTTGCCAA-3' and 5'-ACCGTGACCTCGCTCAAAT-3'), and siRNA against AP-2β (5'-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

GCAGUCCAAUGACAUUUGATT-3'). SiRNAs were purchased from Shanghai GenePharma Co (Shanghai, China).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was sone using Carey's protocol. Briefly, cells were fixed with 1% formaldehyde and the cross-linking quenched with 100μl of 1.375 M glycine/mL of cell culture media. The samples were then sonicated on ice so as to shear DNA into 200-900bp fragments (Setting parameters of ultrasound machine: 30% power, turn on 2s, interval 3s, 4min). A third of the cell lysate was used as DNA input control, a third was used for immunoprecipitation with anti-RBFOX3 antibodies and a third subjected to non-immune rabbit IgG (Cell Signaling Technology, 3900S). DNA fragments were purified on spin columns (Qiagen, 31014) and PCR used to amplify a 227-bp segment of the HTERT promoter region using the following primers: Fwd: 5'-TGGCCTTCCCCAGGGCCCTTC-3', Rvs: 5'-TGAGGACG GGCAGGGAGTGC-3'. The PCR products were resolved on a 2% agarose gel and visualized by Gel-Red staining.

Lentiviral Construction and Cell Transfection

To generate clones stably overexpressing RBFOX3, we infected SGC-7901 and MGC-803 cells with a lentiviral vector encoding the full length human RBFOX3 gene or an empty lentiviral vector as control. We then selected stable clones after 2 weeks by treating the cells with 0.7-5μg/ml puromycin. After clonal expansion, we quantified RBFOX3 expression by RT-qPCR and western blotting. U6-sh-RBFOX3-EGFP-IRES-puromycin (Genechem, Shanghai, China) were used to knock down RBFOX3 expression. A lentiviral vector containing non-silencing shRNA was used as negative control. SGC-7901 and MGC-803 cells were infected with either the lentiviral vectors carrying respective shRNA sequences or empty vector as control. Silencing efficiency was determined by RT-qPCR and western blotting.

Colony formation and 5-Ethynyl-20-deoxyuridine (Edu) assay

200-400 GC cells/well were onto 6-well plates and cultured for 2 weeks. Next, colonies were stained with 1% crystal violet and counted. Colony formation assays

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

were performed as we previously described[15]. All experiments were performed in independent triplicates.

1×10^5 SGC-7901 and MGC-803 cells/well were seeded onto 6-well plates and cultured to 75%-85% confluence. 100μL of 10uM prewarmed Edu was added into each well and the cells put in culture 2 hours. They were then fixed with 4% PFA for 15 minutes. 50μL of 2mg/mL glycine solution was then decolorized for 5 min. The cells were then permeabilized with 0.3% TriX-100 PBS for 10 minutes, followed by Apollo and Hoechst staining. The cells were then examined by confocal microscopy and the Hoechst nuclear staining imaged.

Wound healing and transwell invasion assays

To evaluate the migration and invasion capacity of the GC cells after BRFOX3 silencing or overexpression, we performed wound healing assay and trans-well invasion assays. GC cells stably overexpressing RBFOX3 or RBFOX3 silenced, and the respective controls were cultured on 6-well plates to confluence. The monolayers were then scratched with a 10μl pipette tip. The cells were then imaged 0 and 24 hours later to monitor migration. These experiments were performed in independent triplicates. Cell invasion assays were done using BD BioCoat matrigel invasion chambers (BD, 354480) by following manufacturer instructions. Invading cells in 5 randomly selected fields of view were counted under a light microscope.

Western blot

Whole cell and nuclear protein extracts extracts were prepared using Complete lysis-M reagent (Roche, 4719956001) and RIPA lysis buffer (Beyotime, P0013B). Protein concentration was determined using BCA assay (ThermoFisher Scientific, 23221-23230). Proteins were then separated by 8%-10% SDS-PAGE and transferred onto 0.45μm PVDF membranes for antibody staining and detection.

Real time PCR (RT-qPCR)

RNA extraction was done using TRIZOL reagent (Invitrogen, 15596-026) by following manufacturer instructions. cDNA synthesis was done using ReverTra Ace qPCR RT kit (Toyobo, FSQ-201C). RT-qPCR was done using a SYBR Green PCR kit (Toyobo, KGA1339-1) on a Bio-Rad CFX96 machine. Relative gene expression was

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

calculated using the $2^{-\Delta\Delta CT}$ with GAPDH as the reference gene. These experiments were done in independent triplicates. Primers were purchased from GeneCopoeia (HTERT: Hs-QRP22639, GAPDH: Hs-QRP20169, RBFOX3: Hs-QRP22696).

Co-immunoprecipitation (CoIP) assay

Equal amounts of nuclei protein extracts from different cell lines were incubated with the indicated antibodies. Next, 50µL agarose-conjugated protein-A/G beads (Merck Millipore, YB36403ES03) were added and the mixture incubated at 4°C overnight. After extensive washing with ice cold PBS 1X, the beads were mixed with loading buffer and heated at 4°C for 5 minutes. The supernatant was then subjected to western blot analysis.

In vivo tumor growth assay

30 nude mice, (6-8 weeks-old) were purchased from Shanghai laboratory animal Co. Ltd. SGC-7901 cells expressing a luciferase reporter (pcDNA3.1-luciferase) were stably transfected with shRB and pLVTHM. Next, about 1×10^6 of SGC-7901 cells (mixed prior with Matrigel, 1:1) were carefully injected into the subrenal capsule. Tumor growth and metastases were monitored weekly using an IVIS system (Caliper Life Sciences). The mice were sacrificed after six weeks and the tumors surgically removed for analyses.

Statistical analysis

Data were presented as mean ± SD of at least three independent experiments. Statistical analyses were done using SPSS version 11.0. * indicates statistical significance. p value <0.05 was considered statistically significant.

Results

RBFOX3 is overexpressed in human GC and positively correlates with tumor progression

To determine the expression pattern of RBFOX3 in human GC tissues, RBFOX3 DNA digestion products were first identified by agarose gel electrophoresis. The DNA digestion products were then stably expressed in SGC-7901 and MGC-803 cells (Figure 1A). RT-qPCR analysis of RBFOX3 expression revealed that it was consistently elevated in GC tissues (p<0.001, Figure 1B). IHC analyses of 89 GC tissues and 89

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

matched adjacent non-cancer gastric tissue revealed elevated RBFOX3 staining in the GC tissues relative to controls (p<0.01, Figure 1C). Similar observations were made upon western blot analysis of RBFOX3 in 37 frozen GC tissues and paired non-cancer control tissues (p<0.05, Figure 1D). Western blot analysis of cell lines revealed that RBFOX3 was poorly expressed in the normal gastric cell line, GES-1 relative to the GC cell lines, MKN45, AGS, SGC-7901, MGC-803, BGC-823 (Figure 1E). Next, we evaluated the association between RBFOX3 protein expression and clinicopathological features of GC (Table 1). This analysis indicated that RBFOX3 expression correlates with tumor differentiation (p=0.017), AJCC clinical stage (p=0.015) and TNM stage (p=0.004). Additionally, Kaplan-Meier survival analysis revealed significantly lower overall survival (OS) and disease-free survival (DFS) rates in patients exhibiting high tumor RBFOX3 levels relative to those expressing low RBFOX3 levels (Figure 1F, 1G). Univariate and multivariate Cox regression analysis showed that advanced TNM stage and high RBFOX3 expression significantly corelated with unfavorable OS and DFS. Taken together, these results indicate that High RBFOX3 protein expression is associated with poor prognosis, highlighting the potential of RBFXO3 as an independent prognostic marker in GC (HR=2.670; 95% CI: 1.471-3.917; p=0.001; Table 2).

RBFOX3 promotes GC cell proliferation and cell cycle progression *in vitro*

Next, we evaluated the function of RBFOX3 in GC cell growth *in vitro* by stably overexpressing RBFOX3 in SGC-7901 cells and stably silencing it in MGC-803 cells. This analysis revealed that RBFOX3 overexpression significantly enhances SGC-7901 cell viability and colony formation (p<0.05**,** Figure 2A, 2D). RBFOX3 silencing effectively suppressed MGC-803 cell viability and colony formation (p<0.05, Figure 2B, 2E). Interestingly, the suppression attained by RBFOX3 knockdown was reversed by HTERT overexpression in MGC-803 cells (p<0.05, Figure 2C, 2F). Flow cytometry revealed that RBFOX3 silencing significantly arrests MGC-803 cell cycle in the G1 phase (p<0.05, Figure 2H). RBFOX3 overexpression in SGC-7901 cells had similar results (p<0.05, Figure 2G). Taken together, these data show that exogenous expression of RBFOX3 may promote GC cell proliferation *in vitro*.

 doi:10.20944/preprints202004.0264.v1

RBFOX3 regulates GC cell migration and invasion *in vitro*

The HTERT signaling pathway has been reported as a modulator of cancer cell migration and invasion[16–18]. We therefore investigated the role of RBFOX3 on GC cell migration and invasion using wound healing and transwell invasion assays. These analyses revealed that RBFOX3 overexpression enhances cell migration and invasion in SGC-7901 cells. Conversely, RBFOX3 silencing suppresses MGC-803 cell migration (p<0.05**,** Figure 3A, 3D, 3B and 3E). Moreover, HTERT overexpression in RBFOX3-silenced MGC-803 cells enhanced their migration and invasion relative to RBFOX3-silenced cells transfected with empty vector (p<0.05, Figure 3C, 3F). To elucidate the factors driving RBFOX3-mediated metastasis, we evaluated the expression some oncogenes and tumor suppressors in RBFOX3 knockdown (sh-RB, MGC-803) and overexpression (RBFOX3, SGC-7901) contexts. this analysis revealed that E-cadherin, an important protein involved in tumor metastasis, was downregulated, while β-catenin was markedly elevated upon RBFOX3 overexpression (SGC-7901 cells) (p<0.05, Figure 3G), suggesting perturbation in the expression of these proteins may be involved in RBFOX3-mediated malignant progression. An opposite effect was also observed upon RBFOX3 silencing (sh-RB, MGC-803) (p<0.05, Figure 3H).

RBFOX3 inhibition enhabces 5-Fu sensitivity in GC cells

Although 5-Fu (5-fluorouracil) is commonly used to treat GC, it's dosage is limited by its negative side effects[19]. We hypothesized that given the effect of RBFOX3 on GC growth and proliferation, its knockdown might enhance sensitivity GC sensitivity to 5-Fu. We observed that 5-Fu inhibits GC cell viability and colony formation in a dose dependent manner. Additionally, these effects were significantly stronger in the background RBFOX3 silencing (p<0.05, Figure 4A). Next, we first generated MGC-803 stably expressing RBFOX3 shRNA (shRB-1 and shRB-2) or the negative control (sh-NC). This analysis revealed that cell viability in RBFOX3 knockdown cells treated with 5-Fu was significantly lower relative to mock knockdown cells treated with 5-Fu (p<0.05, Figure 4B). Moreover, western blot analysis revealed that RBFOX3 expression was significantly reduced upon 5-Fu treatment relative the controls (p<0.05, Figure 4C). Taken together, this suggested that RBFOX3 regulates GC cells sensitivity

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

to 5-Fu. However, further studies are needed to fully understand the mechanisms of RBFOX3's regulation of drug resistance.

RBFOX3 binds to the HTERT promoter in GC cells

Previous studies have uncovered novel regulators of the HTERT promoter in lung and liver cancer using streptavidin-agarose bead pull down assays[17, 20]. To establish whether RBFOX3 interacts with the HTERT promoter in GC as well, we pulled down cytoplasm proteins bound at the HTERT promoter in GC cells using the 5'-biotinylated HTERT promoter probes and streptavidin-agarose beads. We then evaluated the presence of RBFOX3 in the cytoplasmic protein/DNA complex by western blot using a RBFOX3-specific antibody. High levels of RBFOX3 were bound to the HTERT promoter probe in GC cells (MKN45, AGS, SGC-7901 and MGC-803，BGC-823) (Figure 5A, upper panel). All the GC cell lines express high levels of RBFOX3 (Figure 5A, lower panel). To confirm RBFOX3 interaction with the HTERT promoter in vivo, we performed a ChIP assay on GC cells. This analysis revealed RBFOX3 protein to be bound to the endogenous HTERT promoter in the cytoplasm of all the GC. Importantly, strong RBFOX3 binding to the HTERT promoter was observed in all five human GC cells (MKN45, AGS, SGC-7901, MGC-803，BGC-823) (Figure 5B). To identify the binding site of RBFOX3 on the HTERT promoter, 3 different fragments of 5'-biotinylated HTERT promoter (Figure 5C) were constructed and incubated with SGC-7901 cell lysates for a pulldown assay. This analysis indicated that the -371 to -305 (5'-C

GCTGACCCCACCGTTCTAGGCAAGGCGTTCACCCGCCCTCTTGGGGCCCTC

GCTGGCGTCCCTGC-3') region of HTERT promoter is critical for RBFOX3 binding. To further study if RBFOX3 regulates HTERT gene expression, we constructed RBFOX3 overexpressing plasmids and synthesized 2 RBFOX3 short hairpin RNAs (shRNAs). Western blot result revealed that overexpression of RBFOX3 elevates HTERT protein levels in SGC-7901 and MGC-803 cells, while RBFOX3 silencing inhibits HTERT expression in SGC-7901 and MGC-803 cells (p<0.05, Figure 5D-E). Additionally, HTERT siRNA silencing did not significantly affect RBFOX3 expression

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

(Figure 5F-5G). Next, we investigated how elevated RBFOX3 and HTERT expression in GC tissues (n=52) affects clinical outcomes. This analysis revealed that RBFOX3 expressinon positively correlates with HTERT expression in human GC tissues (Figure 5H-I). These results were consistent with Figure 5D-G. To further investigate the effects of RBFOX3 on HTERT, we restored RBFOX3 expression in lentivirus-stabilized RBFOX3 knockdown in SGC-7901 cells. These analyses revealed that in the background of stably silenced RBFOX3 expression, RBFOX3 overexpression restores the expression of both RBFOX3 and HTERT in SGC-7901 cells (p<0.05, Figure 6A**)**. Similar results were obtained in MGC-803 stably silenced for RBFOX3 expression (p<0.05, Figure 6B). Additionally, cell proliferation, viability and invasion capacity were restored (p<0.05, Figure6 C-H). Together, these findings further confirm the regulation of HTERT expression by RBFOX3.

RBFOX3 interacts with AP-2β to regulate HTERT expression

Next, we investigated whether RBFOX3 bound to the HTERT promoter interacts with other transcription factors. Previous studies have found that HTERT promoter-binding proteins include KLF4[21], RFPL3[22], CPSF4[23] and AP-2β[17]. To test this possibility, we performed a CoIP pull-down experiment and using a RBFOX3-specific antibody observed that RBFOX3 interacts with AP-2β (Figure 7A-B). Moreover, we found that AP-2β binds to the HTERT promoter (Figure 7C). A co-immunofluorescence analysis of SGC-7901 and MGC-803 cells revealed that RBFOX3 primarily localizes in the cytoplasm while AP-2β was primarily found in the nucleus (Figure 7D). The co-localization between AP-2β and RBFOX3 was observed in the cytoplasm (Figure 7D). Additionally, we found that AP-2β overexpression enhances RBFOX3 to the HTERT promoter (Figure 7E). Conversely, AP-2β knockdown weakened the binding of RBFOX3 to the HTERT promoter even in the context of RBFOX3 overexpression (SGC-7901 cells) (Figure 7E). A luciferase reporter analysis showed that AP-2β overexpression enhanced the HTERT promoter activity while AP-2β knockdown suppressd HTERT promoter activity (Figure 7G). To test the effect of AP-2β on HTERT expression, we performed western blot analysis and found that AP-2β overexpression enhanced HTERT expression. Connversely, AP-2β knockdown suppressed HTERT

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

expression (Figure 7F). Moreover, AP-2β knockdown suppressed RBFOX3 overexpression driven GC cell invasion. Conversely, AP-2β overexpression partially rescued the inhibition of GC invasion caused by RBFOX3 knockdown (Figure 7H). These findings suggest that RBFOX3 interacts with AP-2β to regulate the HTERT expression and GC metastasis.

Deregulation of RBFOX3 suppresses tumor growth in GC orthotopic xenografts

To evaluate whether RBFOX3 has oncogenic functions, we created GC orthotopic mouse xenografts using SGC-7901 cells carrying a luciferase reporter and stably overexpressing RBFOX3, as well as MGC-803 cells carrying as luciferase reported and stably knocked down for RBFOX3. As controls, we used vector groups and sh-NC groups, respectively. The transfected cells and the control cells were inoculated into the right and left renal capsule of the same mouse, respectively (8 mice per group). Tumor growth was monitored using IVIS. 6 weeks into the experiment, we observed that RBFOX3 knockdown tumors were significantly smaller than the controls while RBFOX3 overexpression ones were significantly bigger than the controls (Figure 8A and 8D). Analysis of tumore size revealed that RBFOX3 up-regulation significantly promoted tumor volume and weight (Figure 8B and 8C). However, knocking down RBFOX3 observed the opposite result (Figure 8E and 8F). Taken together, these results indicate that deregulation of RBFOX3 may suppresses tumor progression *in vivo*.

Discussion

Limitless self-renewal is a hallmark of cancer[24]. Telomere maintenance and telomerase activation has been reported to promote cancer cell proliferation[25]. The transcriptional regulation of HTERT is believed to modulate telomerase activation in human cancers. It is thought that a contributing factor to cancer development is their acquired ability to overcome senescence by maintaining telomere length[26, 27]. HTERT has been found to be upregulated in various tumors via genetic and epigenetic means including HTERT amplifications, HTERT structural variation, HTERT promoter mutations and HTERT promoter methylation[28, 29]. As the catalytic subunit of telomerase, HTERT plays a decisive role in cell unlimited replication[29–31]. Recent findings have implicated HTERT in various human diseases, including cancer[17, 30, 32].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

Currently, HTERT deregulation is considered a hallmark of cancer and a potential therapeutic target[30, 31, 33]. Mounting evidence indicates that tumor-specific cellular factors may be differentially expressed and specifically bind to the HTERT promoter, thereby modulating HTERT expression and tumor development[32, 34]. Here, we found that RBFOX3 highly expressed in GC tissues and cell lines, and that RBFOX3 dunctions as an oncogene. Our data showed that patients expressing high RBFOX3 levels exhibit significantly shorter OS and DFS. Furthermore, univariate and multivariate analyses showed that high RBFOX3 expression may independently predict poor GC prognosis.

Next, we investigated significance of high RBFOX3 expression in GC. Functional analysis revealed that RBFOX3 overexpression promotes proliferation, invasion and migration GC cells. However, in the background of stably silenced RBFOX3, the proliferation, invasion and migration of GC cells were suppressed. Additionally, we also observed that RBFOX3 influences GC cell sensitivity to 5-Fu. It has been previously reported that RBFOX3 binds to the promoter region of HTERT, thereby regulating HTERT signaling and tumor growth. Interestingly[17, 33], we find that the RBFOX3 knockdown-mediated suppression of both GC proliferation and invasion is rescued by HTERT overexpression.

Next, we wondered if the RBFOX3 binds to the HTERT promoter region in gastric cancer cells. To this end, we performed biotin-streptavidin-agarose pull-down and proteomics analysis and found identified RBFOX3 as a novel HTERT promoter-binding protein in gastric cancer cells. Importantly, we, for the first time found that RBFOX3 binds to the promoter region of HTERT and activates it. Additionally, our data show that HTERT expression is regulated at the transcriptional level by RBFOX3. Further analysis revealed that RBFOX3 expression is frequently upregulated in primary human GC cell lines (MKN45, AGS, SGC-7901, MGC-803 and BGC-823). To further investigate whether RBFOX3 regulates HTERT gene expression, we constructed RBFOX3 overexpressing plasmids and synthesized 2 shRNAs against RBFOX3. Western blot revealed that RBFOX3 overexpression elevates HTERT protein levels in SGC-7901 and MGC-803 cells, while its silencing RBFOX3 inhibits HTERT

 doi:10.20944/preprints202004.0264.v1

expression at the translational levels in SGC-7901 and MGC-803 cells. Next, we investigated the impact of elevated RBFOX3 and HTERT activity on GC clinical outcomes. We found that RBFOX3 expression correlates with HTERT expression and that both are highly expressed in gastric cancer tissues relative to matching paracancerous tissues. overexpression of RBFOX3, HTERT or both, correlates with advanced disease and poor prognosis, suggesting that RBFOX3 may contribute GC tumorigenesis.

To further evaluated HTERT regulation by RBFOX3, we restored RBFOX3 expression in stably silenced GC cell lines. This analysis revealed that in the background of stably suppressed RBFOX3 expression, reintroduction of RBFOX3 restores expression of both RBFOX3 and HTERT in SGC-7901 and MGC-803. Additionally, HTERT suppression did not affect RBFOX3 levels. In addition, cell proliferative and invasion were restored. Taken together, these experiments further indicated that HTERT expression is regulated by RBFOX3.

Finally, we observed that RBFOX3 interacts with AP-2β to regulate HTERT expression. AP-2β binding to the HTERT promoter was confirmed by ChIP. AP-2β Overexpression enhanced RBFOX3 binding to HTERT promoter. Conversely, AP-2β knockdown weakened RBFOX3 binding to the HTERT promoter even in RBFOX3 overexpressing SGC-7901 cells. A luciferase reporter assay revealed that AP-2β overexpression enhances HTERT promoter activity, which is onhibited by AP-2β knockdown. To evaluate the effect of AP-2β on HTERT expression, we perfomed western blot analysis and observed AP-2β upregulates HTERT while HTERT downregulation is achieved by AP-2β knockdown. Analysis of the effect of AP-2β on cell viability by MTS assay, revealed that AP-2β knockdown inhibits the RBFOX3 overexpression driven GC cell growth. On the other hand, AP-2β overexpression partially rescued RBFOX3 knockdown-mediated growth inhibition. Together, these data show that RBFOX3 interacts with AP-2β to regulate HTERT expression and GC cell growth.

RBFOX3 is an antigen of the neuronal marker antibody NeuN[35], which makes its involvement in GC surprising. Recent studies indicate that RBFOX3 has a wide range

of physiological functions on top of its traditional role as a an alternative splicing factor[17, 36]. RBFOX3 has been found to bind DNA in vitro and to control transcription of a subset of microRNAs[12, 14]. However, its role in mammalian development and homeostasis has remained elusive. RBFOX3 possesses an RNA recognition motif (RRM)-type RNA binding domain (RBD) which enables it to regulate splicing events on various transcripts by binding the (U)GCAUG sequence on RNA[14, 37]. Here, have presented strong evidence that RBFOX3 binds to the HTERT promoter to regulate its expression and GC cell growth. However, it is not clear whether the RNA-binding function of RBFOX3 in these processes. Even so, our findings reveals a novel role for RBFOX3 and further examination of its roles in tumorigenesis is meritted.

In summary, our study reveals that RBFOX3 functions as an oncogene, driving GC cell proliferation, migration and invasion. Furthermore, we have find that RBFOX3 exerts its oncogenic effects by up regulating HTERT signaling in GC. Our findings provide novel insights into the regulation of HTERT during GC tumorigenesis, revealing RBFOX3 as an important regulator of tumor progression via modulation of HTERT signaling. Our findings highlight RBFOX3 as a potential novel therapeutic target against GC.

Acknowledgements

The study was supported by grants from National Natural Science Foundation of China (81560389, 81860433, 81960436, 81560396), Natural Science Foundation of Jiangxi Province (20181BBG70015, 2018BBG70019), the Natural Science Youth Foundation of Jiangxi Province(20192BAB215036), the Foundation for Fostering Young Scholar of Nanchang Universiy(PY201822). the Project of Jiangxi Provincial Innovation fund for graduate students (YC2019-B014). General Science and Technology Program of Jiangxi Health and Family Planning Commission (20171068; 20204359).

Author's contribution

Project design and conception: ZZM, ZXJ.

Performed the research and data collection: LC, ZXJ, LQL, BFQ, HC, LK, ZJF, ZWJ, ZZ, LHL, HJ, HCG and ZJF.

Data statistical and analysis: ZXJ, LC, and BFQ.

Drafted the manuscript: ZXJ and LC.

All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Siegel R L, Miller K D, Jemal A. Cancer statistics, 2019[J]. CA: A Cancer Journal for Clinicians,2019,69(1).

[2] Chen W, Zheng R, Baade P D, et al. Cancer statistics in China, 2015[J]. CA: a cancer journal for clinicians,2016,66(2):115-132.

[3] Thrift A P, El-Serag H B. Burden of Gastric Cancer[J]. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association,2020,18(3):534-542.

[4] Finke J, Engelhardt R. Chemotherapy for Advanced Gastric Cancer[J]. Digestive Surgery,11(2):118-120.

[5] Conboy J G. Developmental regulation of RNA processing by Rbfox proteins[J]. Wiley Interdiscip Rev RNA,2017,8(2).

[6] Yeo G W, Coufal N G, Liang T Y, et al. An RNA code for the FOX2 splicing regulator revealed by mapping RNA-protein interactions in stem cells[J]. Nature structural & molecular biology,2009,16(2):130-137.

[7] Gallagher T L, Arribere J A, Geurts P A, et al. Rbfox-regulated alternative splicing is critical for zebrafish cardiac and skeletal muscle functions[J].,359(2):251-261.

[8] Dredge B K, Jensen K B. NeuN/Rbfox3 nuclear and cytoplasmic isoforms differentially regulate alternative splicing and nonsense-mediated decay of Rbfox2[J]. PloS one,2011,6(6):e21585.

[9] Kim K K, Kim Y C, Adelstein R S, et al. Fox-3 and PSF interact to activate neural cell-specific alternative splicing[J]. Nucleic acids research,2011,39(8):3064-3078.

[10] Lee Y, Rio D C. Mechanisms and Regulation of Alternative Pre-mRNA Splicing[J]. Annual review of biochemistry,2015,84:291-323.

[11] Kim K K, Adelstein R S, Kawamoto S. Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors[J]. The Journal of biological chemistry,2009,284(45):31052-31061.

[12] Mullen R J, Buck C R, Smith A M. NeuN, a neuronal specific nuclear protein in vertebrates[J]. Development (Cambridge, England),1992,116(1):201-211.

[13] Preissl S, Fang R, Huang H, et al. Single-nucleus analysis of accessible chromatin in developing mouse forebrain reveals cell-type-specific transcriptional regulation[J]. Nature neuroscience,2018,21(3):432-439.

[14] Kim K K, Yang Y, Zhu J, et al. Rbfox3 controls the biogenesis of a subset of microRNAs[J]. Nature structural & molecular biology,2014,21(10):901-910.

[15] Zhu X, Luo C, Lin K, et al. Overexpression of DJ-1 enhances colorectal cancer cell proliferation through the cyclin-D1/MDM2-p53 signaling pathway[J]. Biosci Trends,2020.

[16] Maggisano V, Celano M, Lombardo G E, et al. Silencing of hTERT blocks growth and migration of anaplastic thyroid cancer cells[J]. Molecular and cellular endocrinology,2017,448:34-40.

[17] Liu T, Li W, Lu W, et al. RBFOX3 Promotes Tumor Growth and Progression via hTERT Signaling and Predicts a Poor Prognosis in Hepatocellular Carcinoma[J]. Theranostics,2017,7(12):3138-3154.
[18] Hu C, Ni Z, Li B, et al. hTERT promotes the invasion of gastric cancer cells by enhancing FOXO3a ubiquitination and subsequent ITGB1 upregulation[J]. Gut,2017,66(1):31-42.
[19] Lorenzen S, Pauligk C, Homann N, et al. Feasibility of perioperative chemotherapy with infusional 5-FU, leucovorin, and oxaliplatin with (FLOT) or without (FLO) docetaxel in elderly patients with locally advanced esophagogastric cancer[J]. Br J Cancer,2013,108(3):519-526.
[20] Kim Y, Kim J O, Park K, et al. Transforming Growth Factor-β-Induced RBFOX3 Inhibition Promotes Epithelial-Mesenchymal Transition of Lung Cancer Cells[J]. Molecules and cells,2016,39(8):625-630.
[21] Hsieh M, Chen Y, Chen Y, et al. PARP1 controls KLF4-mediated telomerase expression in stem cells and cancer cells[J]. Nucleic acids research,2017,45(18):10492-10503.
[22] Lin C, Qin Y, Zhang H, et al. EGF upregulates RFPL3 and hTERT via the MEK signaling pathway in non small cell lung cancer cells[J]. Oncology reports,2018,40(1):29-38.
[23] Chen W, Qin L, Wang S, et al. CPSF4 activates telomerase reverse transcriptase and predicts poor prognosis in human lung adenocarcinomas[J]. Molecular oncology,2014,8(3):704-716.
[24] Zhang H, Li S. Molecular mechanisms for survival regulation of chronic myeloid leukemia stem cells[J]. Protein Cell,2013,4(3):186-196.
[25] Temime-Smaali N, Guittat L, Wenner T, et al. Topoisomerase IIIalpha is required for normal proliferation and telomere stability in alternative lengthening of telomeres[J]. EMBO J,2008,27(10):1513-1524.
[26] Jafri M A, Ansari S A, Alqahtani M H, et al. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies[J]. Genome Med,2016,8(1):69.
[27] De Vitis M, Berardinelli F, Sgura A. Telomere Length Maintenance in Cancer: At the Crossroad between Telomerase and Alternative Lengthening of Telomeres (ALT)[J]. Int J Mol Sci,2018,19(2).
[28] Ludlow A T, Slusher A L, Sayed M E. Insights into Telomerase/hTERT Alternative Splicing Regulation Using Bioinformatics and Network Analysis in Cancer[J]. Cancers,2019,11(5):666.
[29] Leão R, Apolónio J D, Lee D, et al. Mechanisms of human telomerase reverse transcriptase (hTERT) regulation: clinical impacts in cancer[J]. Journal of biomedical science,2018,25(1):22.
[30] Zhao X, Zheng F, Li Y, et al. BPTF promotes hepatocellular carcinoma growth by modulating hTERT signaling and cancer stem cell traits[J]. Redox biology,2019,20:427-441.
[31] Sayed M E, Yuan L, Robin J D, et al. NOVA1 directs PTBP1 to hTERT pre-mRNA and promotes telomerase activity in cancer cells[J]. Oncogene,2019,38(16):2937-2952.
[32] Yan T, Ooi W F, Qamra A, et al. HoxC5 and miR-615-3p target newly evolved genomic regions to repress hTERT and inhibit tumorigenesis[J]. Nature communications,2018,9(1):100.
[33] Choi M J, Cho K H, Lee S, et al. hTERT mediates norepinephrine-induced Slug expression and ovarian cancer aggressiveness[J]. Oncogene,2015,34(26):3402-3412.
[34] Song J H, Kang H, Luevano L A, et al. Small-Molecule-Targeting Hairpin Loop of hTERT Promoter G-Quadruplex Induces Cancer Cell Death[J]. Cell chemical biology,2019,26(8):1110-1121.
[35] Duan W, Zhang Y P, Hou Z, et al. Novel Insights into NeuN: from Neuronal Marker to Splicing Regulator[J]. Mol Neurobiol,2016,53(3):1637-1647.
[36] Tang Y, Sun X, Huang L, et al. Melatonin inhibits MLL-rearranged leukemia via RBFOX3/hTERT and NF-κB/COX-2 signaling pathways[J]. Cancer letters,2019,443:167-178.
[37] Kim K K, Nam J, Mukouyama Y, et al. Rbfox3-regulated alternative splicing of Numb promotes

 doi:10.20944/preprints202004.0264.v1

neuronal differentiation during development[J]. The Journal of cell biology,2013,200(4):443-458.

Table 1 Correlation of the expression of RBFOX3 with clinical features in gastric cancer

Characteristics	RBFOX3 expression		*p*-value
	Low group (N = 89)	High group (N = 89)	
Age (y)			$p = 0.759$
<60	36	33	
≥60	53	56	
Gender			$p = 0.294$
Male	42	50	

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

FeMale	47	39	
Diameter (cm)			$p = 0.122$
<5	50	61	
≥5	39	28	
Location			$p = 0.552$
Proximal	24	24	
Middle	21	27	
Distal	44	38	
Differentiation			**$p = 0.017$***
Poor	23	41	
Moderately	29	19	
Highly	37	29	
Lauren type			$p = 0.563$
Intestinal	71	74	
Diffuse or mixed	18	15	
AJCC clinical stage			**$p = 0.015$***
I	49	37	
II + III	40	62	
Drinking alcohol			$p = 0.118$
No	20	12	
Yes	69	77	
Smoking			$p = 0.563$
No	18	15	
Yes	71	74	
TNM stage			**$p = 0.004$****
I + II	50	31	
III + IV	39	58	
Lymphatic metastasis			$p = 0.602$
N0 + N1	44	38	
N2	25	26	
N3	20	25	

***p<0.05; **p<0.01**

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

Table 2 Univariate and multivariate regression analyses of parameters associated with prognosis of GC patients

Characteristics	Subset	Univariate analysis		Multivariate analysis	
		p-value	HR (95% CI)	*p*-value	HR (95% CI)
Age	<60/≥60	0.137	1.354(0.968-1.994)	-	-
Gender	Male/female	0.741	0.931(0.338–1.835)	-	-
Diameter	<5cm/ ≥5cm	0.630	1.335(0.634–2.567)	-	-
Location	Proximal/Middle/Distal	0.224	1.016(0.512–2.016)	-	-
Drinking alcohol	No/Yes	0.318	2.445(1.145–5.345)	-	-
Smoking	No/Yes	0.377	8.474(1.223–52.031)	-	-
Lauren	Intestinal/Diffuse or mixed	0.914	0.527(0.361-1.158)	-	-
Lymphatic metastasis	N0+N1/N2+N3	0.506	0.716(0.434-1.098)	-	-
Differentiation	Poor+Moderately/Highly	**0.014***	1.204(0.432-1.943)	0.062	1.341(1.015-2.578)
Ajcc clinical stage	I+II/III	**0.001****	2.179(1.588-3.493)	0.129	4.083(0.549-10.240)
TNM stage	I+II/III+IV	**0.002****	3.118(2.090-5.567)	**0.006****	2.118(0.837-4.651)
RBFOX3	High/Low	**0.001****	3.521(2.084-4.747)	**0.001****	2.670(1.471-3.917)

***p*<0.05; ****p*<0.01**

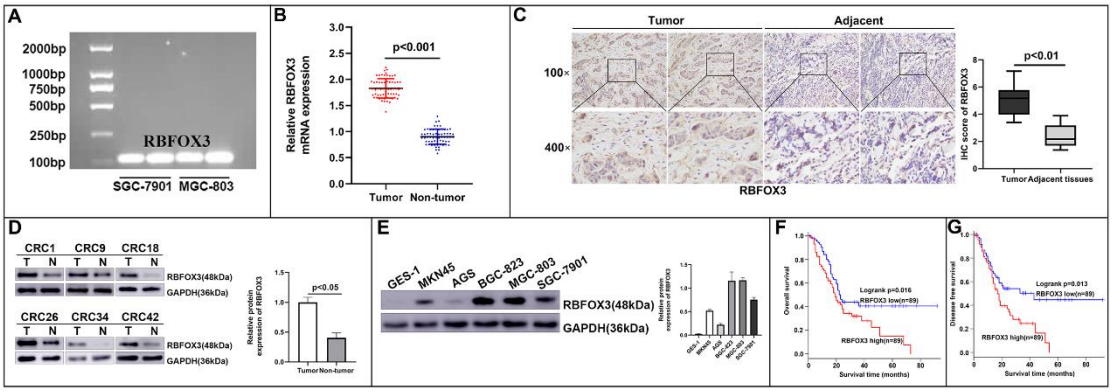


Figure 1. High RBFOX3 protein expression is linked to dismal prognosis GC patients. A RBFOX3 DNA digested fragment expression was examined via agarose gel electrophoresis in SGC-7901 as well as MGC-803 cells. **B** RT-qPCR was conducted in GC tissues samples (n=47) and the corresponding precancerous tissue samples (n=47) to determine RBFOX3 mRNA expression. **C** The representative images of immune-histochemical findings of RBFOX3 in normal gastric mucosa and GC tissues (n=89). **D** The expression of RBFOX3 protein was assessed using Western blot analysis in 37 human GC tissues as well as the adjacent tissues. **E** The protein expression level of

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

RBFOX3 in normal gastric epithelial cells (GES-1) compared with GC cell lines (MKN45, AGS, SGC-7901, MGC-803, and BGC-823). **F-G** Kaplan-Meier survival analysis of the OS and DS in two groups designated by low and high expression of RBFOX3 in patients with GC.

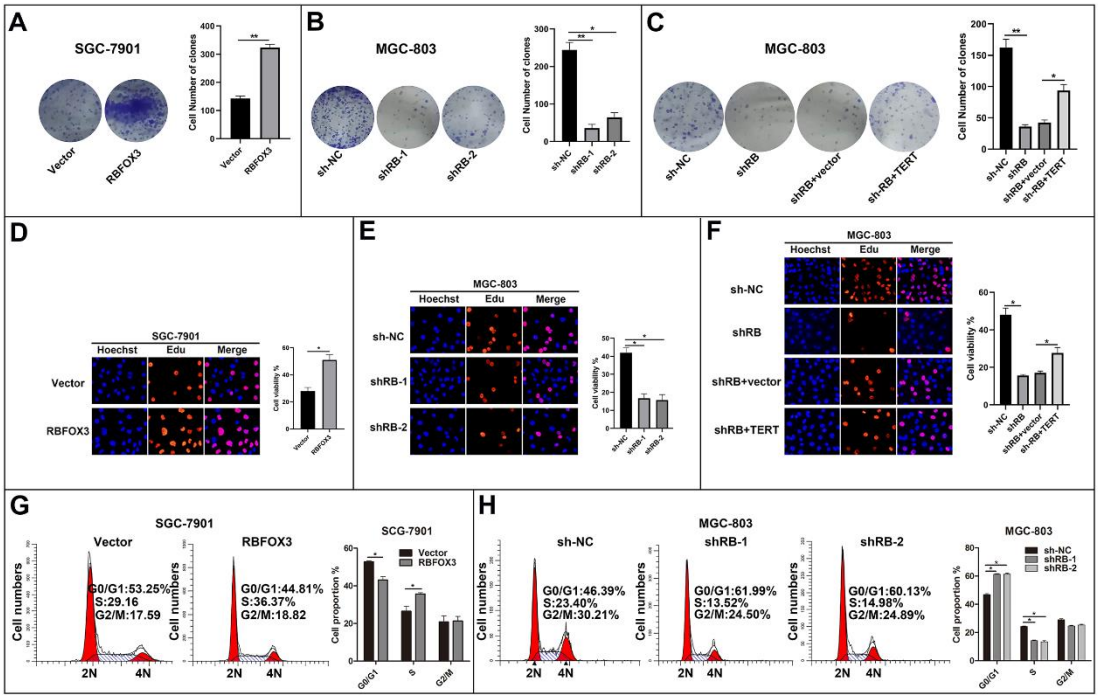


Figure 2. RBFOX3 promoted GC cell multiplication and cell cycle progression *in vitro*. A and D RBFOX3 overexpression increased propagative viability of cells as well as the formation of colonies in SGC-7901 cells. **B and E** RBFOX3 silencing decreased the viability of cells as well as the capacity of formation of colonies in MGC-803 cells. **C and F** Overexpression of HTERT reverted the repression of viability of cells and colony formation moderated by the silencing of RBFOX3 in MGC-803 cells. **G-H** Flow-cytometric analyses of the cell cycle of the indicated GC cells transfected with Vector/RBFOX3 and sh-NC/shRB-1/shRB-2. (*p<0.05, **p<0.01)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

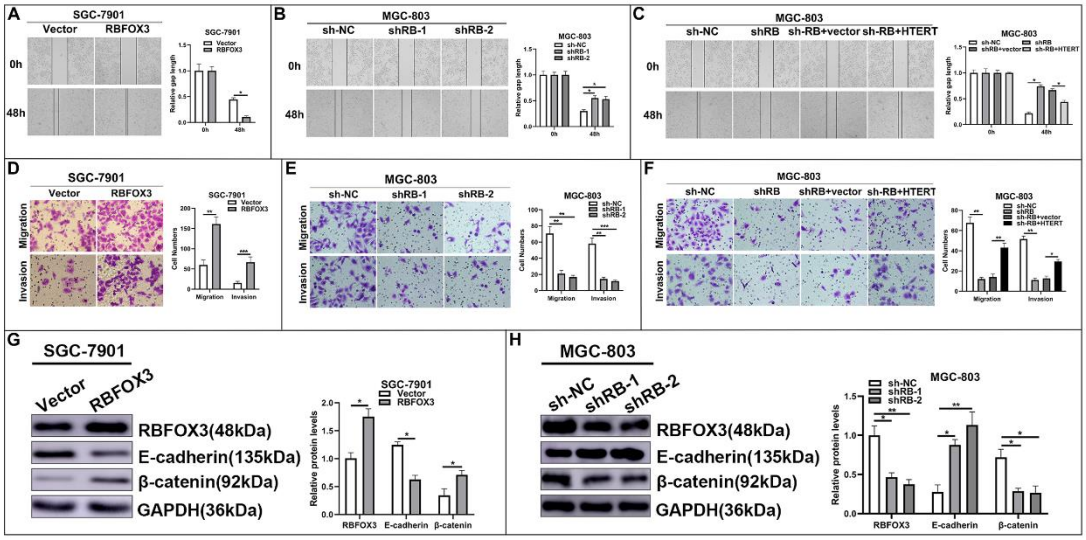


Figure 3. RBFOX3 facilitated the migration and invasion of GC cells *in vitro*. A-C We utilized the wound-healing assay in investigating the horizontal migration potential with RBFOX3 overexpression or silencing in SGC-7901 (left panel), MGC-803 cells (middle panel). Overexpression of HTERT reverted the suppression of cell viability and formation of colonies moderated by RBFOX3 silencing in MGC-803 cells (right panel). **D-F** We utilized the migration as well as invasion assays in investigating vertical migration and invasion capacities with RBFOX3 overexpression or silencing in SGC-7901 (left panel), MGC-803 (middle panel) cells. **G-H** We used the Western blot assay in exploring the expression of metastasis-related proteins with the RBFOX3 overexpression or silencing in SGC-7901 as well as MGC-803 cells. (*p<0.05, **p<0.01, ***p<0.001)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

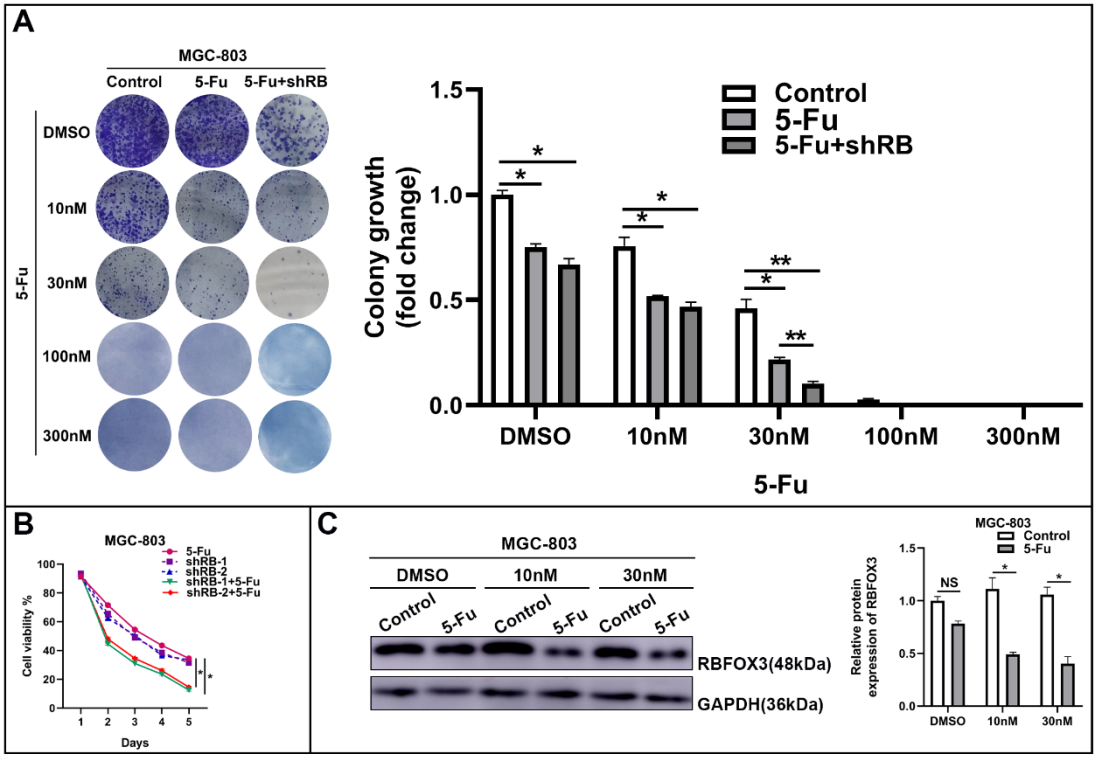


Figure 4. RBFOX3 regulated the sensitivity of GC to 5-Fu. A An image representing the clonogenic assay of MGC-803 cells with RBFOX3 knockdown or control groups. The graph denotes the mean of number of colonies ± SD with respect to the vehicle control, 3 biological replicates with 2 technical replicates; two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test, DMSO, dimethyl sulfoxide. **B** The effect of RBFOX3 repression on the response of MGC-803 cells to 5-Fu treatment. Data are indicated as means ± SEM for 3 biological replicate experiments with 3 technical replicates. We transfected the MGC-803 cells with RBFOX3 shRNA (shRB-1 and shRB-2). We measured the viability of cells using the MTS assay with or without 5-Fu treatment with respect to time. **C** Western blot was conducted to profile the expression of RBFOX3 protein in MGC-803 cells treated with DMSO/10nM/30nM 5-Fu arms compared with the controls. (*p<0.05, **p<0.01)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

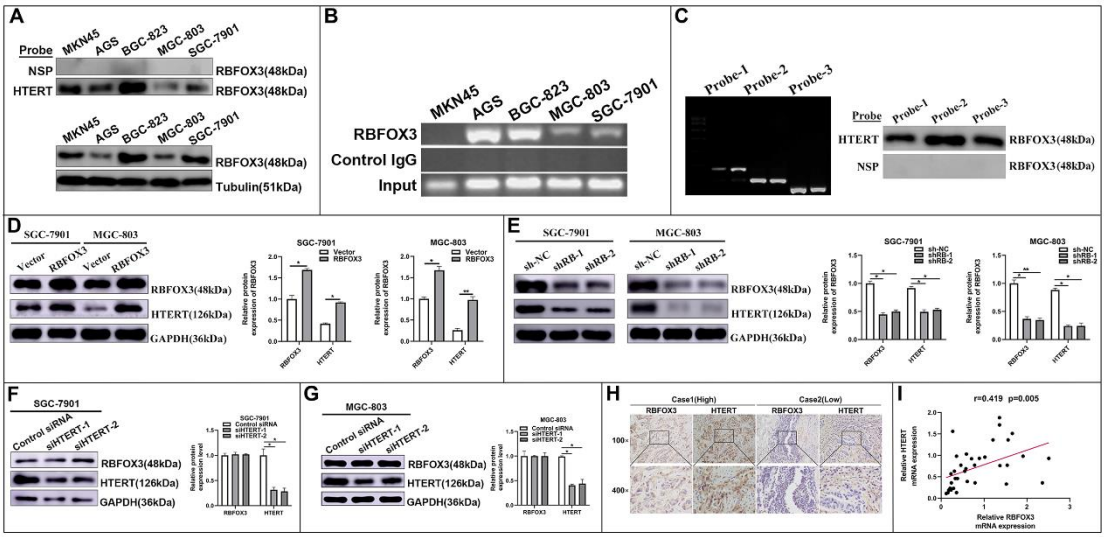


Figure 5. RBFOX3 acts as an HTERT promoter binding protein in GC cells. A We detected the binding of RBFOX3 on the 5'-biotin labeled HTERT promoter probe or a nonspecific probe (NSP) via Western blot using anti-RBFOX3 antibody. We detected RBFOX3 protein in the cytoplasm protein-HTERT probe-streptavidin bead complexes in MKN45, AGS, SGC-7901, MGC-803, and MGC-823 cells (upper panel). We analyzed the expression of total RBFOX3 proteins in GC cells using Western blot, and utilized Tubulin as a loading control (lower panel). **B** Chromatin immunoprecipitation (ChIP) assays were conducted in GC cells using RBFOX3 antibody and the HTERT promoter primers. We separated the PCR products on 2% agarose gels. We used IgG as a negative control. **C** We incubated the 5'-biotin labeled probes corresponding with the diverse fragments of HTERT promoter (probe 1-3, left panel) or a nonspecific probe (NSP) with MGC-803 cell lysates and streptavidin beads. Western blot results using anti-RBFOX3 antibody (right panel) showing the detection of the protein bound proteins. **D-E** HTERT protein expression was down-regulated in RBFOX3 silenced SGC-7901 as well as MGC-803 cells, and up-regulated in RBFOX3 overexpressing SGC-7901 and MGC-803 cells. **F** Immuno-histochemical results of RBFOX3 and HTERT protein levels from GC tissues represented by two images "Low" denotes low expression of both RBFOX3 and HTERT and "High" denotes high protein expression of both RBFOX3 and HTERT. **H** Association between the expressions of RBFOX3 and HTERT in 37 GC paraffin section samples from GC tissues. Pearson correlation test, n=37, r=0.419, p=0.005. (*p<0.05, **p<0.01)

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

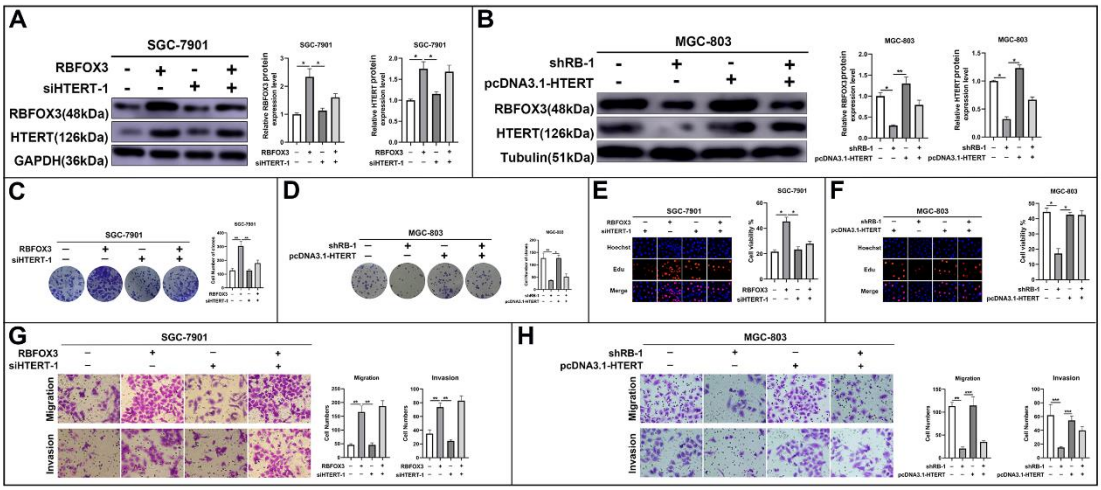
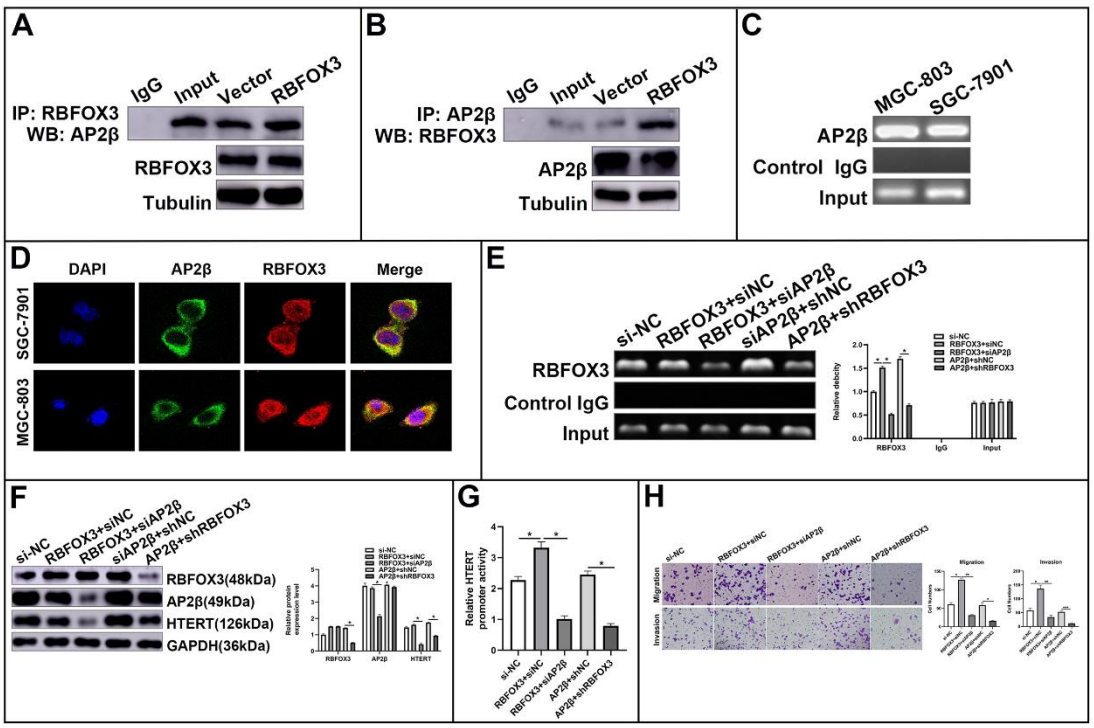
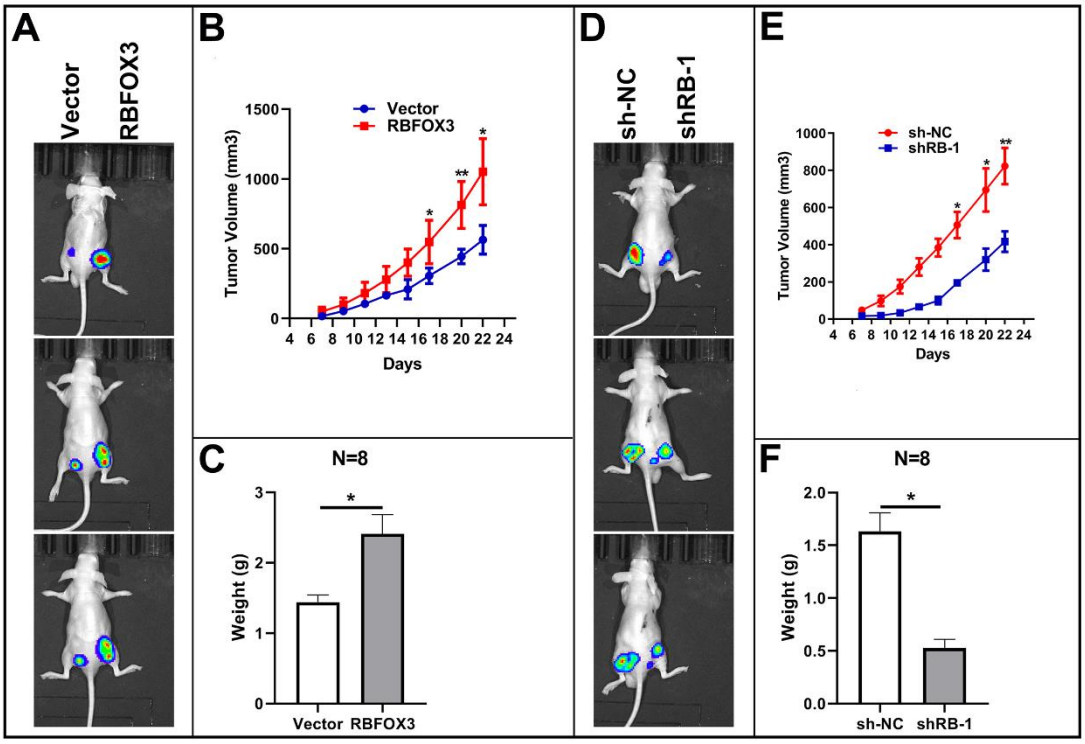


Figure 6. RBFOX3 promotes GC multiplication and invasion by upregulating HTERT expression. A The silencing of HTERT (siHTERT-1) repressed the upregulation of HTERT expression in SGC-7901-RBFOX3 cells. **B** The upregulation of HTERT (pcDNA3.1-HTERT) attenuated HTERT expression in MGC-803-shRB cells. **C-H** Colony formation, Edu, and invasion assays showing that knockdown or overexpression of ectopic HTERT significantly rescues the RBFOX3-overexpressed or RBFOX3-silenced cell division and invasion observed in SGC-7901 and MGC-803 cells. (*p<0.05, **p<0.01)



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

Figure 7. RBFOX3 interacted with AP-2β and modulated the expression of HTERT. A-B We transfected SGC-7901 cells with RBFOX3 overexpression or empty vector. CoIP analysis was used to detect the interaction between RBFOX3 and AP-2β. **C** We performed the ChIP assays using the antibody against AP-2β. We separated the PCR products of the HTERT promoter (-378 to -157) on 2% agarose gels. **D** We cultured the SGC-7901 as well as the MGC-803 cells grown on chamber slides for 24h, and examined the subcellular localization and the co-localization of RBFOX3 with AP-2β via confocal microscopy analysis. **E** We performed that the ChIP assays using the HTERT promoter primers and the respective RBFOX3 antibodies in SGC-7901 cells transfected with RBFOX3, RBFOX3, and siAP-2β, AP-2β, AP-2β and shRBFOX3, respectively. **F** The expression of HTERT in SGC-7901 cells transfected with RBFOX3, RBFOX3 and siAP-2β, AP-2β, AP-2β and shRBFOX3, respectively. **G** Relative HTERT promoter activity in SGC-7901 cells transfected with RBFOX3, RBFOX3 and siAP-2β, AP-2β, AP-2β and shRBFOX3, respectively. **H** We performed the Trans-well invasion assay in SGC-7901 cells transfected with RBFOX3, RBFOX3 and siAP-2β, AP-2β, AP-2β and shRBFOX3, respectively. (*p<0.05, **p<0.01)



Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 16 April 2020 doi:10.20944/preprints202004.0264.v1

Figure 8. Dysregulation of RBFOX3 suppressed cell multiplication in GC orthotopic xenografts. Stable transfection of the SGC-7901 and MGC-803 cells with pcDNA3.1-RBFOX3 plasmid, or empty plasmid (Vector) and RBFOX3 shRNA, or negative control vector (sh-NC), respectively. Next, we subcutaneously injected the transfected GC cells into nude mice and xenograft tumor growth was determined for 25 days. **A, B and C** IVIS representative images showing tumor size (**A**), growth curves of tumor volumes (**B**) and Tumor weight (**C**) in RBFOX3 overexpressed arm vs control arm. **D, E and F** IVIS representative images showing tumor size (**D**), growth curves of tumor volumes (**E**) and Tumor weight (**F**) in shRB-1 arm vs controls. n=8. (*p<0.05, **p<0.01)