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Highly expressed lncRNA FAL1 promotes the progression of gastric cancer by inhibiting PTEN

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Abstract. – OBJECTIVE: The aim of this study was to investigate the function of FAL1 in gastric cancer (GC) development and to examine its underlying mechanism. Our study might provide a the-oretical basis for developing novel diagnostic markers for GC.

PATIENTS AND METHODS: FAL1 expression in GC tissues and adjacent tissues was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The serum level of FAL1 in GC patients with different pathological grades was further detect-ed. The effects of FAL1 on cell proliferation and cell cycle were detected by cell count-ing kit-8 (CCK-8) assay and flow cytometry, respectively. Meanwhile, Western blot was used to detect the protein expression of PTEN after FAL1 overexpression or knock-down in GC cells. In addition, rescue experiments were conducted to verify the regulatory effect of FAL1 on PTEN.

RESULTS: QRT-PCR results showed that the expression of FAL1 in GC tissues was remarkably higher than that of adjacent tissues. FAL1 expression was correlated with pathological grades of GC patients. Meanwhile, FAL1 overexpression promoted the proliferation and cell cycle of BGC-823 and MGC-803 cells. Western blot analysis demonstrated that FAL1 could inhibit the protein expression of PTEN in GC cells. In addition, rescue experiments indicated that the overexpression of PTEN could partially reverse the effect of FAL1 on the proliferation and cell cycle of BGC-823 and MGC-803 cells.

CONCLUSIONS: The overexpression of FAL1 can promote cell proliferation and cell cycle of GC via inhibiting PTEN.

Key Words:
FAL1, Gastric cancer, Proliferation, Cell cycle, PTEN.

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide, with high morbidity and mortality. According to previous epidemiological investigations, GC may be associated with *Helicobacter pylori* infection, nitrous compounds intake, long-term chronic gastric diseases, dietary habits and environmental factors[1,2]. With the development of advanced gastroscopy and drug intervention, clinical outcomes of GC patients have been greatly improved. However, early-stage GC is hardly to be diagnosed due to occult symptoms. As a result, many GC patients have already developed to the advanced stage accompanied by lymph node and distant metastasis when diagnosed. Studies have shown that metastasis of tumor cells is an important cause of poor prognosis of tumor patients[3]. Meanwhile, multiple mechanisms are involved in the regulatory network of tumor metastasis[4]. Therefore, it is of great significance to elucidate the underlying mechanism of GC, to provide therapeutic targets for effective diagnosis and treatment of GC[5,6].

Long non-coding RNA (lncRNA) is a class of RNA molecules with more than 200 nucleotides in length. With no protein-coding function, lncRNA was initially considered as "noise" of genomics[7]. In recent years, it has been found that lncRNA can regulate gene expression at epigenetic, transcriptional and post-transcriptional levels. Meanwhile, lncRNA is also reported to be involved in the occurrence and progression of tumors[7,8]. For example, differentially expressed lncRNA participates in the regulation of apoptosis, invasion and metastasis of GC, which may eventually serve as a diagnostic and prognostic marker[9-11].

Recent studies have shown that lncRNA FAL1 is differentially expressed in a variety of tumors. Meanwhile, FAL1 expression is closely related to the metastasis, progression and prognosis of tumor patients. *In vitro* experiments have found

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that FAL1 promotes cell proliferation, invasion, migration and other functions, suggesting that FAL1 is expected to be a therapeutic target for tumor treatment[12-15]. Although it has been studied in multiple diseases, the specific role of FAL1 in GC remains to be explored.

Patients and Methods

Sample Collection

From July 2013 to July 2017, 66 GC patients admitted to the First People's Hospital of Wenling were enrolled in this study. GC tissues and adjacent tissues were surgically resected and collected. GC tissues were harvested in avoidance of the necrosis area, while adjacent tissues were selected 5 cm away from the cancer tissues. All collected tissues were immediately preserved in liquid nitrogen until use. Enrolled patients were pathologically diagnosed as GC and did not receive any preoperative treatments. Informed consent was obtained from each patient before the study. This study was approved by the Ethics Committee of the First People's Hospital of Wenling.

Cell Culture and Transfection

GES-1, SGC-7901, BGC-823, and MGC-803 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA), and were maintained in a 37°C, 5% CO_2 incubator.

When the confluence was up to 50-60%, cells were first washed with phosphate- buffered saline (PBS) (Beyotime, Shanghai, China) and then seeded into culture plates. Cell transfection was performed in accordance with the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Corresponding plasmids used in this study were constructed by Gene Pharma (Shanghai, China).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in treated cells was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, the extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) in accordance with the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). Three replicates were set in each group. The specific qRT-PCR reaction parameters were: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. Primers used in the study were as follows: FAL1, F: 5'-GCAAGCGGAGACTTGTCTTT-3', R: 5'-TTGAACTCCTGACCTCGTGA-3'; PTEN, F: 5'-TGGATTCGACTTAGACTTGACCT-3', R: 5'-GGTGGGTTATGGTCTTCAAAAGG-3'; GAPDH, F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'.

Cell Counting Kit-8 (CCK-8) Assay

Transfected BGC-823 or MGC-803 cells were first seeded into 96-well plates at a density of 1×10^4 cell/μL. 0, 24, 48 and 72 h after culture, 10 μL cell counting kit-8 solution (CCK-8, Dojindo, Kumamoto, Japan) was added in each well, followed by incubation at 37°o for 2 h in the dark. The absorbance at the wavelength of 450 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Each group had 5 replicates.

Cell Cycle Detection

Transfected cells were collected, and the cell density was adjusted to 1×10^5 cells/mL. Subsequently, the cells were fixed with pre-cooled ethanol overnight. After washing with PBS twice, the cells were incubated with 100 μL RNaseA at 37°C in the dark. 25 min later, the cells were stained with 400 μL propidium iodide (PI). Cell cycle was detected by flow cytometry (Partec AG, Arlesheim, Switzerland) at the wavelength of 488 nm. Each experiment was repeated three times.

Western Blot

Total protein of treated cells was extracted by radio-immunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The concentration of extracted protein was detected by the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Subsequently, extracted protein samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were then incubated with primary antibodies at 4°C overnight. After washing with phosphate buffered saline-tween (PBST), the membranes

incubated with corresponding secondary antibody at room temperature for 1 h. Finally, immunoreactive bands were exposed by the enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 Software (IBM, Armonk, NY, USA) was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. The *t*-test was applied to compare the differences between the two groups. Pearson test was used for correlation analysis. $p < 0.05$ was considered statistically significant.

Results

FAL1 was Highly Expressed in GC Patients

QRT-PCR results showed that the expression of FAL1 in GC tissues was significantly higher than that of adjacent tissues (Figure 1A and 1B). Subsequently, we investigated the relationship between FAL1 expression and pathological features of GC patients. Results found that the serum levels of FAL1 in GC patients with stage III and IV were significantly higher than those with stage I and II (Figure 1C). Besides, the serum levels of FAL1 in GC patients with T3 and T4 were also significantly higher than those with T1 and T2 (Figure 1D). Results also demonstrated that the serum level of FAL1 was positively correlated with the degree of lymph node metastasis, whereas not correlated with the degree of distant metastasis (Figure 1E and 1F). The above results all indicated that FAL1 was highly expressed in GC tissues. Meanwhile, the serum level of FAL1 was markedly associated with pathological grades of GC patients.

FAL1 Promoted the Proliferation and Cell Cycle of GC Cells

We then detected the expression levels of FAL1 in gastric mucosal cell line GES-1 and GC cell lines SGC-7901, BGC-823 and MGC-803 by qRT-PCR. Results showed that FAL1 was highly expressed in GC cells when compared with that of normal cells (Figure 2A). Particularly, BGC-823 and MGC-803 cells expressed the lowest and highest level of FAL1, which were selected for the following experiments. Transfection efficiencies of FAL1-NC, FAL1-OE, and FAL1-siRNA were first verified by qRT-PCR (Figure 2B and 2C). Subsequently, the regulatory effects of FAL1 on cell proliferation and cell cycle were detected by CCK-8 and flow cytometry, respectively. CCK-8 results indicated that FAL1 overexpression remarkably promoted the proliferation of GC cells (Figure 2D). Meanwhile, flow cytometry results suggested that FAL1 up-regulation promoted cell cycle (Figure 2F). However, knockdown of FAL1 obtained the opposite results on the proliferation and cell cycle of GC cells (Figure 2E and 2G).

FAL1 Inhibited PTEN Expression

Previous studies have shown that FAL1 overexpression promotes tumorigenesis *via* inhibiting PTEN expression. In the present study, we found that the expression of PTEN was significantly decreased in GC tissues (Figure 3A). Transfection of FAL1-NC or FAL1-OE downregulated PTEN expression, whereas FAL1-siRNA transfection upregulated PTEN expression (Figure 3B and 3C). Western blot results demonstrated that the protein level of PTEN was negatively regulated by FAL1 (Figure 3D and 3E). Moreover, PTEN expression in GC tissues was also negatively correlated with FAL1 expression (Figure 3F). Subsequently, pcDNA-NC and pcDNA-PTEN were constructed and transfected into BGC-823 and MGC-803 cells for the following rescue experiments. Transfection efficiencies were verified by qRT-PCR (Figure 3G and 3H).

PTEN Overexpression Partially Reversed the Effects of FAL1 on the Proliferation and Cell Cycle of GC Cells

To explore the regulatory effect of FAL1 on PTEN, rescue experiments were conducted. Results showed that the overexpression of FAL1 significantly promoted the proliferation of GC cells, which could be partially reversed by PTEN overexpression (Figure 4A and 4B). Similarly, promoted cell cycle induced by FAL1 overexpression was partially reversed after the overexpression of PTEN (Figure 4C and 4D).

Discussion

As one of the most common malignant tumors over the world, early symptoms of GC are not evident. Currently, the main diagnostic approaches for GC include upper gastrointestinal

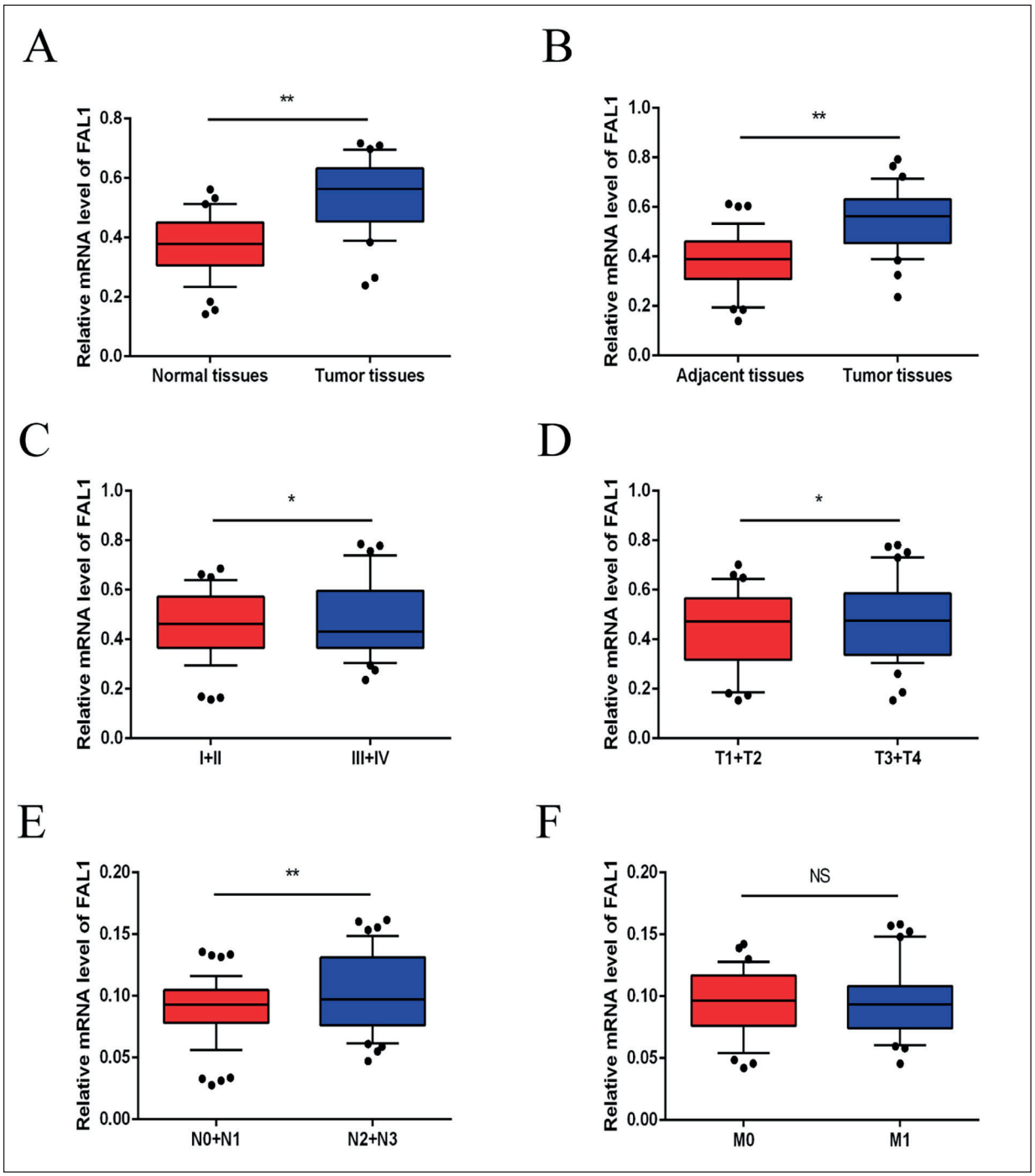

Figure 1. FAL1 was highly expressed in GC. ***A, B,*** QRT-PCR results showed that FAL1 expression in GC tissues was significantly higher than that of adjacent tissues. ***C,*** Serum level of FAL1 in GC patients with stage III and IV was significantly higher than those with stage I and II. ***D,*** Serum level of FAL1 in GC patients with T3 and T4 was significantly higher than those with T1 and T2. ***E, F,*** Serum level of FAL1 was positively correlated with the degree of lymph node metastasis, whereas not correlated with the degree of distant metastasis.

contrast X-ray and endoscopic biopsy. However, the sensitivity of upper gastrointestinal contrast X-ray in the early diagnosis of GC is relatively low. Meanwhile, an endoscopic biopsy is uncommonly performed due to its invasive characteristic. Many GC patients cannot be operated since

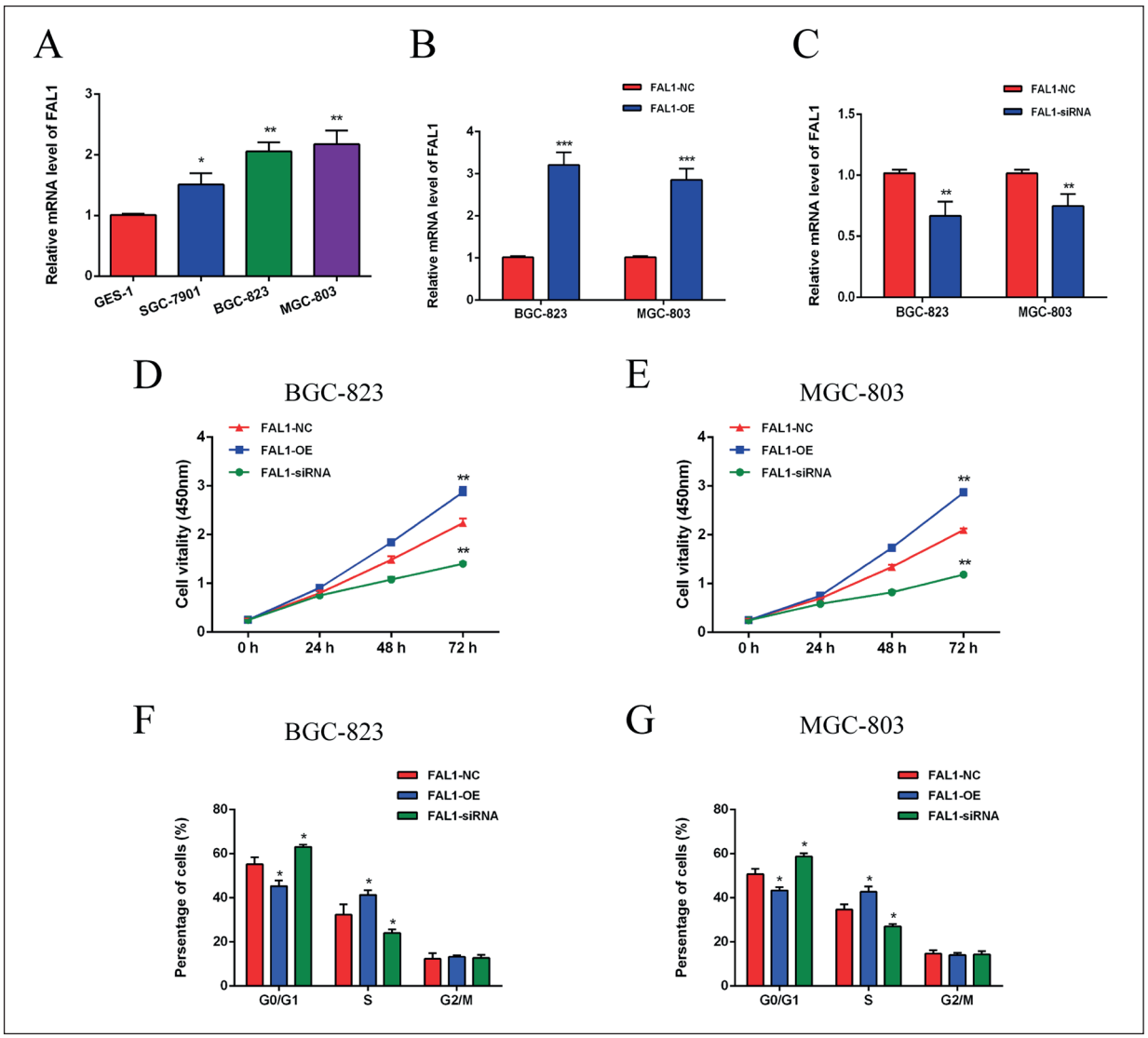

Figure 2. FAL1 promoted the proliferation and cell cycle of GC. ***A,*** FAL1 was highly expressed in GC cells when compared with that of normal cells. ***B, C,*** Transfection efficiencies of FAL1-NC, FAL1-OE, and FAL1-siRNA were verified by qRT-PCR. ***D, E,*** CCK-8 results indicated that FAL1 overexpression remarkably promoted the proliferation of GC cells. However, FAL1 knockdown remarkably inhibited the proliferation of GC cells. ***F, G,*** Flow cytometry results suggested that cell cycle was promoted by FAL1 overexpression. However, FAL1 knockdown inhibited cell cycle of GC cells.

they are already in the advanced stage and lose the opportunities of surgery[1]. Some studies have indicated that a large number of GC patients die from postoperative recurrence and metastasis. The 5-year survival rate of advanced GC is less than 20%, which is over than 90% in early-stage GC patients. Hence, the improvement of early diagnosis rate may contribute to better clinical outcomes of GC patients[16].

The Human Genome Project reveals that 98% of human gene sequences do not encode proteins. With the development of in-depth studies, it has been found that lncRNAs exert various biological functions[17]. It's reported that lncRNA participates in gene transcription and translation, as well as cell differentiation and development. In particular, lncRNA plays an important role in tumor development[18]. For example, lncRNA MALAT1 promotes the proliferation of GC *via* recruiting SFD2/ASF[9]. LncRNA GHET can promote the proliferation of GC cells by enhancing the mRNA expression level of c-Myc[11]. Meanwhile, lncRNA LEIGC inhibits the proliferation of tumor cells through suppressing epithelial-mesenchy-

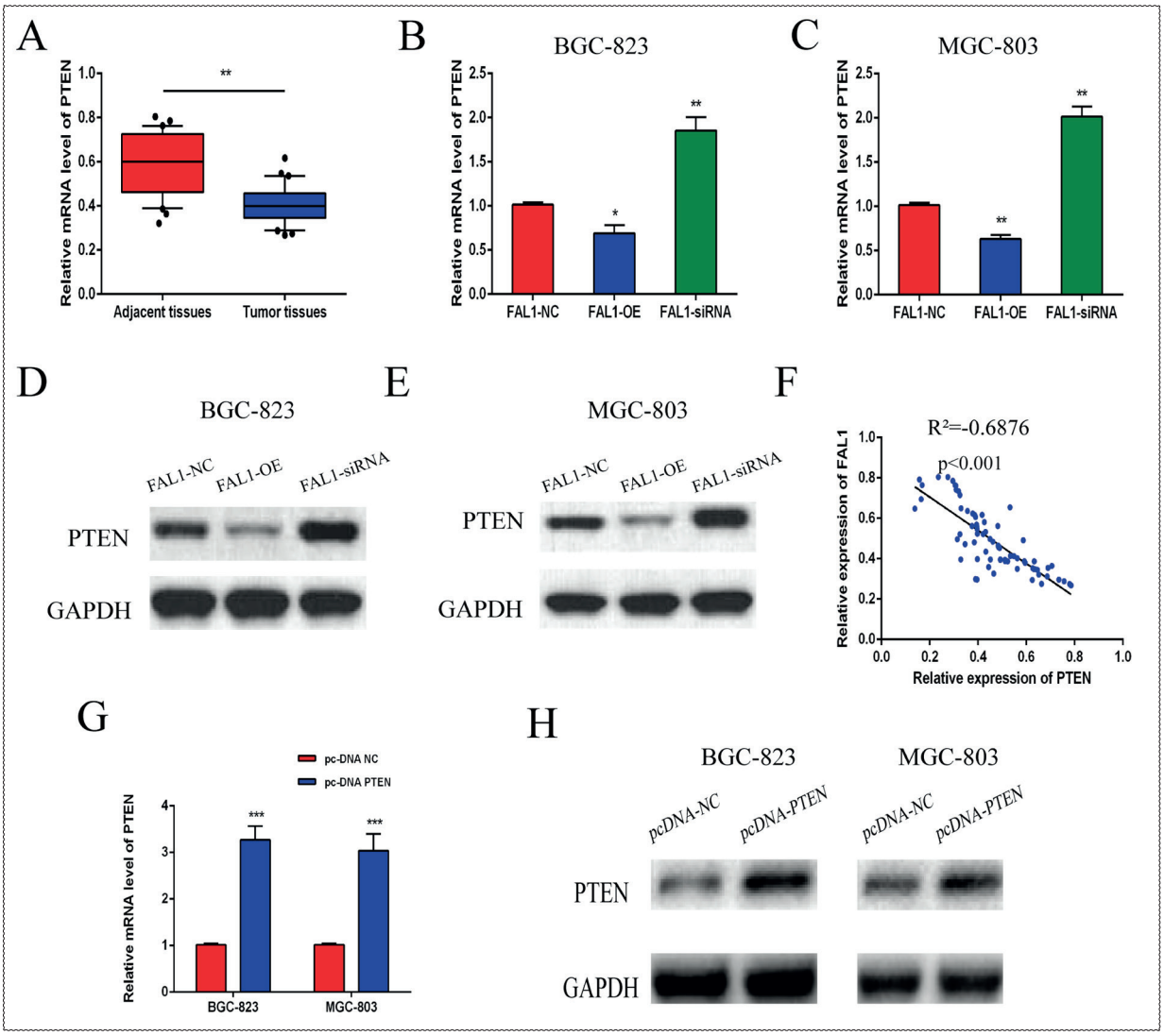

Figure 3. FAL1 inhibited PTEN expression. ***A,*** PTEN expression was significantly decreased in GC tissues. ***B, C,*** Transfection of FAL1-NC or FAL1-OE remarkably downregulated PTEN expression, whereas FAL1-siRNA transfection significantly upregulated PTEN expression. ***D, E,*** Western blot results showed that the protein expression level of PTEN was negatively regulated by FAL1. ***F,*** PTEN expression in GC tissues was negatively correlated with FAL1 expression. ***G, H,*** Transfection efficiencies of pcDNA-NC and pcDNA-PTEN in BGC-823 and MGC-803 cells were verified by qRT-PCR.

mal transition[19]. Another study has demonstrated that lncRNA HOTAIR knockdown in GC cells inhibits tumor growth by arresting cells in the G0/G1 phase and inducing cell apoptosis[10].

NcRNA in serum is not sensitive to room temperature, repeated freezing and thawing conditions, which can be stabilized in blood[20]. Advantages in blood sample collection provide theoretical and operational possibilities for ncRNA to serve as tumor biomarkers[21]. It is reported that ncRNA can be detected in GC tissues, serum, and gastric juice. However, the specific role of FAL1 in serum and tumor samples are rarely reported. In the present study, we found that FAL1 overexpression could promote the proliferation and cell cycle of GC cells, which was significantly correlated with the pathological grades of GC patients.

Our findings indicated that FAL1 exerted its biological function by downregulating PTEN expression, which was further verified by qRT-PCR and Western blot. PTEN is a tumor-suppressor gene with dual-phosphorylase activities, and it may be a target gene for FAL1. Meanwhile, it

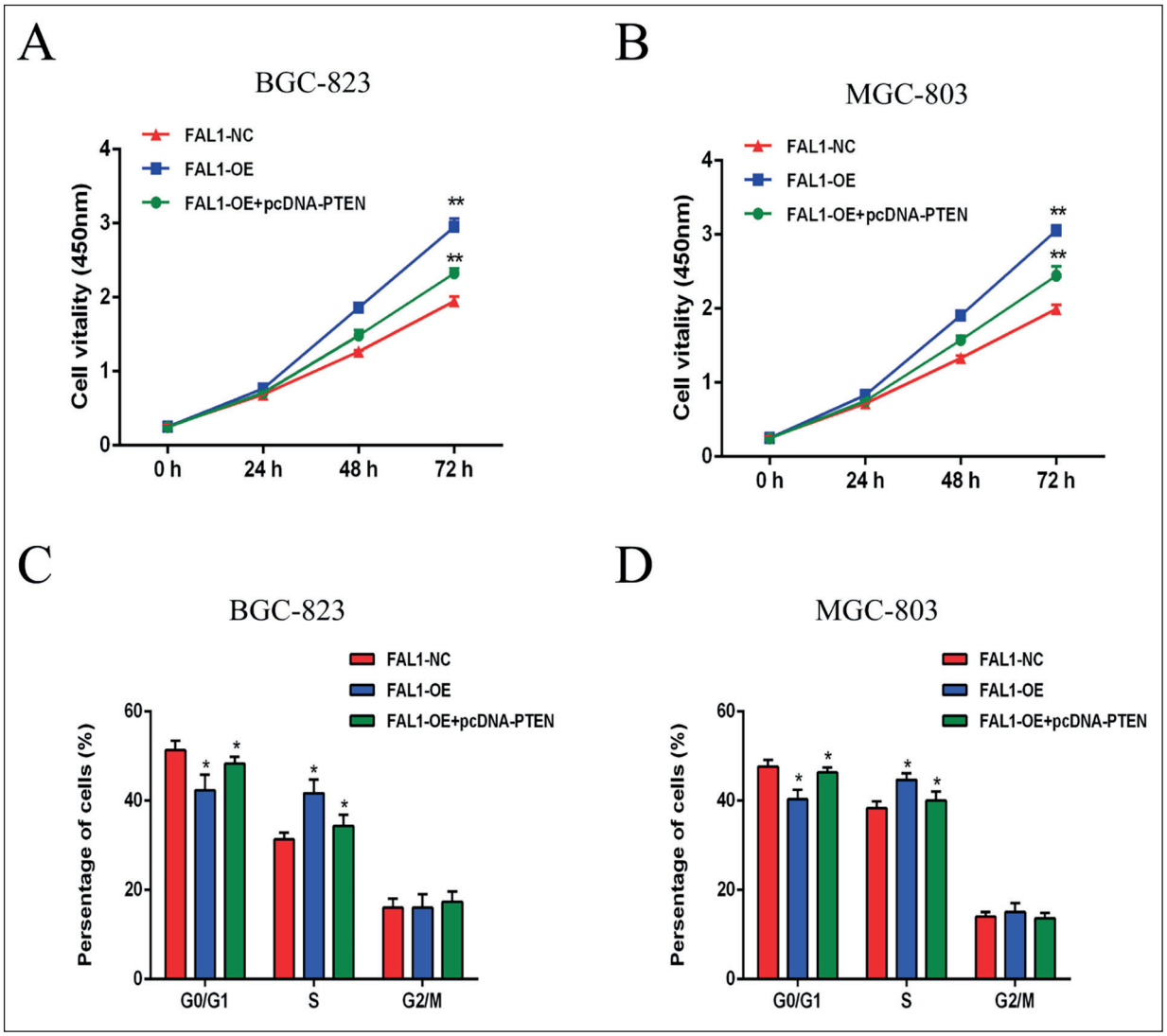

Figure 4. PTEN overexpression partially reversed the effects of FAL1 on the proliferation and cell cycle of GC cells. ***A, B,*** Overexpression of FAL1 significantly promoted the proliferation of GC cells, which was partially reversed by PTEN overexpression. ***C, D,*** The promoted cell cycle induced by FAL1 overexpression was partially reversed after the overexpression of PTEN.

is a natural inhibitor of the PI3K/AKT pathway. PTEN deficiency frequently occurs in a variety of malignant tumors[22]. By the de-phosphorylation of PIP3, PTEN downregulates related genes in the PI3K/AKT pathway and inhibits a series of downstream anti-apoptotic, proliferative and invasive pathways. Previous studies have confirmed that PTEN is capable of promoting cell apoptosis and inhibiting cell proliferation and invasion by reducing the phosphorylation levels of survival kinases[23]. Additionally, PTEN is involved in maintaining chromosome stability, inhibiting mutant genes accumulation and suppressing cell transformation[24]. In this study, we found that the expression of PTEN was remarkably decreased in GC tissues than that of adjacent tissues. Moreover, the overexpression of PTEN partially reversed the promotive effects of FAL1 on the proliferation and cell cycle of GC cells.

Conclusions

We observed that the overexpression of FAL1 can promote the proliferation and cell cycle of GC by inhibiting PTEN. Moreover, the serum level of FAL1 may serve as a novel marker for the progress of GC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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