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Original Article

EIF4A3 induced circABCA5 promotes the gastric cancer progression by SPI1 mediated IL6/JAK2/STAT3 signaling

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Abstract: Gastric cancer is one of the most common malignancies of the digestive system with high mortality rates. Recent studies have demonstrated that circRNAs are novel noncoding RNAs that play vital roles in the tumorigenesis and development of gastric cancer. Our study found a novel circRNA, namely, hsa_circ_0107595 (also called circABCA5), that is overexpressed in gastric cancer based on circRNA sequencing. qPCR demonstrated its overexpression in gastric cancer specimens. The overexpression or knockdown of circABCA5 in gastric cancer cell lines was achieved by lentiviral-mediated transfection. All MTS, EdU, Transwell and migration assays and xenograft experiments demonstrated that circABCA5 could promote gastric cancer proliferation, invasion, and migration in vitro and in vivo. Mechanistically, both RIP and RNA pulldown assays confirmed that circABCA5 could bind to the SPI1 protein, upregulate SPI1 expression, and promote its nuclear translocation. SPI1 could further promote the malignant phenotype of gastric cancer by activating IL6/JAK2/STAT3 signaling. In addition, EIF4A3 could directly bind to circABCA5, promoting its stability and expression. Our study reveals that circABCA5 plays a vital role in the diagnosis and prognosis of gastric cancer and may even be developed as a molecular target for the treatment of gastric cancer.

Keywords: Gastric cancer, circABCA5, SPI1, IL6, progression

Introduction

Gastric cancer is one of the most common malignancies of the digestive system, and it has the highest incidence rate in humans. According to the most recent epidemiological statistics in 2018, the incidence rate of gastric cancer ranks fifth among the major malignancies in the world (5.7%), and its mortality rate is as high as 8.3%, ranking third among the major malignancies [1, 2]. Although the five-year survival rate of patients with early stage gastric cancer is as high as 90.9%-100%, the five-year survival rate of most patients with gastric cancer is still less than 30%, even if they receive active and standardized surgery combined with radiotherapy and chemotherapy. Most patients with early stage gastric cancer have atypical or no symptoms, most are diagnosed in the middle and late stages of disease [3, 4]. Since gastric cancer is a highly invasive malignant tumor, it is difficult to eradicate gastric cancer with conventional radical gastrectomy. Distant invasion and metastasis occur during treatment, which leads to the inevitable recurrence of gastric cancer in most patients [5]. Therefore, how to effectively intervene and block the invasion and metastasis of gastric cancer is an important factor in improving the treatment and prognosis of patients with gastric cancer.

Circular RNAs (circRNAs) are a unique kind of single-stranded RNA with a closed circular structure, which is mainly derived from the reverse shearing of mRNA precursors [6]. Compared with traditional linear RNA, the circular structure is more stable, and its relative sealing degree in various cells is significantly higher [7]. Research on circRNAs has notably increased in recent years. Many studies have

confirmed that circRNAs are involved in many tumor biological processes, such as tumorigenesis, tumor cell proliferation, invasion, and apoptosis. For example, hsa_circ_0005230 is upregulated in gastric cancer and promotes cell invasion and migration by regulating the miR-1299/RHOT1 axis [8]. Hsa_circ_0008434 promotes gastric cancer growth, migration and invasion via miR-6838-5p/USP9X [9]. However, due to the substantial volume of circRNAs, there are still many circRNAs with unknown functions in gastric cancer and other tumors, which need to be further studied. The identification of circRNAs that participate in the occurrence and development of gastric cancer will help to further understand the molecular mechanism underlying gastric cancer and provide new targets for molecular targeted therapy.

SPI1 is a member of the ETS transcription factor family and was initially found to play an essential role in the differentiation and development of myeloid and B lymphocytes [10]. SPI1 can promote lung cancer cell proliferation, invasion, and migration by regulating lncRNA SNHG6 [11]. In cervical cancer, lncRNA SNHG6 can also regulate PARP9 by inducing SPI1 transcription and promoting the proliferation and tumorigenesis of cervical cancer cells [12]. In gastric cancer, it was reported that lncRNA NR2F1-AS1 plays a carcinogenic role by recruiting the transcription factor SPI1 to upregulate ST8SIA1 expression [13]. However, there is no research report on the regulation of circRNAs and SPI1 in gastric cancer.

IL6 is a kind of cytokine that participates in inflammation and the immune system [14]. A large number of studies have found that inflammation is involved in the occurrence and development of tumors, and IL6 was found to be involved in the occurrence and development of multiple malignant tumors. In gastric cancer, RBMS1 promotes metastasis through autocrine IL6/JAK2/STAT3 signaling [14]. In glioma, IL6 can promote the proliferation of glioma stem cells via the JAK2/STAT3 signaling pathway [15].

In this study, we first found a novel circRNA hsa_circ_0107595 (also called circABCA5), which was overexpressed in gastric cancer according to circRNA sequencing and qPCR analysis of specimens. We further discovered that circABCA5 could promote gastric cancer proliferation, invasion, and migration via SPI1 and IL6/JAK2/STAT3 signaling in vitro and in vivo. In addition, EIF4A3 could directly bind to circABCA5, promoting its stability and expression. Therefore, circABCA5 plays an important role in the diagnosis and prognosis of gastric cancer and may even be developed into a molecular target for the treatment of gastric cancer.

Materials and methods

Clinical specimens and ethical statement

A total of 60 cases of gastric cancer tissues and the adjacent normal tissues were collected in the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University from 2015 to 2016. All patients received resection first time without previous chemotherapy or radiotherapy. All surgically resected specimens were stored immediately at -80°C until RNA extraction. Informed consent was obtained from each patient before enrolling them in the study. This study was approved by the Ethics Committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University.

Cells culture

The human gastric cancer cell lines MKN-45 and GES-1 were purchased from the Chinese Academy of Sciences cell bank (Shanghai, China) and validated by STR (short tandem report) sequencing. All cell lines were maintained in Roswell Park Memorial Institute 1640 culture medium (RPMI-1640, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco) at 37°C with 5% CO_2. IL-6-neutralizing antibody (R&D Systems, Minneapolis, MN, USA) was used to block the biological function of IL6 at a concentration of 0.5 mg/ml.

Total RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from gastric cancer tissues and cells was extracted using the Mini-BEST Universal RNA Extraction kit (TaKaRa, Kyoto, Japan). The

qRT-PCR assays were detected using TIANGEN Master Mix SYBR Green RT-PCR SuperMix UDG reagents with PCR LightCycler480 (Roche Diagnostics, Basel, Switzerland). Moreover, RNase R assay was used to eliminate the effect of linear RNAs and confirm the circular structure of circRNAs. Its treatment concentration is 10 μg total RNA incubated with 40 U RNase R (Epicentre Technologies, Madison, WI, USA) for 15 min at 37°C. The β-actin was used as an endogenous control. Primers used in this study are listed as follows: circABCA5: F': 5'-GCTGTGGTTCCCATCAAACT-3'; R': 5'-TGACTGTCTAGGGCAGAAAACA-3'. SPI1: F': 5'-GTGCCCTATGACACGGATCTA-3', R': 5'-AGTCCCAGTAATGGTCGCTAT-3'. IL6: F': 5'-ACTCACCTCTTCAGAACGAATTG-3', R': 5'-CCATCTTTGGAAGGTTCAGGTTG-3'. β-actin: F': 5'-CATGTACGTTGCTATCCAGGC-3', R': 5'-CTCCTTAATGTCACGCACGAT-3'.

Lentiviral vector construction and transfection

The lentiviral vector for RNAi or overexpression of circABCA5 and SPI1 was generated by Gene-Chem (Shanghai, China). Two siRNA sequences were designed for circABCA5 silencing: forward 5'-UUGUUUUGUAUUUAUGUGGUU-3', reverse 5'-CCACAUAAAUACAAAACAAGU-3' and forward 5'-AAAAAUACGUCUUCCAAAGUC-3', reverse 5'-CUUUGGAAGACGUAUUUUUAA-3'. The siRNA sequences for SPI1 silencing were: forward 5'-UAUAGAUCCGUGUCAUAGGGC-3', reverse 5'-CCUAUGACACGGAUCUAUACC-3' and forward 5'-UGAGAUAGGGGUAAUACUCGU-3', reverse 5'-GAGUAUUACCCCUAUCUCAGC-3'. The transfected cells were furtherly selected by puromycin (Sigma, Santa Clara, CA, USA) at a 10 μg/ml concentration for 15 days. The transfection effectiveness of silencing and overexpression were validated by qPCR and western blotting.

Western blotting

The total protein of cell lines was lysed in RIPA lysis buffer (RIPA, Beyotime, Beijing, China). The nuclear and cytoplasmic protein was isolated using a NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The proteins were quantified by bicinchoninic acid analysis (Beyotime). Equal amounts of protein were separated by 4 to 20% SDS-PAGE (Genscript, Nanjing, China) and transferred to a polyvinylidene fluorid (PVDF) membrane (Millipore, Darmstadt, Germany). Then the membranes were blocked with 2% bovine serum albumin (KeyGen Biotechnology) and incubated with primary antibody at 4°C overnight. After secondary antibody (ProteinTech, Chicago, Illinois, USA) incubation, the Chemiluminescence ECL kit (Beyotime) was used to detect the bands, ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for quantifying.

MTS proliferation assay

The gastric cancer cell lines were plated in 96-well plates at a density of 1,000 cells/well for 0, 24, 48, 72, 96, and 120 hours. The cell viability was determined using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay Kit (MTS, Promega, Madison, WI, USA). The MTS reagent was added to each well for 3 h at 37°C. The absorbance at 495 nm was detected using an ultraviolet spectrophotometer (ThermoFisher, Waltham, MA, USA).

EdU assay

According to the manufacturer's instructions, the proliferation of gastric cancer cell lines was detected using an EdU assay kit (Beyotime). Briefly, 1×10^5 cells were seeded into 24-well plates for 24 h. Then 10 μM reagent was added to each well at 37°C for 2 h. The laser scanning confocal microscope (Olympus) was used to calculate the percentage of positive cells.

Transwell assay and migration assay

For the Transwell assay, the polycarbonate membrane of the upper Transwell chamber (pore size diameter 8 μm, Corning, Corning, NY, USA) was treated with a Matrigel filter (BD Biosciences, San Jose, CA, USA) for 30 min incubation at 37°C. Then 3×10^4 cells were suspended in the upper chamber, and the medium with 20% fetal bovine serum was added to the lower chamber. After 24 h incubation, the invaded cells were fixed with 4% paraformaldehyde and stained with crystal violet (Beyotime, Biotechnology). The invaded cell numbers were counted under the light microscope (Olympus). For the migration assay, the upper Transwell chamber was not pretreated

with a Matrigel filter, and the other steps were the same as the Transwell assay.

Immunofluorescence

The gastric cancer cells under different conditions were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 and 5% bovine serum albumin to block the antigens. Then the cells were incubated with primary antibodies against SPI1 (1:100; Abcam) at 4°C overnight, followed by treatment of fluorescein isothiocyanate-conjugated secondary antibodies (ProteinTech). The nucleus was counterstained using DAPI (Sigma-Aldrich). Finally, the gastric cells were visualized via a laser scanning confocal microscope (Olympus).

RNA immunoprecipitation (RIP) assay

The RIP assay was performed using the EZ-magna RIP RNA-binding Protein Immunoprecipitation Kit (Millipore, Darmstadt, Germany) according to the manufacturer's suggestions. The gastric cancer cells were lysed in RIP buffer followed by proteinase K treatment. Then the complexes were incubated with magnetic beads conjugated with anti-SPI1 antibodies or negative control IgG. The immunoprecipitated RNAs were isolated, washed, and purified, and qRT-PCR was used to examine the precipitants.

RNA pulldown assay

The RNA pulldown assay was performed using the Pierce Magnetic RNA Protein pulldown Kit (Thermo Fisher Scientific), according to the manufacturer's suggestions. The circABCA5 mutant probe were designed via CatRAPID dataset to found the candidated circABCA5-SPI1 protein binding sites. The most likely circABCA5 binding sites are 201-252 bp and 192-243 bp. Therefore, we mutate circABCA5 mainly in its sequences from 192 to 252 bp. Briefly, the RNA probes (positive control (input), negative control (antisense RNA), and biotinylated circABCA5) were co-incubated with gastric cancer cells' proteins at room temperature. Then the magnetic beads were added to form a probe-magnetic bead complex. The complexes were immunoprecipitated, washed, purified and finally detected by western blotting, using β-actin as a control.

Enzyme-linked immunosorbent (ELISA)

According to the manufacturer's suggestions, ELISA assays were performed using commercially available ELISA kits (Cusabio, Stratech, Suffolk, UK). The media supernatant of the gastric cells was collected, followed by reagents treatment, and the absorbance at 450 nm was detected using an ultraviolet spectrophotometer (ThermoFisher). The concentrations of IL6 were calculated via normalizing to the protein concentration in the control group.

Luciferase reporter assay

Luciferase reporter assays were performed using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The luciferase reporter plasmids (SPI1-wt and SPI1-mt) were constructed using a pGL3 plasmid vector, and the luciferase reporter plasmids were co-transfected into gastric cancer cells for 48 h. The relative luciferase activity was detected and calculated as the ratio of firefly luciferase activity to Renilla luciferase activity.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the ChIP Assay Kit (Beyotime) according to the manufacturer's instructions. Anti-SPI1 or negative control IgG immunoprecipitated the chromatin complexes, followed by DNA purifying. Then the DNA samples were analyzed by qRT-PCR. The primers for ChIP qPCR: IL6: forward 5'-CCTGAACCTTCCAAAGATGGC-3', reverse 5'-TTCACCAGGCAAGTCTCCT CA-3'.

Xenograft experiments

Five-week-old female BALB/c nude mice (Shanghai Laboratory Animal Center) were used for the xenograft experiments. All animal experiments were performed in accordance with the Animal Care Committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. The mice were bred in the Laboratory Animal Center of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. The mice were treated under a 12 h light/12 h dark cycle with free access to water and a standard mouse diet. The gastric cancer cell suspensions (2×10^6

cells/200 ml) were injected into the back flanks of the nude mouse. A Vernier caliper detected the tumor size, and the tumor volume was calculated using the following formula: V = (D × d)/2 mm, where D is the longest diameter and d is the shortest diameter of the tumor. After 5 weeks after implantation, all the mice were sacrificed, and the tumors were weighed and photographed.

Immunohistochemical (IHC) staining

According to the manufacturer's instructions, IHC was performed using an immunohistochemistry kit (Beyotime). Briefly, the paraffin-embedded tumor specimens were cut into 4-mm sections, endogenous peroxidase was removed, and the antigen was repaired. After being blocked by 10% normal goat serum, the primary antibodies against Ki-67, SPI1 and IL6 (Abcam) were incubated at 4°C overnight, followed by secondary antibodies (Abcam). The sections were finally treated with 3,3'-diaminobenzidine (Sigma), counterstained with hematoxylin and photographed with a light microscope (Olympus). The results were semi-quantified via the German immunohistochemical score [16].

Bioinformatics analysis

The RNA-protein binding between circABCA5 and SPI1 protein was predicted by CatRAPID (http://service.tartaglialab.com/page/catrapid_group). The data on mRNA expression and the clinical material of gastric patients were obtained from the Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov). Gene set enrichment analysis (GSEA, http://www.broadinstitute.org/gsea/index.jsp) was used to detect the significant enrichment of signalings between higher and lower SPI1 expression groups.

Statistical analysis

The statistical analysis was used via SPSS 23.0 software (IBM, Armonk, NY, USA) and GraphPad Prism 9.0 (GraphPad Software Inc., San Diego, CA, USA). All experiments were repeated more than 3 times, and the results were expressed as mean ± standard error. The chi-square test or t-test was used to compare between different groups relatively. The survival status of patients was detected by Kaplan-Meier analysis was used to analyze. Statistical significance is defined as a two-tailed *P*-value < 0.05.

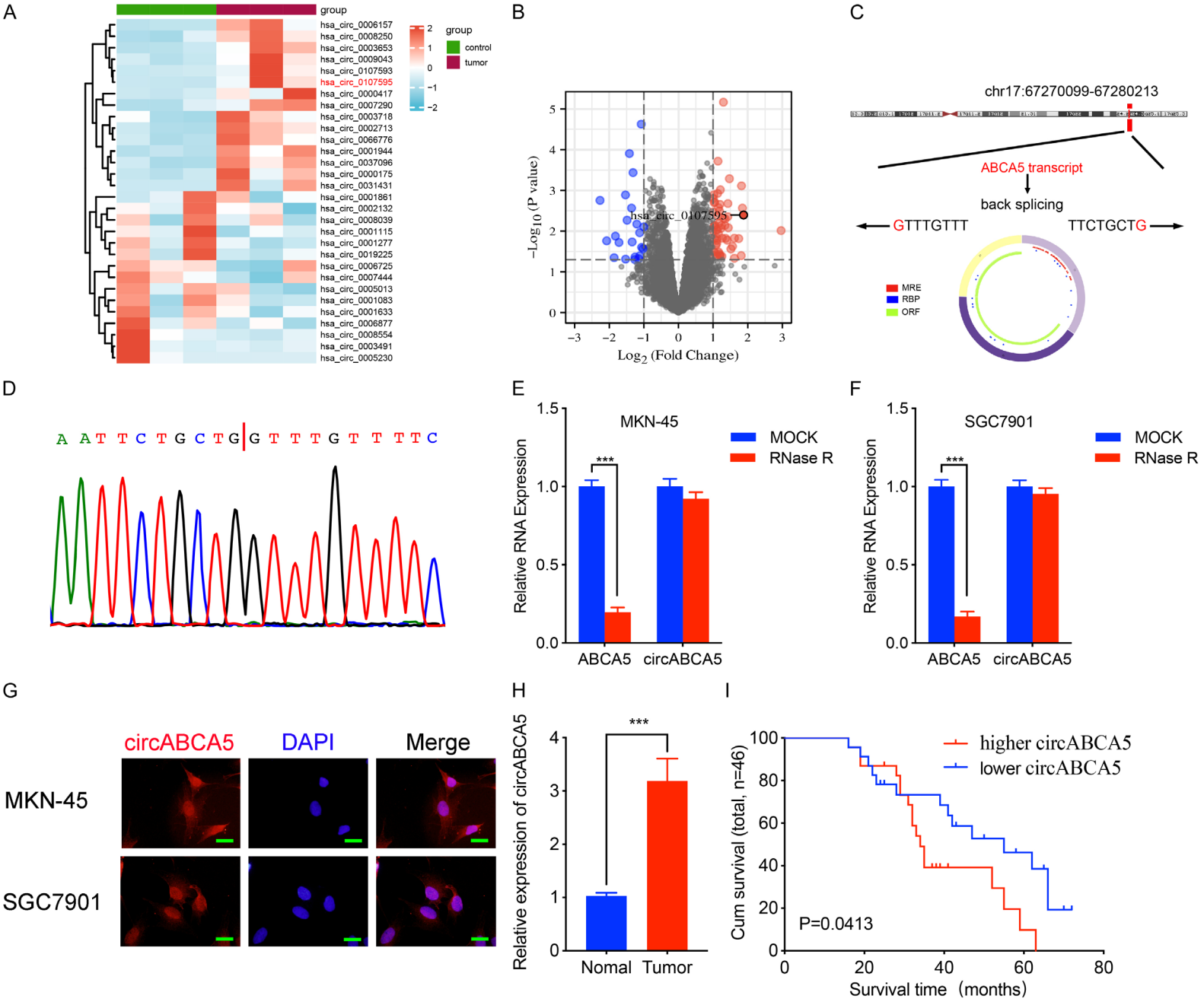
Results

CircABCA5 is upregulated in gastric cancer tissues and correlated with progression and poor prognosis

To identify circRNAs with significant expression in gastric cancer, we analyzed the Gene Expression Omnibus (GEO) dataset GSE121445 via limma analysis. We found that a novel circRNA, hsa_circ_0107595 (circABCA5), was the fourth most overexpressed circRNA in gastric cancer tissues compared with normal tissues (**Figure 1A**, **1B**). A diagram of its structure is shown in **Figure 1C**. CircABCA5 is located on chr17:67270099-67280213 and is composed of 21, 22 and 23 exons of ABCA5 mRNA transcript 1 (**Figure 1C**). The specific junction between the head and tail of circABCA5 was verified by Sanger sequencing (**Figure 1D**). RNase R was used to remove the linear mRNA of ABCA5, and the results showed that circABCA5 was resistant to RNase R treatment, without obvious changes, in MKN-45 and SGC7901 cells (**Figure 1E**, **1F**). FISH assays were used to determine the subcellular location of circABCA5 in gastric cancer cells, and the results showed that circABCA5 was mainly located in the nucleus and cytoplasm (**Figure 1G**). Moreover, we further measured circABCA5 expression in our 60 gastric cancer tissues and adjacent normal tissues. The results confirmed its overexpression in gastric cancer (**Figure 1H**). Subsequently, Kaplan-Meier survival analysis also showed that the average survival time of patients with higher circABCA5 expression was significantly shorter than that of patients with lower expression among the 60 gastric cancer patients (qPCR quantification, cutoff: median, **Figure 1I**).

CircABCA5 promoted the malignant phenotype of gastric cancer in vitro

To study the biological role of circABCA5 in gastric cancer cells, we constructed gastric cell lines with stable circABCA5 overexpression or knockdown and verified its effect by qPCR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tumor
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Log2 (Fold Change)
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ABCA5 transcript
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TTCTGCTG
MRE
RBP
ORF
D
A A T T C T G C T G G T T T G T T T C
E
MKN-45
MOCK
RNase R
Relative RNA Expression
***
ABCA5
circABCA5
F
SGC7901
MOCK
RNase R
Relative RNA Expression
***
ABCA5
circABCA5
G
circABCA5
DAPI
Merge
MKN-45
SGC7901
H
Relative expression of circABCA5
***
Nomal
Tumor
I
higher circABCA5
lower circABCA5
Cum survival (total, n=46)
P=0.0413
Survival time (months)

Figure 1. CircABCA5 is up-regulated in gastric cancer tissues and correlated with poor prognosis. A, B: Heatmap and volcano diagram showing the differential expressed circRNAs in gastric cancer tissues and adjacent normal tissues based on GSE121445 via limma analysis. C: Schematic diagram illustrated the circular structure of circABCA5. D: The junction site of circABCA5 was verified by Sanger sequencing. E, F: qPCR showing the RNA levels of ABCA5 or circABCA5 with or without RNase R treatment. G: FISH assays showing the subcellular location of circABCA5 in the gastric cancer cells. Scale bars = 50 μm. H: qPCR showing the expression of circABCA5 in the gastric cancer tissues and the adjacent normal tissues. I: Kaplan-Meier analysis showing the survival of gastric patients between higher or lower circABCA5 group. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(**Figure 2A**, **2B**). MTS assays showed that the absorbance increased significantly after circABCA5 overexpression but decreased after its knockdown (**Figure 2C**, **2D**), indicating that circABCA5 can promote the viability of MKN-45 and SGC7901 cells. EdU assays also showed that the EdU-positive cell rates increased after circABCA5 overexpression but decreased after circABCA5 knockdown (**Figure 2E**, **2F**), indicating that circABCA5 can promote the proliferation of MKN-45 and SGC7901 cells. Furthermore, both Transwell and migration assays showed that the number of invading and migrating cells increased after circABCA5 overexpression but decreased after circABCA5 knockdown (**Figure 2G-J**). The above results demonstrate that circABCA5 could promote the viability, proliferation, invasion and migration of gastric cancer cells in vitro.

CircABCA5 can bind to and upregulate SPI1 expression and promote nuclear translocation of SPI1

At present, there are several kinds of mechanisms by which circRNAs promote cancer, and we mainly focused on the mechanism by which circRNAs directly bind to and regulate target proteins. We analyzed the CatRAPID dataset and found that the SPI1 protein was a candidate target protein of circABCA5 (**Figure 3A**). We performed both RIP and RNA pulldown assays to further study the possible relationship. The RIP assay results showed that anti-SPI1 treatment enriched the expression of circABCA5 more than IgG treatment (**Figure 3B**, **3C**). Moreover, the enrichment of circABCA5 was increased after circABCA5 overexpression and downregulated after circABCA5 knockdown (**Figure 3B**, **3C**). RNA pulldown assays also confirmed that the circABCA5-wt probe could pull down the SPI protein in MKN-45 and SGC7901 cells (**Figure 3D**, **3E**). We also performed both FISH assays and immunofluorescence staining to determine the colocalization of circABCA5 and SPI1 in gastric cancer cells. The results showed that both were colocalized in the nucleus (**Figure 3F**). Then, we measured the mRNA expression of SPI1 via qPCR, and surprisingly, the results were almost unchanged after circABCA5 overexpression or knockdown (**Figure 3G**). However, western blotting showed that SPI1 expression was obviously increased after circABCA5 overexpression and downregulated after circABCA5 knockdown (**Figure 3H**). We further analyzed the nuclear and cytoplasmic fractions of cells to examine the subcellular location of SPI1 via western blotting. The results demonstrated that circABCA5 overexpression upregulated SPI1 expression in the nucleus and downregulated its expression in the cytosol, while the opposite results were observed after circABCA5 knockdown (**Figure 3I**, **3J**). We also studied the distribution of SPI1 in gastric cancer cells via immunofluorescence assays. The results showed that circABCA5 knockdown obviously decreased SPI1 distribution in the nucleus and increased its distribution in the cytosol, while circABCA5 overexpression promoted SPI1 distribution in the nucleus and decreased its distribution in the cytosol (**Figure 3K**). In addition, we also analyzed SPI1 expression in the TCGA gastric cancer dataset. The results showed that SPI1 expression was higher in gastric cancer tissues than in normal tissues (**Figure 3L**, **3M**). Kaplan-Meier survival analysis also showed that the average survival time of patients with higher SPI1 expression was significantly shorter than that of patients with lower expression in TCGA datasets (**Figure 3N**). Together, these results suggested that circABCA5 can bind to the SPI1 protein, maintain its stability and promote its nuclear translocation. SPI1 is also a reasonable downstream target in promoting the malignant phenotype of gastric cancer.

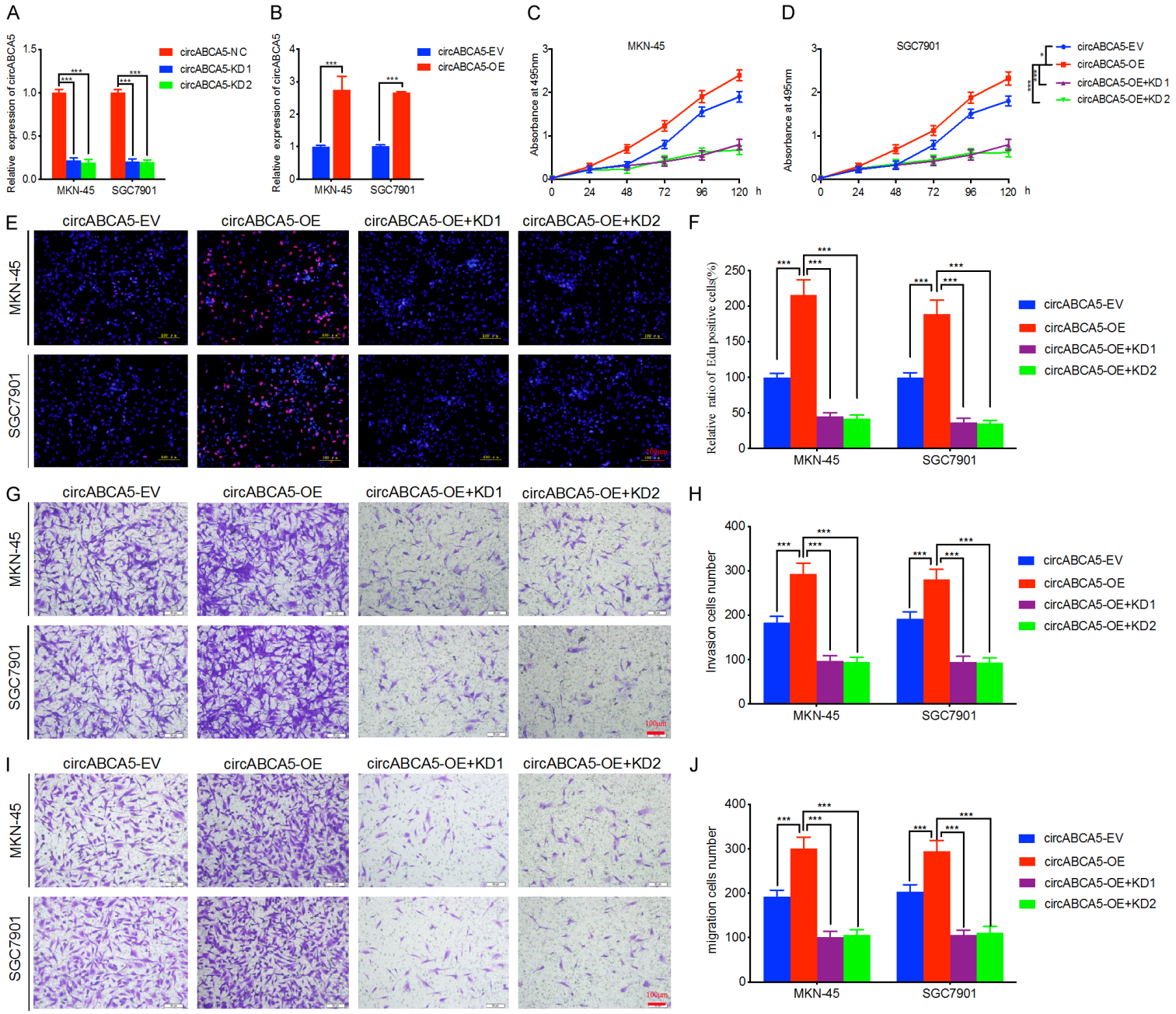

A
Relative expression of circABCA5
circABCA5-NC
circABCA5-KD1
circABCA5-KD2
MKN-45
SGC7901
B
Relative expression of circABCA5
circABCA5-EV
circABCA5-OE
C
MKN-45
Absorbance at 495nm
h
D
SGC7901
circABCA5-EV
circABCA5-OE
circABCA5-OE+KD1
circABCA5-OE+KD2
E
F
Relative ratio of Edu positive cells(%)
G
H
Invasion cells number
I
J
migration cells number

Figure 2. CircABCA5 promoted the malignant phenotype of gastric cancer in vitro. (A, B) qPCR showing the expression of circABCA5 after knockdown (A) or overexpressed (B) in MKN-45 and SGC7901. (C, D) MTS assays show the gastric cancer cells' viability after circABCA5 overexpression or knockdown. (E, F) EdU assays show gastric cancer cell proliferation after circABCA5 overexpression or knockdown. Scale = 100 μm. (G, H) Transwell assays showing the invasion cell numbers after circABCA5 overexpression or knockdown. Scale = 50 μm. (I, J) Migration assays showing the migration cell numbers after circABCA5 overexpression or knockdown. Scale = 50 μm. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

SPI1 knockdown can abolish the circABCA5-induced malignant phenotype of gastric cancer

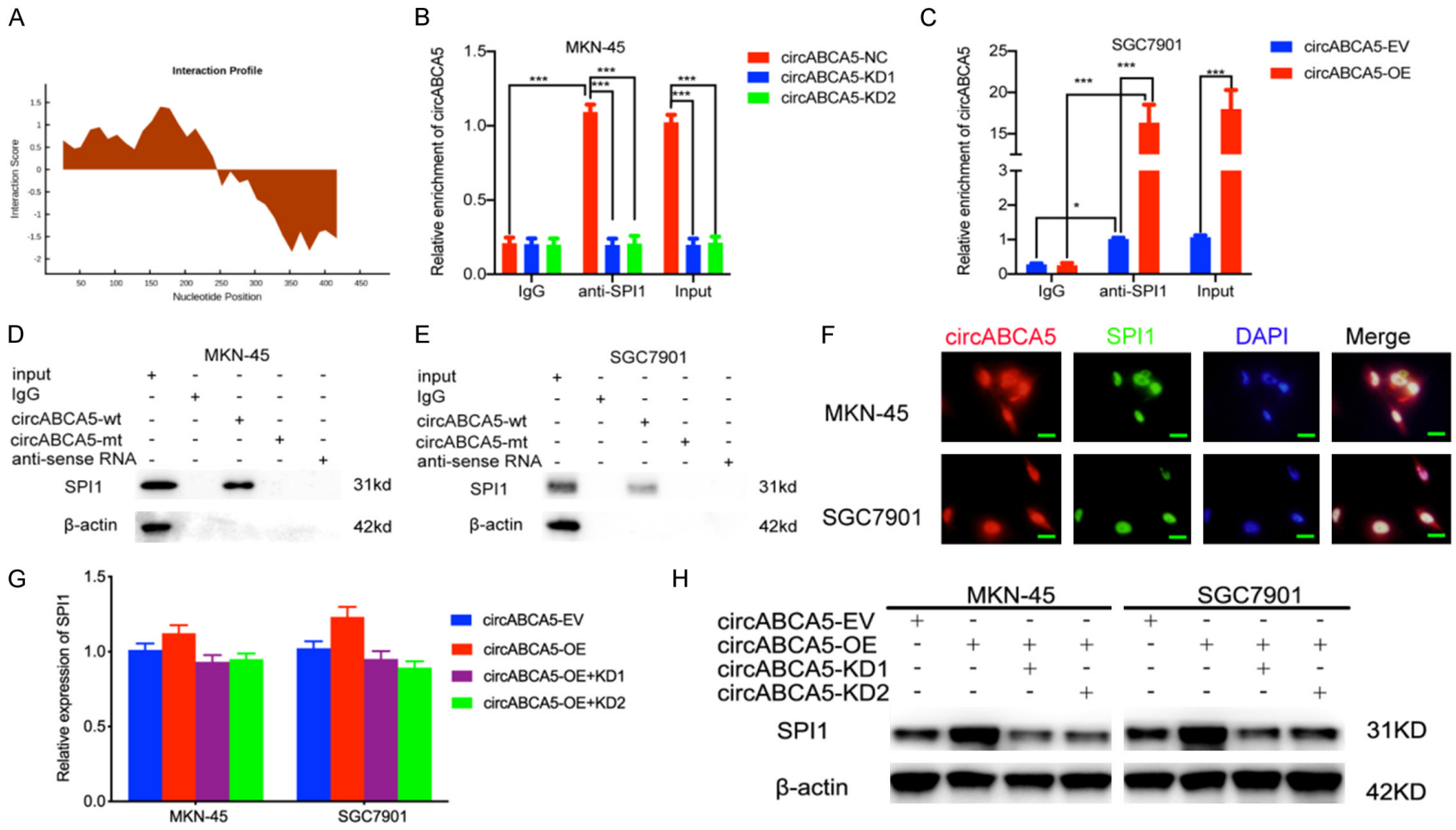
To confirm the mechanism by which circABCA5 promotes the malignant progression of gastric cancer by regulating SPI1, we also constructed stable SPI1 overexpression or knockdown gastric cell lines and verified the effect by qPCR (**Figure 4A**, **4B**). MTS assays showed that the absorbance increased significantly after circABCA5 overexpression but decreased after SPI1 knockdown (**Figure 4C**, **4D**). EdU assays also showed that the EdU-positive cell rates increased after circABCA5 overexpression but decreased after SPI1 knockdown (**Figure 4E**, **4F**). Furthermore, both Transwell and migration assays also showed that the number of invading and migrating cells increased after circABCA5 overexpression and decreased after SPI1 knockdown (**Figure 4G-J**). The above results demonstrate that SPI1 is the downstream target gene of circABCA5 and plays a crucial role in the promotion of the viability, proliferation, invasion and migration of gastric cancer cells by circABCA5. SPI1 knockdown can abolish the effects of circABCA5 in promoting gastric cancer.

SPI1 can transcriptionally upregulate IL6 expression and activate IL6/JAK/STAT3 signaling

To identify the different downstream mechanisms by which SPI1 participates in the occurrence and development of gastric cancer, we performed GSEA based on SPI1 expression in the TCGA gastric cancer dataset. The results showed that the higher SPI1 expression group was enriched with activation of the IL6/JAK2/STAT3 pathway, one of the most critical signaling pathways in the development of tumors [14] (**Figure 5A**). Since SPI1 is a transcription factor, we examined whether SPI1 can transcriptionally regulate the expression of IL6 in gastric cancer (**Figure 5B**). According to the prediction of the binding site from the Jaspar dataset, we designed luciferase reporter assays to assess the interaction between SPI1 and IL6, and the mutant luciferase plasmid included two mutated binding sites (**Figure 5C**). The results showed that the relative luciferase activities decreased after cotransfection with the SPI1 knockdown and wild-type plasmids but increased after cotransfection with the SPI1 knockdown plasmid and wild-type plasmid. However, there were almost no changes in luciferase activities after cotransfection with a mutant plasmid (**Figure 5D**, **5E**). Furthermore, ChIP assays also showed that the enrichment of IL6 was obviously decreased after SPI1 knockdown but increased after SPI1 overexpression (**Figure 5F**, **5G**). In addition, both qPCR and ELISAs showed that SPI1 knockdown downregulated the mRNA expression and secretion of IL6 in MKN-45 and SGC7901 cells, but the expression and secretion or IL6 were upregulated after SPI1 overexpression (**Figure 5H-K**). Moreover, western blotting was used to measure the expression of genes downstream of IL6/JAK2/STAT3 signaling. The results showed that the expression of IL6, p-JAK2 and p-STAT3 was downregulated after SPI1 knockdown but upregulated after SPI1 overexpression (**Figure 5L**, **5M**). Altogether, these results demonstrated that SPI1 could transcriptionally upregulate IL6 expression and activate IL6/JAK2/STAT3 signaling in gastric cancer.

SPI1 can promote the malignant phenotype of gastric cancer via IL6/JAK/STAT3 signaling

We further studied whether SPI1 can promote the malignant phenotype of gastric cancer via IL6/JAK/STAT3 signaling. An IL-6-neutralizing antibody was used to block the function of IL6 in gastric cancer cells. MTS assays showed that the absorbance increased significantly after SPI1 overexpression but decreased after anti-IL6 treatment (**Figure 6A**, **6B**). EdU assays also showed that the EdU-positive cell proportions increased after SPI1 overexpression but decreased after anti-IL6 treatment (**Figure 6C**, **6D**). Furthermore, both Transwell and migration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circABCA5-KD1
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circABCA5-EV
circABCA5-OE
IgG
anti-SPI1
Input
D
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circABCA5-KD2
SPI1
31KD
β-actin
42KD

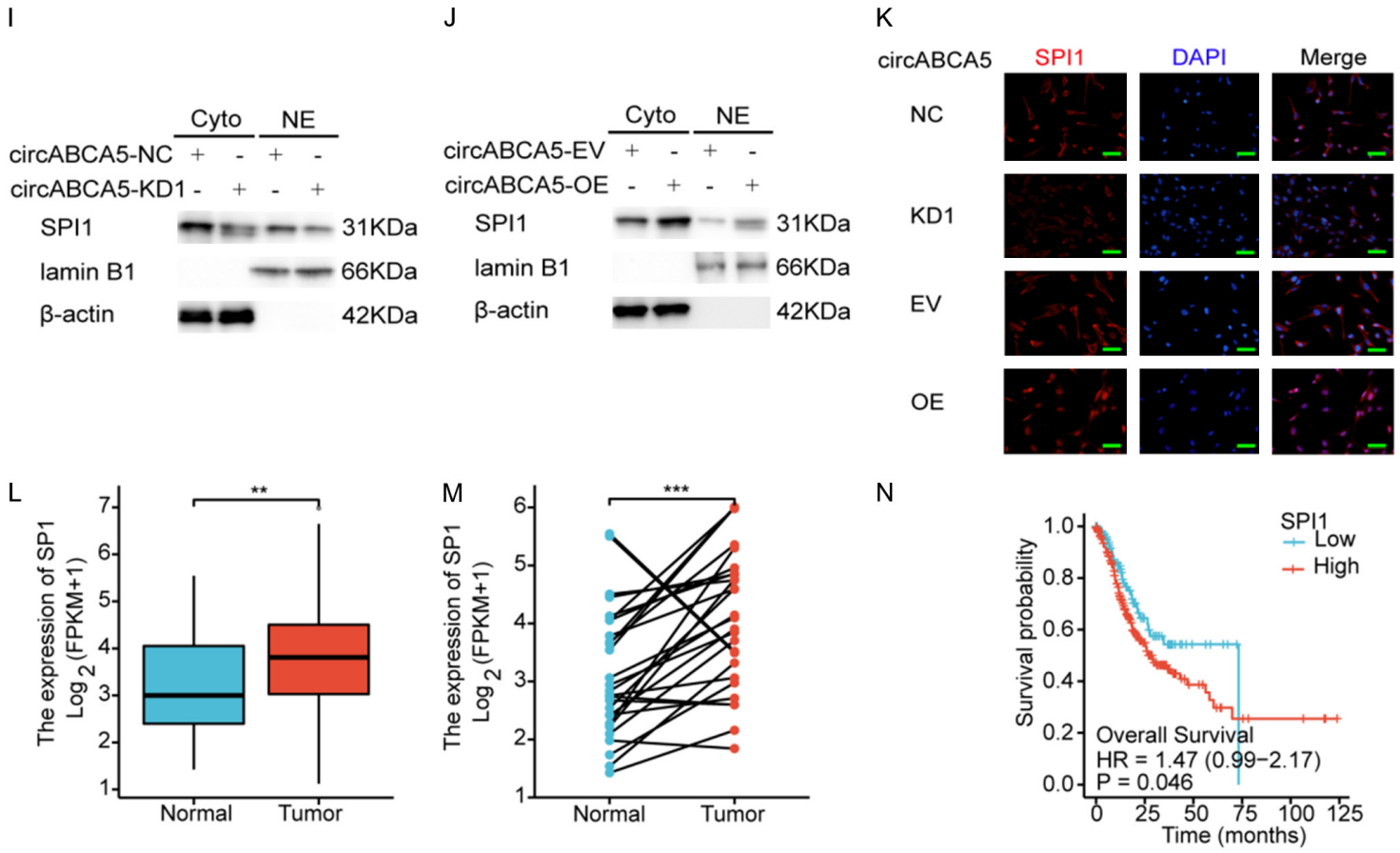


Figure 3. CircABCA5 can bind to and up-regulate SPI1 expression and promote nuclear translocation of SPI1. (A) CatRAPID predicts the possible binding between circABCA5 and SPI1 protein. (B, C) RIP assays showing the anti-SPI1 treatment led to the enrichment of circABCA5. (D, E) RNA pulldown assays showing circABCA5 can bind to SPI1 proteins. (F) FISH assay and immunofluorescence showed the colocalization of circABCA5 and SPI1 in gastric cancer cells. Scale bars = 50 μm. (G, H) qPCR assays (G) and western blotting (H) showing the expression of SPI1 mRNA after circABCA5 overexpression or knockdown. (I, J) Western blotting showing the expression changes in nuclear and cytoplasmic after circABCA5 knockdown (I) or overexpression (J). (K) Immunofluorescence showing the subcellular localization of SPI1 after circABCA5 overexpression or knockdown. Scale bars = 50 μm. (L) The expression of SPI1 in gastric cancer tissues compared with normal tissues in the TCGA dataset. (M) The expression of SPI1 in gastric cancer tissues compared with the adjacent normal tissues in the TCGA dataset. (N) Kaplan-Meier analysis showing the survival of gastric patients between higher or lower circABCA5 group in TCGA dataset. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

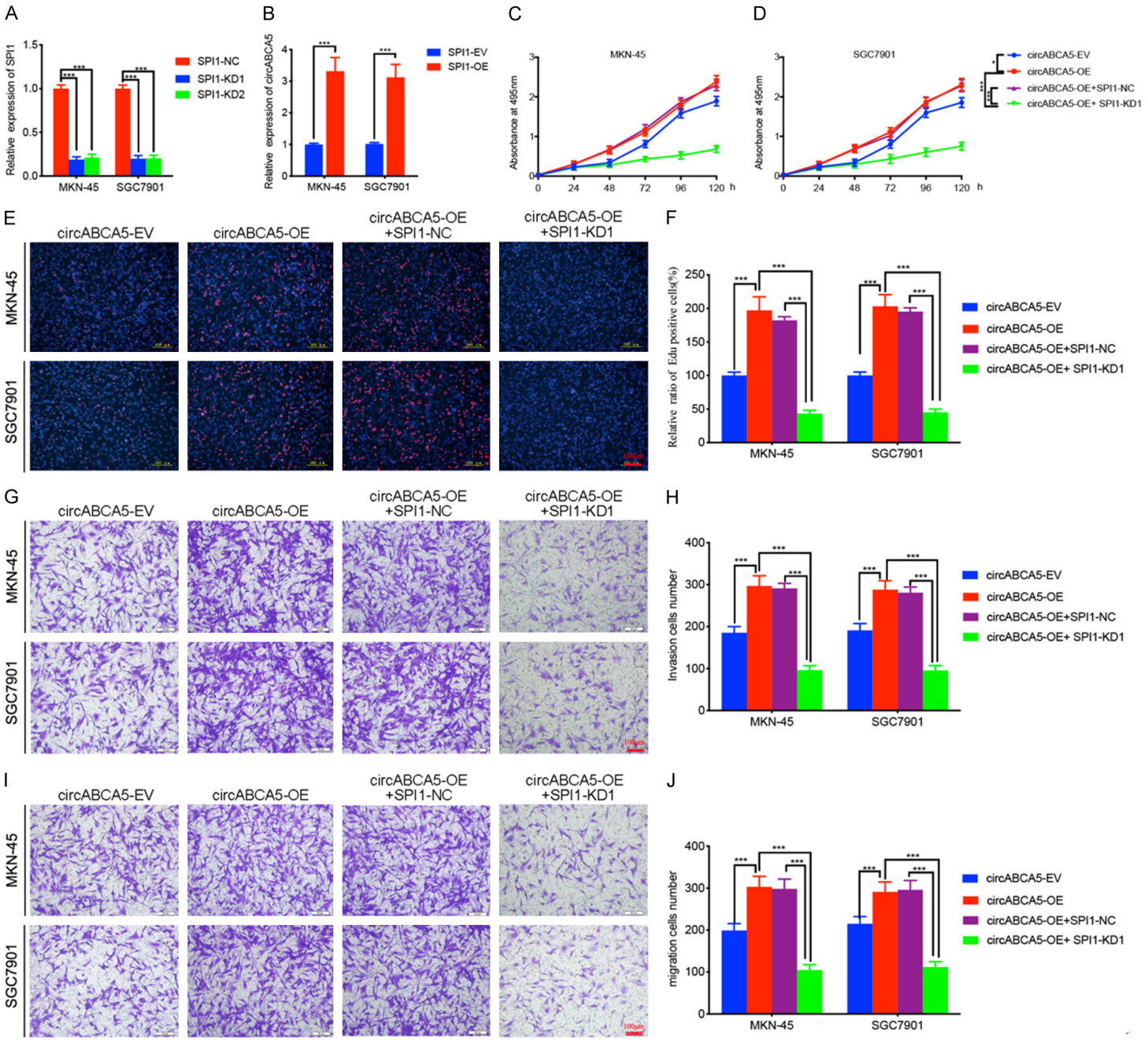

A
Relative expression of SPI1
SPI1-NC
SPI1-KD1
SPI1-KD2
MKN-45
SGC7901
B
Relative expression of circABCA5
SPI1-EV
SPI1-OE
C
MKN-45
Absorbance at 495nm
D
SGC7901
circABCA5-EV
circABCA5-OE
circABCA5-OE+SPI1-NC
circABCA5-OE+ SPI1-KD1
E
circABCA5-EV
circABCA5-OE
circABCA5-OE +SPI1-NC
circABCA5-OE +SPI1-KD1
F
Relative ratio of Edu positive cells(%)
G
H
Invasion cells number
I
J
migration cells number

Figure 4. SPI1 knockdown can abolish circABCA5 induced malignant phenotype of gastric cancer. (A, B) qPCR showing the expression of SPI1 after knockdown (A) or overexpressed (B) in MKN-45 and SGC7901. (C, D) MTS assays show the gastric cancer cells' viability after SPI1 knockdown in circABCA5 overexpressed cells. (E, F) EdU assays show gastric cancer cells' proliferation after SPI1 knockdown in circABCA5 overexpressed cells. Scale = 100 μm. (G, H) Transwell assays showing the invasion cell numbers after SPI1 knockdown in circABCA5 overexpressed cells. Scale = 50 μm. (I, J) Migration assays showing the migration cell numbers after SPI1 knockdown in circABCA5 overexpressed cells. Scale = 50 μm. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

assays also showed that the number of invading and migrating cells increased after SPI1 overexpression but decreased after anti-IL6 treatment (**Figure 6E-H**). Therefore, these results confirmed that SPI1 promoted the viability, proliferation, invasion and migration of gastric cancer cells via IL6/JAK/STAT3 signaling.

EIF4A3 binds to and maintains the stability of circABCA5 in gastric cancer

Moreover, RBPs are reported to be involved in binding circRNAs, regulating their back-splicing, synthesis, stability, degradation and so on [17]. To identify the possible causes of circABCA5 overexpression in gastric cancer, we predicted the candidate RBP that can regulate circABCA5 via starBase, circIntercome and CSCD (**Figure 7A**). Three intersecting RBPs were found, including EIF4A3, FUS and IGF2BP3. Among them, EIF4A3 was the best candidate RBP with the highest binding site number (**Figure 7B**). In addition, we also measured circABCA5 expression after overexpression or knockdown of EIF4A3, FUS or IGF2BP3 via qPCR. The results showed that only EIF4A3 overexpression upregulated circABCA5 expression, while EIF4A3 knockdown downregulated circABCA5 expression (**Figure 7C**, **7D**).

We also performed immunofluorescence to determine the subcellular location of EIF4A3 in gastric cancer cells, and the results showed that EIF4A3 was mainly located in the nucleus (**Figure 7E**). Then, both FISH assays and immunofluorescence staining were used to determine the colocalization of circABCA5 and EIF4A3, and the results showed that both were colocalized in the nucleus (**Figure 7F**). We further performed RIP assays to study whether there is direct binding between EIF4A3 and circABCA5. The results showed that anti-EIF4A3 treatment enriched the expression of circABCA5 more than IgG treatment (**Figure 7G**, **7H**). Moreover, the enrichment of circABCA5 was increased after circABCA5 overexpression and downregulated after circABCA5 knockdown (**Figure 7G**, **7H**). In addition, we studied whether EIF4A3 could upregulate circABCA5 expression by maintaining its stability. The RNA stability assays showed that the half-life of circABCA5 was significantly extended after EIF4A3 overexpression in comparison with the negative control group (**Figure 7I**, **7J**). In conclusion, EIF4A3 is an RBP that can directly bind to circABCA5, promoting its stability and expression.

CircABCA5 promotes the growth of gastric cancer cells in vivo

Finally, the proliferation-promoting effects of circABCA5 in gastric cancer were further studied in vivo. The circABCA5-overexpressing MKN-45 or circABCA5-knockdown SGC7901 cells were injected into the flank regions of nude mice, and the mice were fed and maintained for five weeks. The results showed that the circABCA5 overexpression group had a higher mean xenograft tumor volume and weight than the control group, while the circABCA5 knockdown group had a lower mean xenograft tumor volume and weight (**Figure 8A-E**). IHC was further performed to determine the proliferation-promoting effects of circABCA5 in tumor tissues. The staining intensity and expression levels of Ki-67, SPI1 and IL6 were all upregulated in the circABCA5-overexpressing group but downregulated in the circABCA5 knockdown group (**Figure 8F**). Taken together, these results demonstrated that circABCA5 promotes the growth of gastric cancer cells in vivo.

Discussion

Gastric cancer is one of the most common malignant tumors in the digestive system. Although most patients are treated with active surgical treatment combined with radiotherapy or chemotherapy after diagnosis, there are still

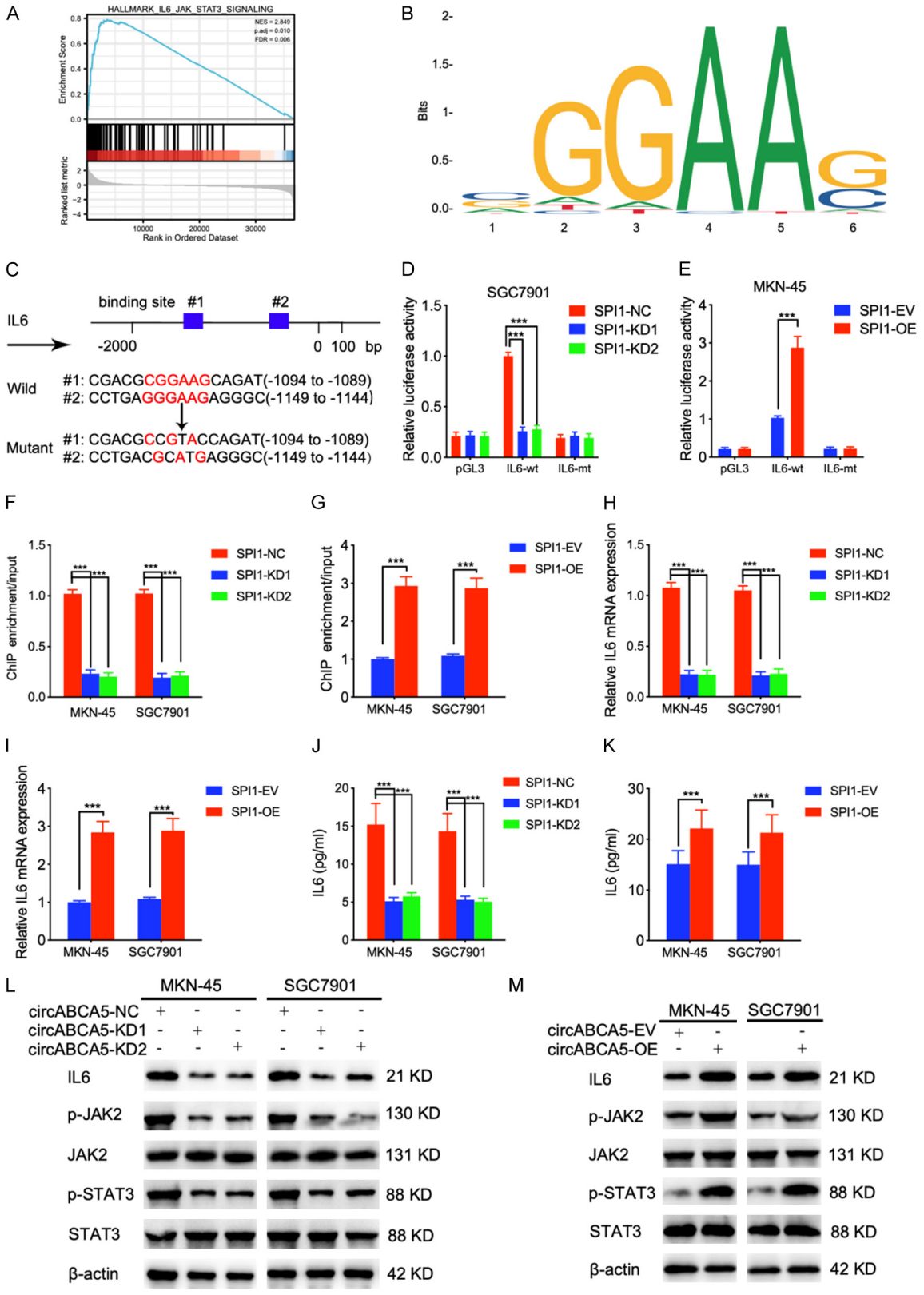


Figure 5. SPI1 can transcriptionally up-regulate IL6 expression and activate IL6/JAK/STAT3 signaling. (A) GSEA analysis showing the enrichment of IL6/JAK2/STAT3 signaling in SPI1 higher expression group. (B) Sequence motif of the consensus SPI1 binding motif in the JASPAR database. (C) Schematic diagram showing the possible bind-

ing sites of SPI1 on the human IL6 promoter region. (D, E) Luciferase assays show the relative luciferase activities changes after SPI1 knockdown (D) or overexpression (E). (F, G) ChIP assays showing the enrichment of IL6 DNA after SPI1 knockdown (F) or overexpression (G). (H, I) ELISA assays showing the secretion of IL6 in the supernatant of the gastric cells after SPI1 knockdown (H) or overexpression (I). (J, K) qPCR assays show IL6 mRNA levels after SPI1 knockdown (J) or overexpression (K). (L, M) Western blotting showing the expression of IL6/JAK2/STAT3 in gastric cancer after SPI1 knockdown (L) or overexpression (M). All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

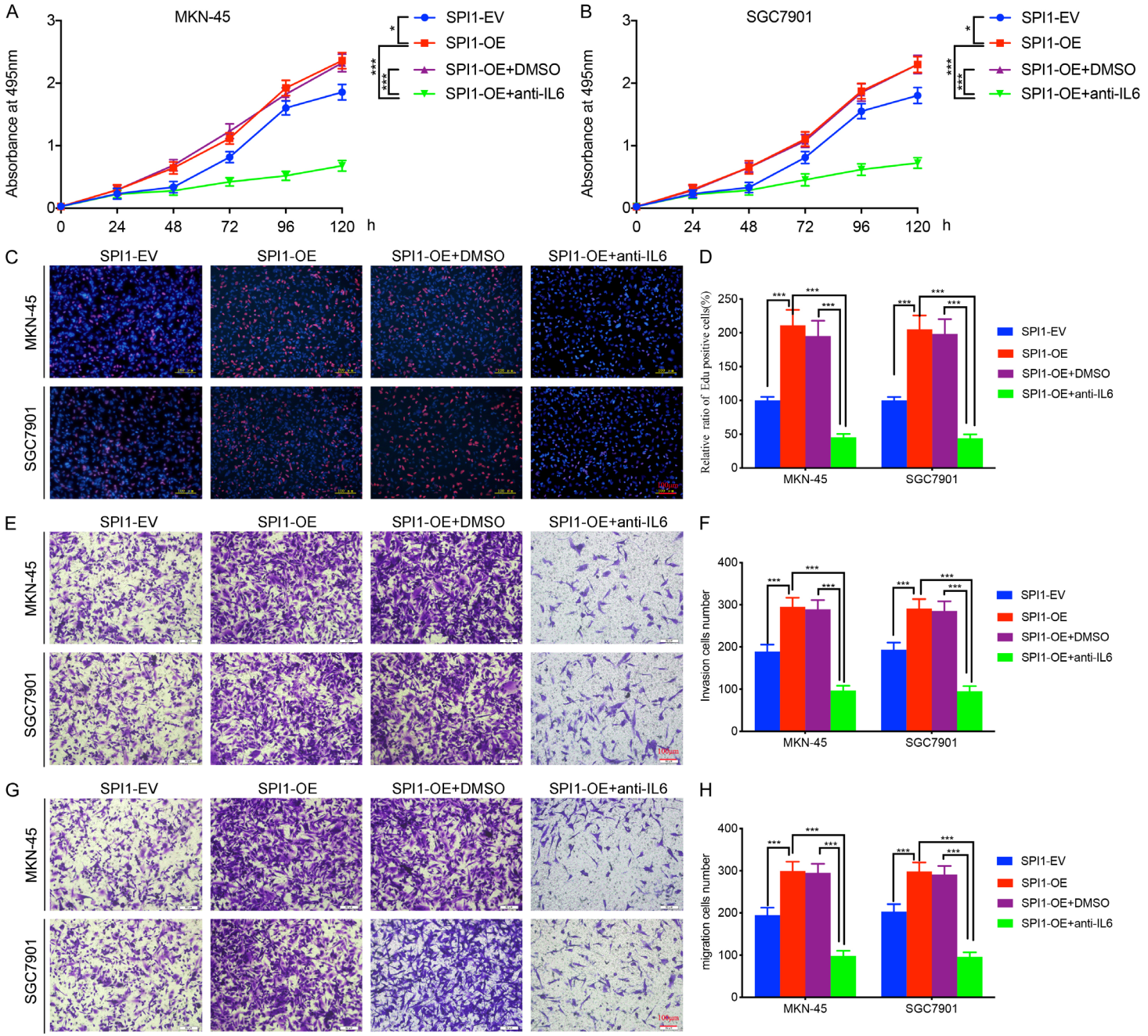
a large number of patients with recurrence, distant metastasis and poor prognosis, which seriously affect the survival time and quality of life of patients. In recent years, with the development of molecular biology techniques, a large number of studies have confirmed that the occurrence, development and prognosis of malignant tumors are closely related to the abnormal expression of genes. Searching for tumor-related genes has become an essential direction of research on malignant tumor therapies, and molecular targeted therapy has been proposed, which is expected to become a vital method for curing malignant tumors. Therefore, the identification of novel gastric cancer-related genes and the determination of their functional mechanisms is essential.

With the completion of the human genome project, the nucleic acid sequence that encodes proteins has been shown to be less than 2% of the entire nucleic acid sequence of the human genome. Most genes in the human genome encode noncoding RNAs (ncRNAs), including common microRNAs (miRNAs), long noncoding RNAs (lncRNAs), and circRNAs [18]. Due to the limitations of previous experimental technology, the role of the substantial numbers of ncRNAs in the body's physiological and pathophysiological processes is insufficiently studied and understood [19]. As a new type of gene related to gastric carcinogenesis, circRNAs have become a research hotspot in recent years [20]. For example, circMAN1A2 is upregulated by *Helicobacter pylori* and promotes the development of gastric cancer via miR-1236-3p/MTA2 [21]. CircRNA circCPM promotes chemoresistance in gastric cancer by activating PRKAA2-mediated autophagy [22]. Based on this, molecular therapies that target circRNA have also been proposed.

In our study, we found for the first time that circRNA circABCA5 was a novel circRNA that is overexpressed in gastric cancer as shown by circRNA sequencing and qPCR analysis of specimens. CircABCA5 is back-spliced from its parental gene ABCA5 and consists of 21, 22 and 23 exons of ABCA5 mRNA transcript 1. Although there is no study about the expression and biological function of ABCA5 in gastric cancer, it was reported to act as a diagnostic marker in prostatic intraepithelial neoplasia and osteosarcoma [23, 24]. Gastric cancer patients with higher circABCA5 expression had a shorter average survival time than those with lower circABCA5 expression. Functionally, our molecular experiments showed that circABCA5 could promote proliferation, invasion and migration in vitro and in vivo. Therefore, we think that circABCA5 plays a vital role in the occurrence of gastric cancer and can be used as a marker for the diagnosis and prognosis evaluation of gastric cancer.

In addition, the mechanism by which circRNAs function in tumorigenesis and development has also been a research hotspot in recent years. Most of the published studies on the functional mechanism of circRNAs are focused on their roles as miRNA sponges. Since circRNAs have many miRNA response elements (MREs), circRNAs can inhibit the negative regulation of miRNAs of their target gene and upregulate target genes via competitive adsorption of miRNA [25]. However, studies on this mechanism lack sufficient innovation, and their reliability also need to be further confirmed. circRNAs can directly bind proteins, promote their stability or degradation, and regulate their distribution in cells [26]. For example, circRNA_102231 was upregulated in gastric cancer and promoted its progression by increasing IRTKS protein stability [27].

Our study found that circABCA5 can bind to and upregulate SPI1 expression and promote its nuclear translocation. SPI1 is a proto-oncogene involved in the progression of glioma, breast cancer, lung cancer and other malignant tumors [28]. SPI1 was reported to be upregulated in gastric cancer, to be related to poor prognosis


A
MKN-45
Absorbance at 495nm
0
1
2
3
0
24
48
72
96
120
h
SPI1-EV
SPI1-OE
SPI1-OE+DMSO
SPI1-OE+anti-IL6
*
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B
SGC7901
Absorbance at 495nm
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1
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SPI1-OE+anti-IL6
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SPI1-EV
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SPI1-OE+DMSO
SPI1-OE+anti-IL6
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D
Relative ratio of Edu positive cells(%)
0
50
100
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200
250
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MKN-45
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SPI1-OE+DMSO
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SPI1-EV
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SPI1-OE+DMSO
SPI1-OE+anti-IL6
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SGC7901
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100
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400
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MKN-45
SGC7901
SPI1-EV
SPI1-OE
SPI1-OE+DMSO
SPI1-OE+anti-IL6

Figure 6. A, B: MTS assays show the gastric cancer cells' viabilities after anti-IL6 treatment in SPI1 overexpressed cells. C, D: EdU assays showing the proliferation of gastric cancer cells after anti-IL6 treatment in SPI1 overexpressed cells. Scale = 100 μm. E, F: Transwell assays showing the invasion cell numbers after anti-IL6 treatment in SPI1 overexpressed cells. Scale = 50 μm. G, H: Migration assays showing the migration cell numbers after anti-IL6 treatment in SPI1 overexpressed cells. Scale = 50 μm. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

and to promote the proliferation, invasion and migration of gastric cancer cells [13, 28]. Our study also demonstrated that SPI1 knockdown could abolish the circABCA5-induced malignant phenotype of gastric cancer. Therefore, it is reasonable that SPI1 might be the candidate downstream gene targeted and regulated by circABCA5.

Although a previous study reported that SPI1 is recruited by NR2F1-AS1 and promotes gastric cancer cells by upregulating ST8SIA1 expression [13], no other functional mechanism of SPI1 in gastric cancer was reported. Our study found that the higher SPI1 expression group was enriched with active IL6/JAK2/STAT3 signaling, and we proposed a new mechanism by which SPI1 functions in gastric cancer. It was reported that IL6/JAK/STAT3 signaling participated in NSCLC brain metastasis, promoting the malignant phenotype of ovarian epithelial carcinoma and gastric cancer [14, 29, 30]. A previous study confirmed that SPI1 could bind to the IL1β promoter and upregulate its transcription and expression [31], while there was no study about its regulation of IL6. Our study demonstrated that SPI1 could transcriptionally upregulate IL6 expression and promote the malignant phenotype of gastric cancer via IL6/JAK/STAT3 signaling. Altogether, our study revealed a novel molecular biological mechanism by which circABCA5 promotes the malignant phenotype of gastric cancer via SPI1-regulated IL6/JAK/STAT3 signaling.

RNA binding proteins (RBPs) are a group of proteins associated with the regulation and metabolism of RNA and RNA binding [32]. The main role of this family is to mediate the maturation, transport, localization and translation of RNA. A particular RBP may have multiple target RNAs, and defects in its expression can cause a variety of diseases, including cancers [33]. EIF4A3 is one of the most common and important RBPs in tumors and has been reported to play key roles in the transcription, maturation and stability of several circRNAs. For example, EIF4A3 can induce circTOLLIP expression in hepatocellular carcinoma and promote its malignant progression [34]. EIF4A3 can also regulate circ_0087429 expression in cervical cancer and promote the epithelial to mesenchymal transition (EMT) [35]. In our study, we found that EIF4A3 can directly bind to circABCA5, promoting its stability and expression. This result is also consistent with the reports of other studies.

Conclusion

Our study found a novel circRNA, circABCA5, that was overexpressed in gastric cancer and correlated with poor prognosis. CircABCA5 can promote gastric cancer proliferation, invasion, and migration in vitro and in vivo. Mechanistically, circABCA5 can bind to and upregulate SPI1 expression and promote its nuclear translocation. The latter can transcriptionally upregulate IL6 expression and activate IL6/JAK/STAT3 signaling. Moreover, EIF4A3 is an RBP that can directly bind to circABCA5, promoting its stability and expression. Therefore, we discovered a novel mechanism by which circRNAs function in the occurrence and development of gastric cancer. CircABCA5 also plays a vital role in the diagnosis and prognosis of gastric cancer and may be a molecular target for the treatment of gastric cancer.

Acknowledgements

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The present study was approved by the ethical review committee of the Affiliated Huaian No. 1 People's Hospital of Nanjing Medical University. Written informed consent was obtained from all enrolled patients.

Disclosure of conflict of interest

None.

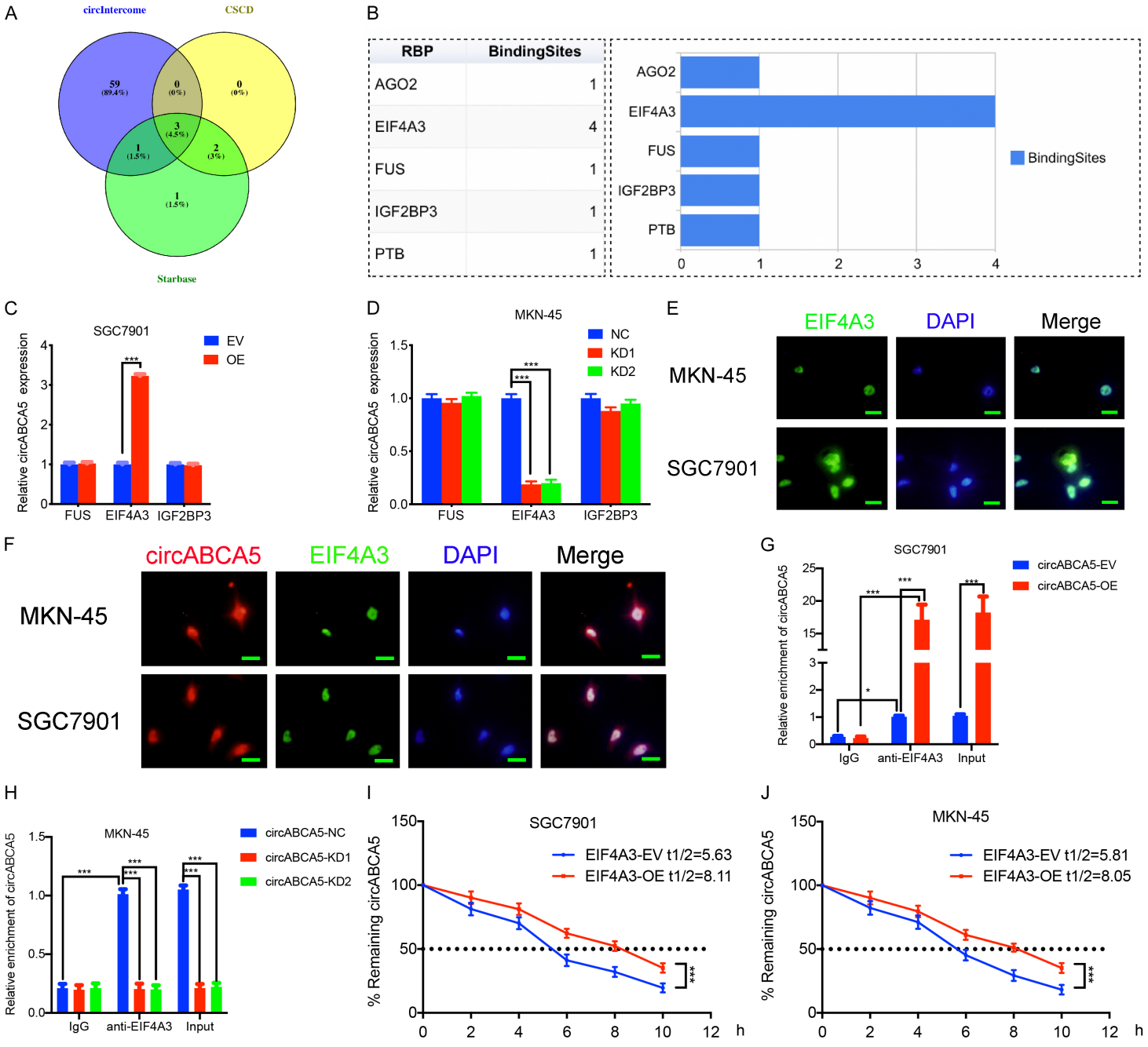
A
circInterome
CSCD
Starbase
59 (89.4%)
0 (0%)
0 (0%)
3 (4.5%)
1 (1.5%)
2 (3%)
1 (1.5%)
B
RBP
BindingSites
AGO2 1
EIF4A3 4
FUS 1
IGF2BP3 1
PTB 1
AGO2
EIF4A3
FUS
IGF2BP3
PTB
BindingSites
0 1 2 3 4
C
SGC7901
EV
OE
Relative circABCA5 expression
FUS EIF4A3 IGF2BP3
D
MKN-45
NC
KD1
KD2
Relative circABCA5 expression
FUS EIF4A3 IGF2BP3
E
EIF4A3
DAPI
Merge
MKN-45
SGC7901
F
circABCA5
EIF4A3
DAPI
Merge
MKN-45
SGC7901
G
SGC7901
circABCA5-EV
circABCA5-OE
Relative enrichment of circABCA5
IgG anti-EIF4A3 Input
H
MKN-45
circABCA5-NC
circABCA5-KD1
circABCA5-KD2
Relative enrichment of circABCA5
IgG anti-EIF4A3 Input
I
SGC7901
EIF4A3-EV t1/2=5.63
EIF4A3-OE t1/2=8.11
% Remaining circABCA5
h
J
MKN-45
EIF4A3-EV t1/2=5.81
EIF4A3-OE t1/2=8.05
% Remaining circABCA5
h

Figure 7. EIF4A3 binds to and maintain the stability of circABCA5 in gastric cancer. (A) The venn diagram showed the prediction of circABCA5's RBP via Starbase, circIntercome and CSCD. (B) The binding sites of RBPs on the sequence of circABCA5. (C, D) qPCR showed the expression of circABCA5 after RBP's (EIF4A3, FUS and IGF2BP3) overexpression (C) or knockdown (D). (E) Immunofluorescence showing the subcellular location of EIF4A3 in the gastric cancer cells. Scale bars = 50 μm. (F) FISH assay and immunofluorescence showed the colocalization of circABCA5 and EIF4A3 in gastric cancer cells. Scale bars = 50 μm. (G, H) RIP assays showed the direct binding between EIF4A3 and circABCA5. (I, J) Relative expression levels of circABCA5 in the EIF4A3 overexpression treated with actinomycin D at different time points were detected using qRT-PCR. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

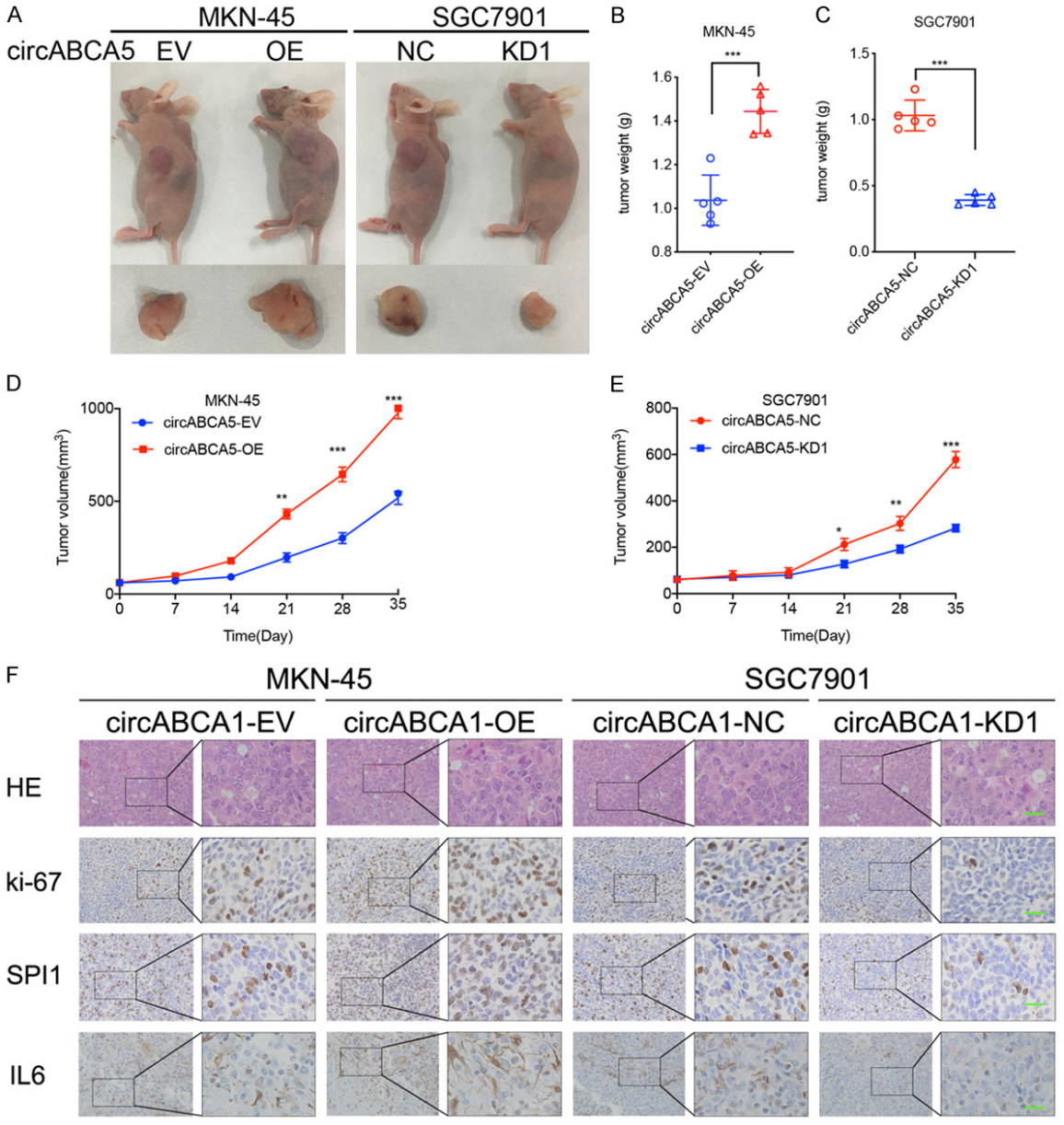

Figure 8. CircABCA5 promotes the growth of gastric cancer cells in vivo. A: Representative images of tumors formed by circABCA5 overexpressed or knockdown gastric cancer cells in nude mice via subcutaneous injection. B, C: The tumor weights were detected in circABCA5 overexpressed, knockdown or control groups. D, E: The tumor volumes were calculated in circABCA5 overexpressed, knockdown or control groups. F: IHC staining showing the expressions of ki-67, SPI1 and IL6 in circABCA5 overexpressed, knockdown or control groups. Scale = 50 μm. All data are shown as the mean ± SD (three independent experiments). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Abbreviations

ncRNAs, non-coding RNAs; GC, Gastric cancer; miRNAs, microRNAs; MREs, microRNA response elements; GEO, Gene Expression Omnibus; H&E, hematoxylin and eosin; IHC, Immunohistochemistry; qRT-PCR/qPCR, Real-Time Quantitative Reverse Transcription PCR.

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