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Circ_001680 aggravates the malignant process of gastric carcinoma by targeting MAP2

H.-D. ZHAO[1], Y. MA[2], X.-S. WU[1], J.-J. CHENG[1], Z.-G. XIA[3], C. KAN[4], X.-J. DING[5], W.-D. CHEN[1], L.-Z. ZHANG[1], X.-Y. WANG[1], Y. CAI[6], S.-Y. WANG[4]

[1]Department of Emergency Surgery, The First Affiliated Hospital of Anhui Medical University, Hefei, China
[2]Department of Radiology, The First Affiliated Hospital of Anhui Medical University, Hefei, China
[3]Department of Wound Repair, Plastic and Aesthetic Surgery, The Fourth Affiliated Hospital of Anhui Medical University, Hefei, China
[4]Department of Pathophysiology, Anhui Medical University, Hefei, China
[5]Department of Microbiology, Anhui Medical University, Hefei, China
[6]Department of Gastroenterology, The First Affiliated Hospital of Anhui Medical University, Hefei, China

Haodong Zhao, Yang Ma and Xuesheng Wu contributed equally to this work

Abstract. – OBJECTIVE: This study aims to explore the expression pattern and clinical significance of circ_001680 in gastric carcinoma (GC) process.

PATIENTS AND METHODS: Circ_001680 levels in 40 pairs of GC and paracancerous ones were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between circ_001680 and GC clinicopathological parameters was analyzed. AGS and SGC-7901 cells were used for constructing circ_001680 knockdown models by shRNA transfection. Proliferative and metastatic abilities in GC cells with circ_001680 knockdown were examined by cell counting kit-8 (CCK-8) and transwell assay, respectively. Dual-Luciferase reporter assay was conducted to clarify the interaction between circ_001680 and MAP2. Their co-regulation on GC process was detected through rescue experiments.

RESULTS: Circ_001680 was highly expressed in GC tissues and cell lines. High level of circ_001680 predicted high incidences of lymphatic and distant metastasis, and poor prognosis in GC patients. Knockdown of circ_001680 suppressed proliferative and metastatic abilities in AGS and SGC-7901 cells. MAP2 was the target gene binding circ_001680, which was lowly expressed in GC. In addition, MAP2 was negatively correlated to circ_001680. Knockdown of MAP2 could abolish the suppressed proliferative and metastatic abilities in GC cells with circ_001680 knockdown.

CONCLUSIONS: Circ_001680 is highly expressed in GC tissues and closely related to metastasis and prognosis in GC patients, which promotes the proliferative and metastatic abilities in GC cells by negatively interacting with MAP2.

Key Words:
Circ_001680, MAP2, Gastric carcinoma.

Introduction

Gastric carcinoma (GC) is a malignant tumor derived from gastric mucosal epithelial cells and glandular epithelial cells. As the most common tumor in the gastrointestinal tract, GC is featured by strong invasiveness and poor prognosis[1-3]. In recent years, the incidence and mortality of GC have been declined due to the effective control of GC-associated risk factors[1,2,4]. Early stage screening of GC has been popularized in China. However, there are still many people suffering from GC because of aging population and deficiency of sensitive tumor biomarkers[5-8]. Novel biomarkers for GC are urgently required, which can be utilized for developing diagnostic markers and targeted drugs[8-10].

CircRNAs are widely expressed in the human body, which is capable of regulating RNAs through multiple mechanisms[11,12]. Their biological functions are used to be ignored in the past decades[13,14]. Thanks to the rapid processes made

Corresponding Authors: Haodong Zhao, Ph.D; e-mail: 18956045499@126.com
Siying Wang, MD; e-mail: sywang@ahmu.edu.cn

on RNA sequencing and bioinformatic analyses, circRNAs have been identified for their vital functions involved in biological activities. Unlike traditional linear splicing, circRNAs are formed by reverse splicing or gene rearrangement[14,15]. Growing evidence has proven the close relationship between circRNAs and human diseases, and they present certain diagnostic and therapeutic values in cancers[16-18]. Liu et al[19] reported that circ_001680 exerts an oncogenic role in colorectal carcinoma. Its potential influence on GC process remains largely unclear.

Through bioinformatic analysis, MAP2 was predicted to be a target gene of circ_001680. This study aims to explore the co-regulation of circ_001680 and MAP2 on the malignant phenotypes of GC.

Patients and Methods

GC Samples

A total of 40 GC patients with surgical resection were retrospectively analyzed. Their cancer tissues and adjacent ones were collected, labeled and stored at -80°C. Recruited patients did not have preoperative chemotherapy or radiotherapy. Tumor node metastasis (TNM) staging of GC was defined by Union for International Cancer Control (UICC) criteria. Inclusion criteria: patients with no severe diseases in other organs and those undergoing no post-operative radiotherapy. Exclusion criteria: patients with distant metastasis or lung metastasis of tumors, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, or those previously exposed to radioactive rays. This investigation was approved by the research Ethics Committee of The First Affiliated Hospital of Anhui Medical University and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell Lines and Reagents

GC cell lines (AGS, BGC-823, SGC-7901, MKN28 and MKN45) and epithelial cells of gastric mucosa (GES-1) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA) at 37°C with 5% CO_2. Medium was replaced every 2-3 days. Cell passage was conducted at 90% confluence using trypsin, and those in the logarithmic growth phase were collected for experiments.

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-60% density in a 6-well plate, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At 24, 48, 72 and 96 h, optical density at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Assay

Cell suspension was prepared at 5×10^5 cells/mL. 200 μL of suspension and 700 μL of medium containing 20% FBS was respectively added on the top and bottom of a transwell insert, and cultured for 48 h. Migratory cells on the bottom were induced with methanol for 15 min, 0.2% crystal violet for 20 min and captured using a microscope. Five random fields per sample were selected for capturing and counting cells. Invasion assay was similarly conducted in a transwell insert pre-coated with Matrigel.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Shiga, Japan), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. circ_001680: forward: 5'-CATGGACATGATCTTCTTTATAA-3'; reverse: 5'-CATGGACATGATCTTCTTTATAA-3'; MAP2: forward: 5'-CAGTTTCTGCGCCCAGATTT-3', reverse: 5'-CCCAATCAATGCTTCCTCGG-3'; GAPDH: forward: 5'-GGACCTGACCTGCCGTCTAG-3', reverse: 5'-GTAGCCCAGGATGCCCTTGA-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice for 15 min, and the mixture was centrifuged at 14000 × g, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples with the adjusted same concentration were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-Luciferase Reporter Assay

HEK293T cells were seeded in a 24-well plate and were co-transfected with pcDNA-MAP2/pcDNA-NC and circ_001680-WT/circ_001680-MUT, respectively. Luciferase activity (Promega, Madison, WI, USA) was measured at 48 h in a standard method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the *t*-test. The relationship between circ_001680 and GC pathology was analyzed by Chi-square test. Kaplan-Meier survival curves were depicted, followed by log-rank test for comparing differences between curves. $p<0.05$ was considered as statistically significant.

Results

Expression Pattern and Clinical Significance of Circ_001680 in GC

Compared with paracancerous tissues, circ_001680 was upregulated in GC tissues (Figure 1A, 1B). Besides, circ_001680 was highly expressed in GC cell lines as well (Figure 1C). Clinical data of recruited GC patients were analyzed by Chi-square test. It is shown that circ_001680 level was positively linked to the incidences of lymphatic metastasis and distant metastasis in GC (Table I). Furthermore, Kaplan-Meier survival curves revealed that high level of circ_001680 predicted poor survival in GC patients (Figure 1D). It is indicated that circ_001680 was an oncogene involved in GC process.

Knockdown of Circ_001680 Inhibited Proliferative and Metastatic Abilities in GC

AGS and SGC-7901 cells were used for constructing circ_001680 knockdown models. Transfection of either sh-circ_001680-1, sh-circ_001680-2 or sh-circ_001680-3 effectively downregulated circ_001680 in GC cells (Figure 2A). Among the

Table I. Association of circ_001680 expression with clinicopathologic characteristics of gastric cancer.

Parameters	No. of cases	circ_001680		*p*-value
		Low (%)	High (%)	
Age (years)				0.775
<60	19	10	9	
≥60	21	12	9	
Gender	1.000			
Male	20	11	9	
Female	20	11	9	
HP behavior				0.412
Negative	15	7	8	
Positive	25	15	10	
T stage				0.324
T1-T2	19	12	7	
T3-T4	21	10	11	
Lymph node metastasis				0.033
No	25	17	8	
Yes	15	5	10	
Distance metastasis				0.033
No	27	18	9	
Yes	13	4	9	

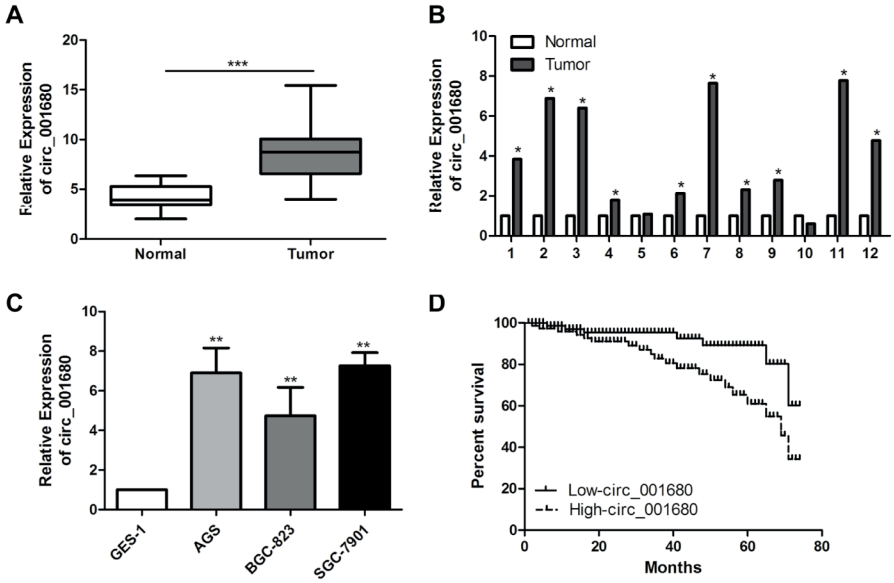

Figure 1. Expression pattern and clinical significance of circ_001680 in GC. **A-B,** Differential levels of circ_001680 in GC and paracancerous tissues; **C,** Circ_001680 levels in GC cell lines; **D,** Overall survival in GC patients expressing high or low level of circ_001680. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

three circ_001680 shRNAs, sh-circ_001680-1 had the best efficacy and it was used in the following experiments. CCK-8 assay showed that knockdown of circ_001680 reduced viability in AGS and SGC-7901 cells, suggesting the attenuated proliferative ability (Figure 2B). Moreover, both migratory and invasive cell numbers were declined by knockdown of circ_001680 in GC cells, indicating the suppressed metastatic ability (Figure 2C).

Circ_001680 Was Bound to MAP2

MAP2 was predicted as a target of circ_001680 using online bioinformatic tools. As shown in Figure 3A, circ_001680 was able to bind MAP2 through the predicted binding sites. Compared with normal tissues, MAP2 was downregulated in GC tissues, and negatively correlated to circ_001680 level (Figure 3B, 3C). Consistently, MAP2 was downregulated in GC cell lines (Figure 3D). To further clarify the involvement of MAP2 in GC process, si-MAP2 was constructed and we tested its efficacy in AGS and SGC-7901 cells (Figure 3E). In AGS and SGC-7901 cells transfected with sh-circ_001680, protein level of MAP2 was remarkably upregulated (Figure 3F).

Co-Regulation of Circ_001680 and MAP2 on GC

Knockdown of circ_001680 inhibited MAP2 level in GC cells, which was reversed by co-silence of circ_001680 and MAP2 (Figure 4A). Of note, the attenuated proliferative and metastatic abilities in AGS and SGC-7901 cells with circ_001680 knockdown were partially reversed by co-knockdown of circ_001680 and MAP2 (Figure 4B, 4C). It is concluded that circ_001680 aggravated the malignant process of GC by targeting MAP2.

Discussion

GC is the third lethal cancer in the world. In China, the incidence of GC ranks the first place in the digestive system tumors. Seriously, the diagnostic rate of early stage GC is as low as 5-15%[1,3,4]. It is of great significance to improve diagnostic and therapeutic efficacies of GC[5-8]. Very recently, accumulating numbers of circRNAs have been discovered and the establishment of circRNA database provides a convenient platform for deepening the research on circRNAs[13-18]. It is reported that circRNAs participate in cardiovascular diseases, neuronal diseases, tumors and other pathological processes[19,20].

Jian et al[20] suggested that circ_001680 affected the proliferation and migration of colorectal cancer and mediated its chemoresistance by regulating BMI1 through miR-340. However, its biological functions in GC are unclear. Our findings showed that circ_001680 was upregulated in GC

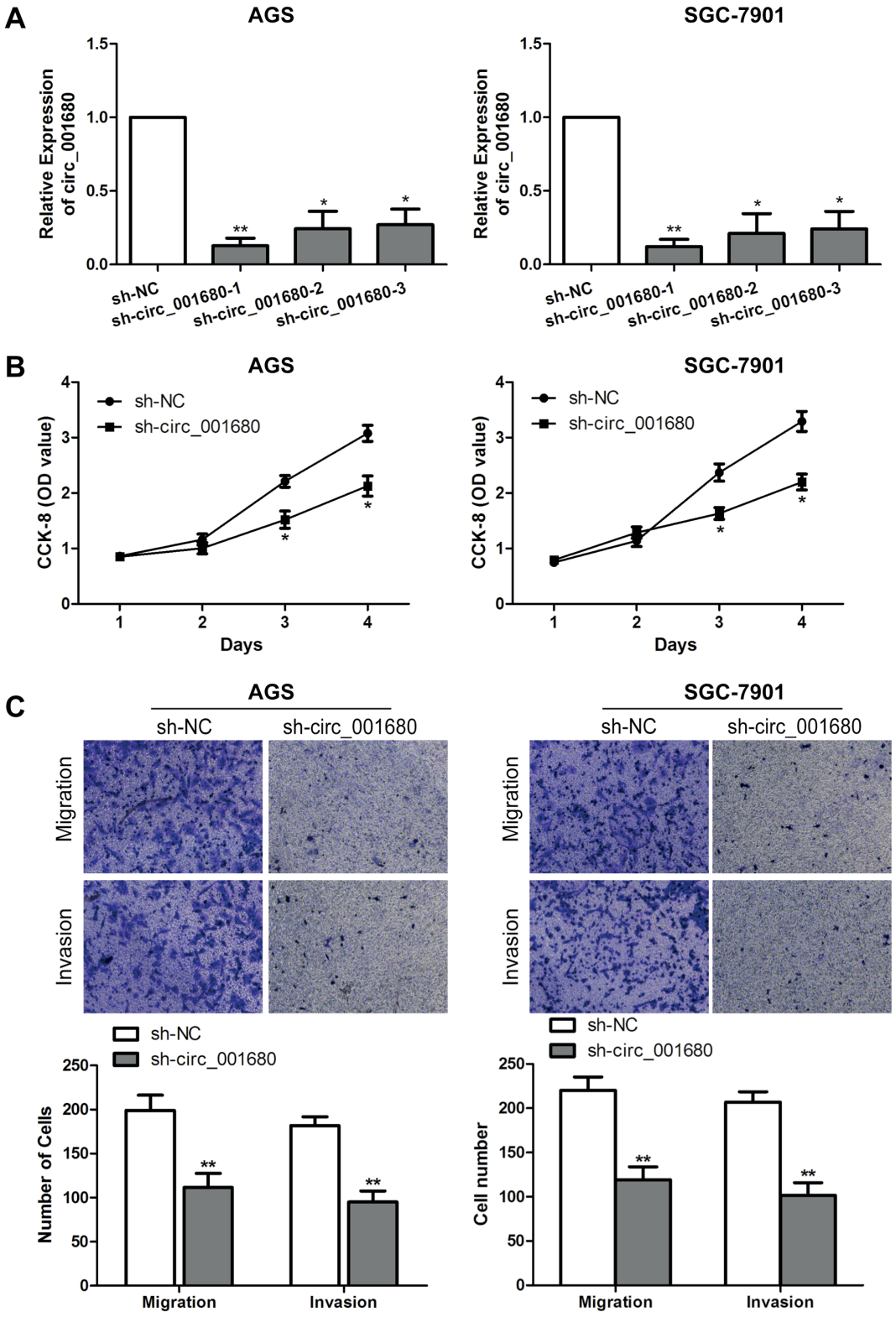

Figure 2. Knockdown of circ_001680 inhibited proliferative and metastatic abilities in GC. **A,** Transfection efficacy of three circ_001680 shRNAs in AGS and SGC-7901 cells; **B,** Viability in AGS and SGC-7901 cells with circ_001680 knockdown; **C,** Migration and invasion in AGS and SGC-7901 cells with circ_001680 knockown (magnification 40×). *$p < 0.05$, **$p < 0.01$.

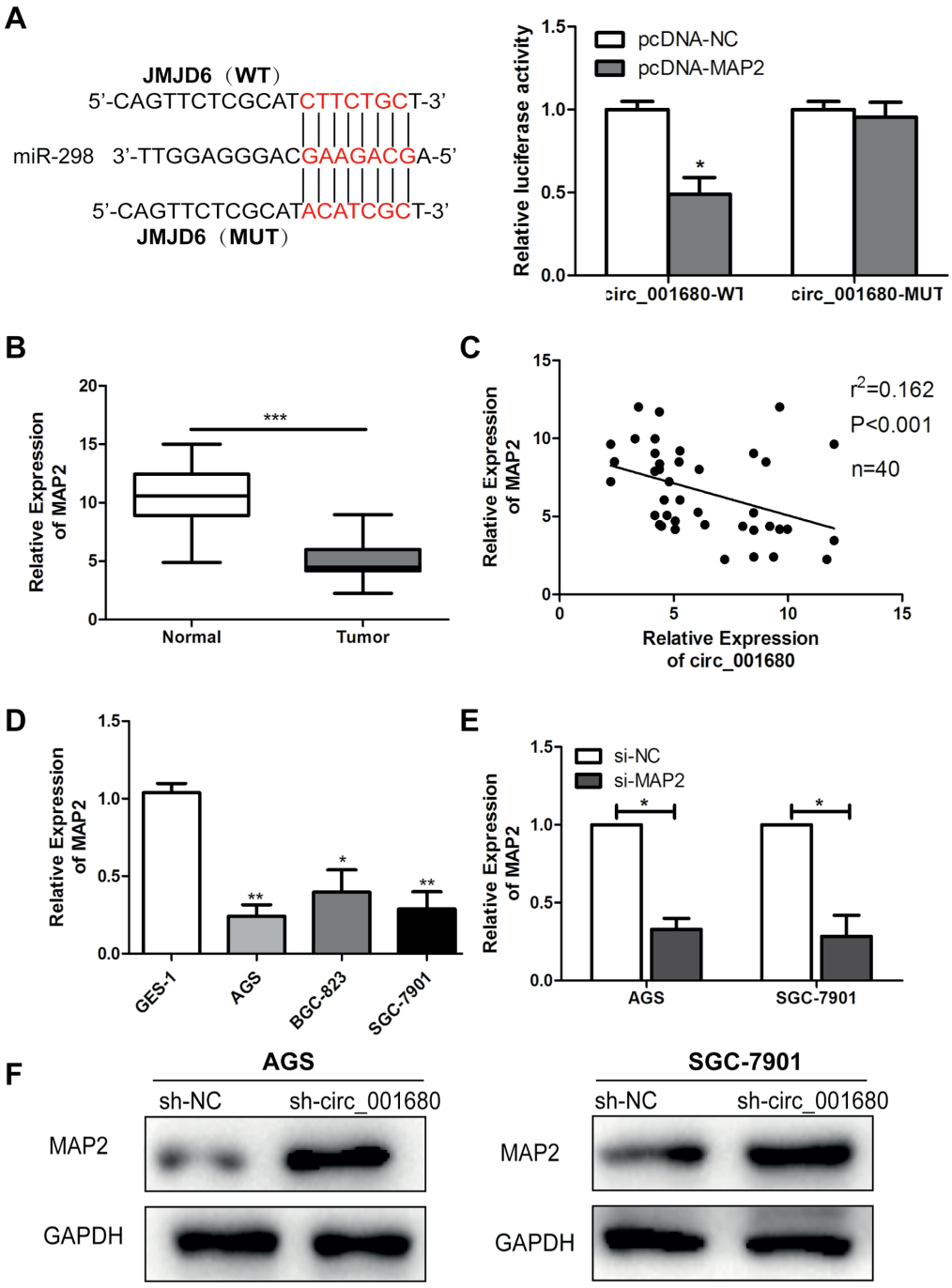

Figure 3. Circ_001680 was bound to MAP2. **A,** Binding relationship between circ_001680 and MAP2; **B,** MAP2 levels in GC and paracancerous tissues; **C,** A negative correlation between circ_001680 and MAP2; **D,** MAP2 levels in GC cell lines; **E,** Transfection efficacy of si-MAP2 in AGS and SGC-7901 cells; **F,** Protein level of MAP2 in AGS and SGC-7901 cells with circ_001680 knockdown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

samples. GC patients overexpressing circ_001680 had higher risks of lymphatic metastasis, distant metastasis and poor prognosis. Thus, we speculated that circ_001680 may serve as an oncogene in GC process. Subsequently, circ_001680 knockdown model was constructed by transfection of sh-circ_001680. Knockdown of circ_001680 remarkably suppressed viability, migratory and invasive cell numbers in AGS and SGC-7901 cells.

CircRNAs are able to post-transcriptionally regulate gene expressions by competitively binding mRNAs, and it is well known as the ceRNA

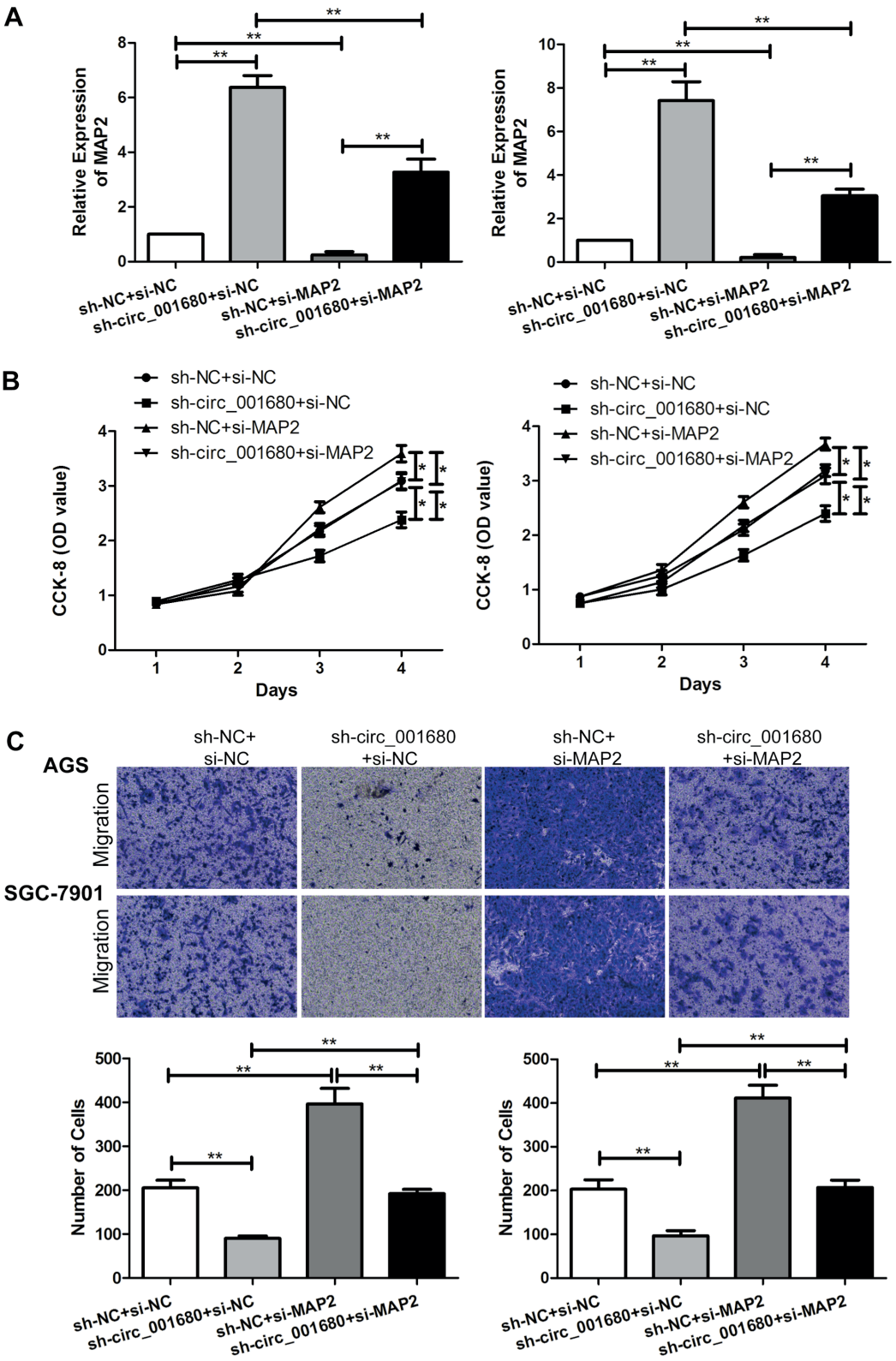

Figure 4. Co-regulation of circ_001680 and MAP2 on GC. **A,** MAP2 levels in AGS and SGC-7901 cells regulated by circ_001680 and MAP2; **B,** Viability in AGS and SGC-7901 cells regulated by circ_001680 and MAP2; **C,** Migration in AGS and SGC-7901 cells regulated by circ_001680 and MAP2 (magnification 40×). *$p < 0.05$, **$p < 0.01$

theory[14-17]. Here, we have proven that MAP2 was the target gene binding circ_001680. MAP2 was detected to be downregulated in GC tissues and cell lines. A negative correlation was identified between circ_001680 and MAP2. Notably, knockdown of MAP2 could abolish the suppressed proliferative and metastatic abilities in GC cells with circ_001680 knockdown. To sum up, as a novel oncogenic gene, circ_001680 could aggravate the malignant development of GC. In addition, a negative feedback loop circ_001680-MAP2 axis has been identified, which became a promising biomarker for diagnosis and treatment for GC.

Conclusions

It has been demonstrated that circ_001680 is highly expressed in GC tissues and closely related to metastasis and prognosis in GC patients. It promotes proliferative and metastatic abilities in GC cells by negatively interacting with MAP2.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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