# Original Article
# Notch1 regulates proliferation and invasion in gastric cancer cells via targeting Fascin1

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**Abstract:** We investigated the role of Notch1 and Fascin1 in the proliferation and invasion of gastric cancer cells to elucidate the detailed mechanism. Notch1 and Fascin1 expression levels were detected by immunohistochemistry in a gastric cancer tissue microarray. The effect of Notch1 on Fascin1 expression and the roles of Notch1 and Fascin1 in the proliferation and invasion of gastric cancer cell lines were investigated through siRNA-mediated knockdown of each gene. In addition, we tested for direct binding between RBP-JK, a Notch1 signaling pathway transcription factor, and the Fascin1 promoter *in vivo* using chromatin immunoprecipitation (ChIP). We found that Notch1 and Fascin1 expression levels were higher in gastric cancer tissues than in normal mucosa tissues adjacent to carcinoma, and their expressions were positively correlated in gastric cancer. After knockdown of Notch1, the expression levels of Fascin1 mRNA and protein measured by real-time PCR and Western blot, respectively, were significantly decreased in two gastric cancer cell lines (SGC-7901 and MGC-803). Knockdown of either Notch1 or Fascin1 inhibited gastric cancer cells proliferation and invasion. Furthermore, ChIP revealed the direct binding of RBP-JK to the Fascin1 promoter *in vivo*. Our results demonstrate that Notch1 regulated Fascin1 expression at the transcriptional level, thereby affecting proliferation and invasion in gastric cancer cells. Our results also suggest the Notch1/RBP-JK/Fascin1 axis is a potential molecular target for gastric cancer therapy.

**Keywords:** Notch1, Fascin, siRNA, gastric cancer

## Introduction

Gastric cancer is the most common malignant tumor in the digestive system and a serious health concern for people around the world [1, 2]. Despite great progress in the treatment of gastric cancer in recent years, the effects of clinical treatment and survival outcomes for patients require further improvement. The rapid proliferation and invasion of gastric cancer cells are important contributors to the low survival rate of patients with gastric cancer. Therefore, inhibiting the proliferation and invasion of gastric cancer cells is an important target for inhibiting the progression of gastric cancer and improving patient prognosis.

Previous studies indicated that the Notch1 signaling pathway was involved in the development and progression of gastric cancer, esophageal cancer, liver cancer, colorectal cancer, and pancreatic cancer [3-7]. Notch1 signaling plays a role in the proliferation and invasion of gastric cancer cells [8] and may regulate cyto-

skeleton formation as a classical developmental pathway. Given the important role of the cytoskeleton reorganization in the invasion and metastasis of tumor cells, particularly in formation of invadopodia, understanding the mechanism of proliferation and metastasis of gastric cancer involving the Notch1 signaling pathway could provide new insights and direction for the treatment of gastric cancer.

The actin cytoskeleton network plays an important role in cell movement, which is important for cell invasion and metastasis [9]. Fascin1 is an actin-binding cytoskeletal protein involved in filopodia formation and may promote the migration of endothelial cells. Previous studies showed that the expression of Fascin1 is closely related to the invasion and metastasis of many tumors, including gastric cancer [10-12].

Therefore, we hypothesized that Notch1 might regulate the proliferation and invasion of gastric cancer cells by regulating the expression of Fascin1. Furthermore, we predicted the direct

binding of the Notch1 signaling pathway transcription factor RBP-JK to the Fascin1 promoter by bioinformatics. The purpose of this study was to test the above assumptions toward elucidating the mechanism of increased proliferation and invasion in gastric cancer cells.

## Materials and methods

### Antibodies and reagents

Fetal bovine serum was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. The chromatin immunoprecipitation (ChIP) kit and Cell Counting Kit-8 (CCK8) were from Beyotime Biotechnology. Lipofectamine 2000 Transfection Reagent was from Invitrogen. Notch1-siRNA, Fascin1-siRNA, and Control-siRNAs were from Santa Cruz Biotechnology. Rabbit anti-human Notch1 polyclonal antibody was from Cell Signaling Technology. Human Fascin1 antibody was from R&D Systems. ChIP-grade anti-RBP-JK antibody was from Abcam. The transwell chamber was from Corning.

### Cell culture and transfection

Human gastric cancer SGC-7901 cells were purchased from the China Center for Type Culture Collection. Human gastric cancer MGC-803 cells were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences. Cells were cultured in DMEM culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution. Cells in the logarithmic growth phase were used for experiments. Firstly, cells were divided into three groups: Normal group (without transfection), Control-siRNA group (transfected with Control-siRNA), and Notch1-siRNA group (transfected with Notch1-siRNA). Secondly, cells were also divided into three groups: Normal group (without transfection), Control-siRNA group (transfected with Control-siRNA), and Fascin1-siRNA group (transfected with Fascin1-siRNA). Transfection was carried out using Lipofectamine 2000 according to the manufacturer instructions.

### Western blot analysis

Cellular proteins were extracted, and protein concentrations were determined using a BCA kit. After adding SDS-PAGE loading buffer to cellular proteins, they were boiled 5 min for denaturation, and 20 μg total proteins were separated in each lane on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membranes, and the membranes were blocked for 2 h using 5% skim milk powder at room temperature. Primary antibodies were added for overnight incubation at 4°C, then secondary antibodies were added for 1 h at room temperature, and protein bands were detected by ECL chemiluminescence.

### Real-time PCR

Total RNA was extracted by Trizol reagent, and the concentration of RNA was detected spectrophotometrically. cDNA was generated by reverse transcription using a TaKaRa reverse transcription kit according to the manufacturer instructions, and PCR amplification was performed using the cDNA as the template. The mRNA level was quantified via real-time PCR using the following primer sequences: Notch1 forward 5'-CGCCTTTGTGCTTCTG-TTCTTCG-3'; and reverse 5'-TTCTTGGTCTCCA-GGTCCTCGTC-3'; Fascin1 forward 5'-GCCAGG-GTATGGACCTGTCTG-3'; and reverse 5'-CACGC-CACTCGATGTCAAAGTA-3'; and β-actin forward 5'-AGCGAGCATCCCCCAAAGTT-3'; and reverse 5'-GGGCACGAAGGCTCATCATT-3'.

### CCK8 assay

Cell proliferation was measured using a CCK8 kit according to the manufacturer instructions. Cells transfected for 24 h were inoculated into 96-well plates at $2 \times 10^3$ cells per well, and the volume in each well was 100 μL. Culture medium alone was used as the negative control. After culturing for 1, 2, or 3 days, 10 μL CCK8 solution was added to each well for 2 h at 37°C. The absorbance for the negative control well was set to zero, and the absorbance of each well (OD value) was determined on an enzymatic reader at 450 nm. Cell proliferation was expressed as the OD ratio. Each sample was run in triplicate.

### Transwell assay

Cell invasion was measured using a transwell assay. Matrigel gel was stored at 4°C overnight and diluted in DMEM culture medium at a 1:8 ratio. Then 100 μL diluted matrigel gel was added to the upper chamber of a transwell plate and incubated at 37°C for 5 h.

Cells were suspended in DMEM culture medium without fetal bovine serum at a concentration of $2 \times 10^5$ cells/mL, and 200 μL was seed-

ed into the upper chamber of a 24-well chemotaxis chamber with 800 μL DMEM culture medium containing 10% fetal bovine serum in the lower chamber. The chambers were incubated at 37°C, 5% $CO_2$ for 24 h. The culture chamber was removed, fixed in 4% paraformaldehyde for 20 min, air-dried, and dyed with 0.1% crystal violet for 10 min. Matrigel on the bottom of the chamber and non-invasive cells were gently removed using cotton swabs. After washing three times with phosphate-buffered saline, five microscope fields (400×) were randomly selected, and the cells were observed and counted.

*Bioinformatics prediction of RBP-JK binding sites*

The potential binding sites of RBP-JK on the Fascin1 promoter were predicted using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3).

*ChIP assay*

ChIP was performed using the ChIP kit according to the manufacturer protocol. MGC-803 cells were plated in a 10-cm culture dish, cultured at 37°C for 24 h, and then fixed in 1% formaldehyde for 10 min at 37°C. After fixing, 1.1 mL 10× Glycine Solution was added to the culture dish and incubated at room temperature for 5 min. Cellular DNA was cut into fragments of 200-1000 bp by ultrasonication, and the primary antibody was added for overnight incubation with gentle rocking at 4°C. The primary antibodies used were anti-RBP-JK for the experimental group, IgG for the negative control group, and anti-RNA Polymerase II for the positive control group. All DNA was purified using a DNA purification kit and then amplified by PCR. The input group, experimental group, and negative control group were amplified with Fascin1 primers (forward, 5'-T-GGAGGGTTGGGATTCTGAGG-3'; reverse, 5'-GT-GAGTGGGCAGGACAGGAA-3'). The positive control group was amplified with GAPDH primers (forward, 5'-TACTAGCGGTTTTACGGGCG-3'; reverse, 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'). The results were analyzed by agarose gel electrophoresis.

*Immunohistochemistry and results determination*

This study was approved by the institutional ethics committee of the Renmin Hospital of Wuhan University. The gastric cancer tissue microarray was purchased from Xian Alina Biological Technology Co., Ltd. The tissue microarray contained primary gastric cancer samples and paired adjacent normal mucosa tissues. The samples were from 29 males and 11 females between 47 and 78 years old (mean: 59.03 ± 8.39 years); none of the patients had been cured by chemotherapy or radiotherapy. A SP immunohistochemistry kit (Fuzhou Maxim Biotech. Co., Ltd.) was used to analyze the samples according to the manufacturer protocol with anti-Notch1 (1:1000) and anti-Fascin1 (1:500) antibodies. Phosphate-buffered saline was used as the negative control.

The results were analyzed using a two-level scoring method. (1) The number of positive cells was scored as 0-4: 0, < 5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, > 75%. (2) The staining intensity was classified as 0-3: 0, no dye; 1, light yellow; 2, yellow; and 3, brown. The result was considered positive when the product of the two scores was more than 4.

*Statistical analysis*

All experiments were repeated at least three times. SPSS 13.0 software was used for statistical analysis. Quantitative data were expressed using mean ± standard deviation. One-way ANOVA was used to compare the different groups. Count data were compared by Chi-square test. Correlation between the levels of Notch1 and Fascin1 protein in gastric cancer tissues was examined using the Spearman rank order correlation test. $P < 0.05$ was considered statistically significant.

**Results**

*Notch1 regulates Fascin1 expression in gastric cancer cells*

To investigate whether Notch1 regulates Fascin1 expression, Notch1-siRNA and Control-siRNA were transfected into MGC-803 and SGC-7901 cells. The mRNA and protein expression of Notch1 and Fascin1 were detected by real-time PCR and Western blot. After siRNA knockdown of Notch1, the mRNA and protein expression levels of Fascin1 significantly decreased in both cell lines (**Figure 1**). These results indicated that Notch1 could positively regulate Fascin1 expression.
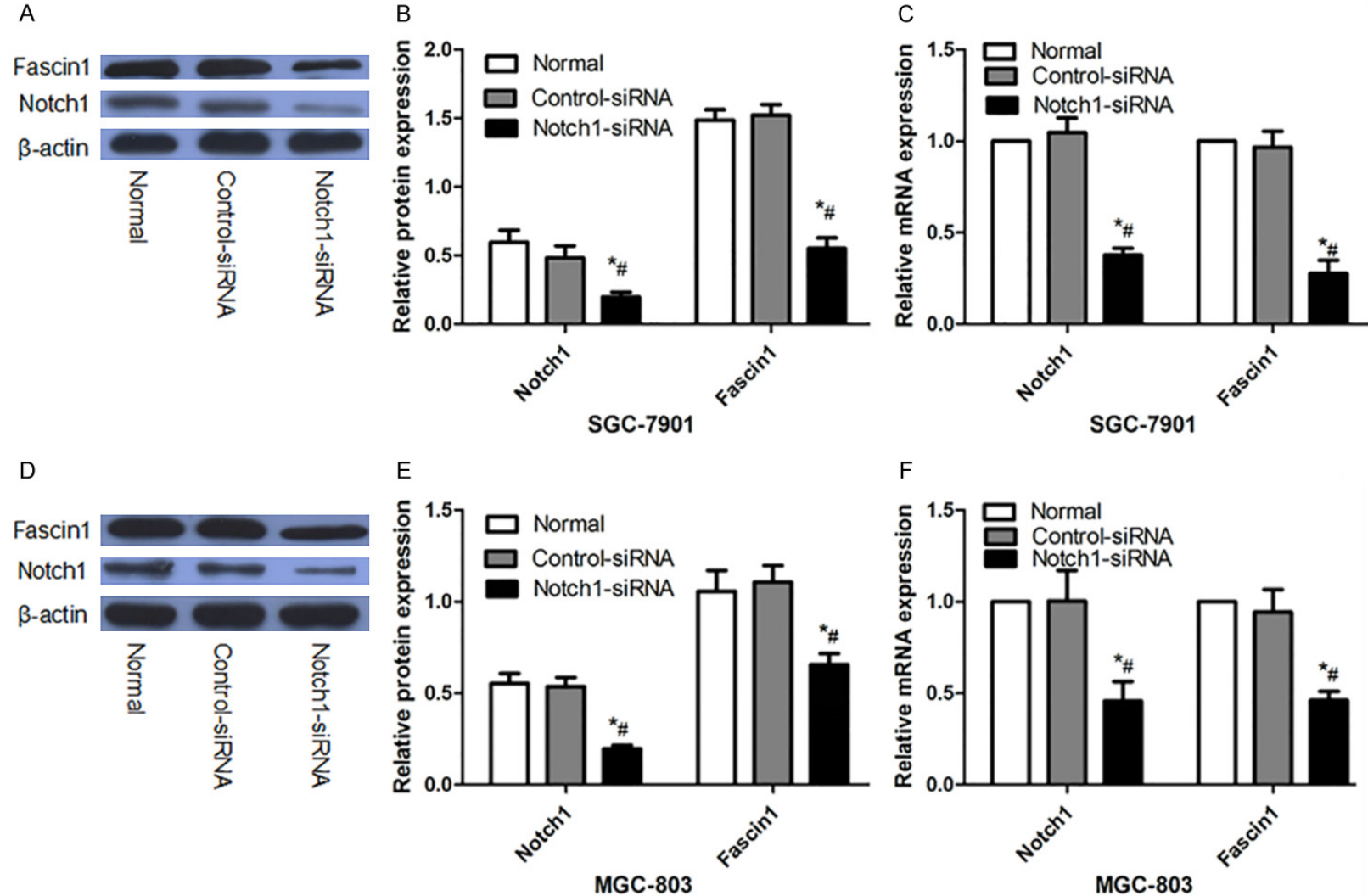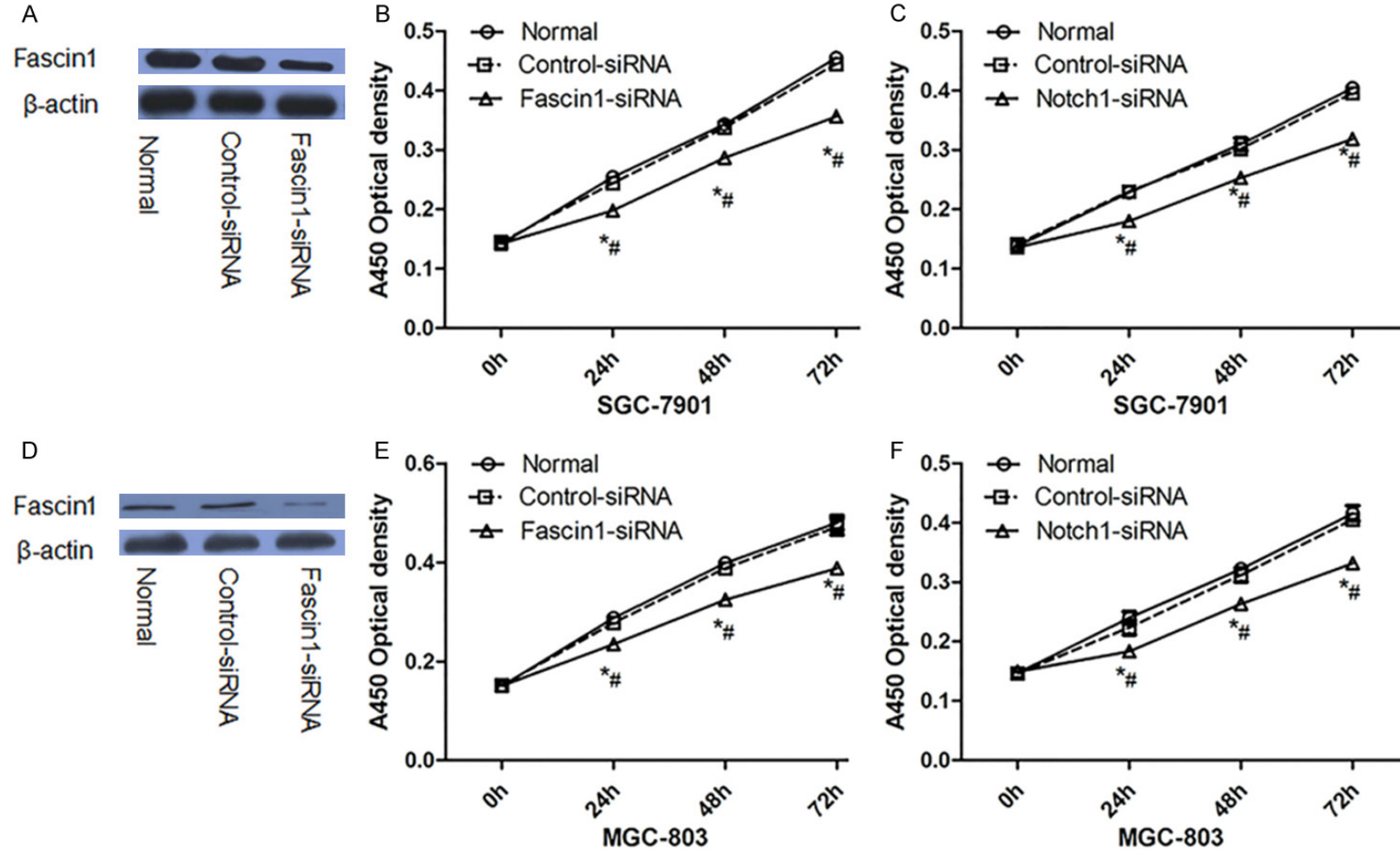
**Figure 1.** Notch1 regulates Fascin1 expression in gastric cancer cells. A. SGC-7901 cells were transfected with Notch1-siRNA and Control-siRNA; B and C. Notch1/Fascin1 protein and mRMA levels were detected by real-time PCR and Western blot analysis. D. MGC-803 cells were transfected with Notch1-siRNA and Control-siRNA; E and F. Notch1/Fascin1 protein and mRMA levels were detected by real-time PCR and Western blot analysis. β-actin was used as loading control. *: Compared with Normal group, $P < 0.05$; #: Compared with Control-siRNA group, $P < 0.05$.
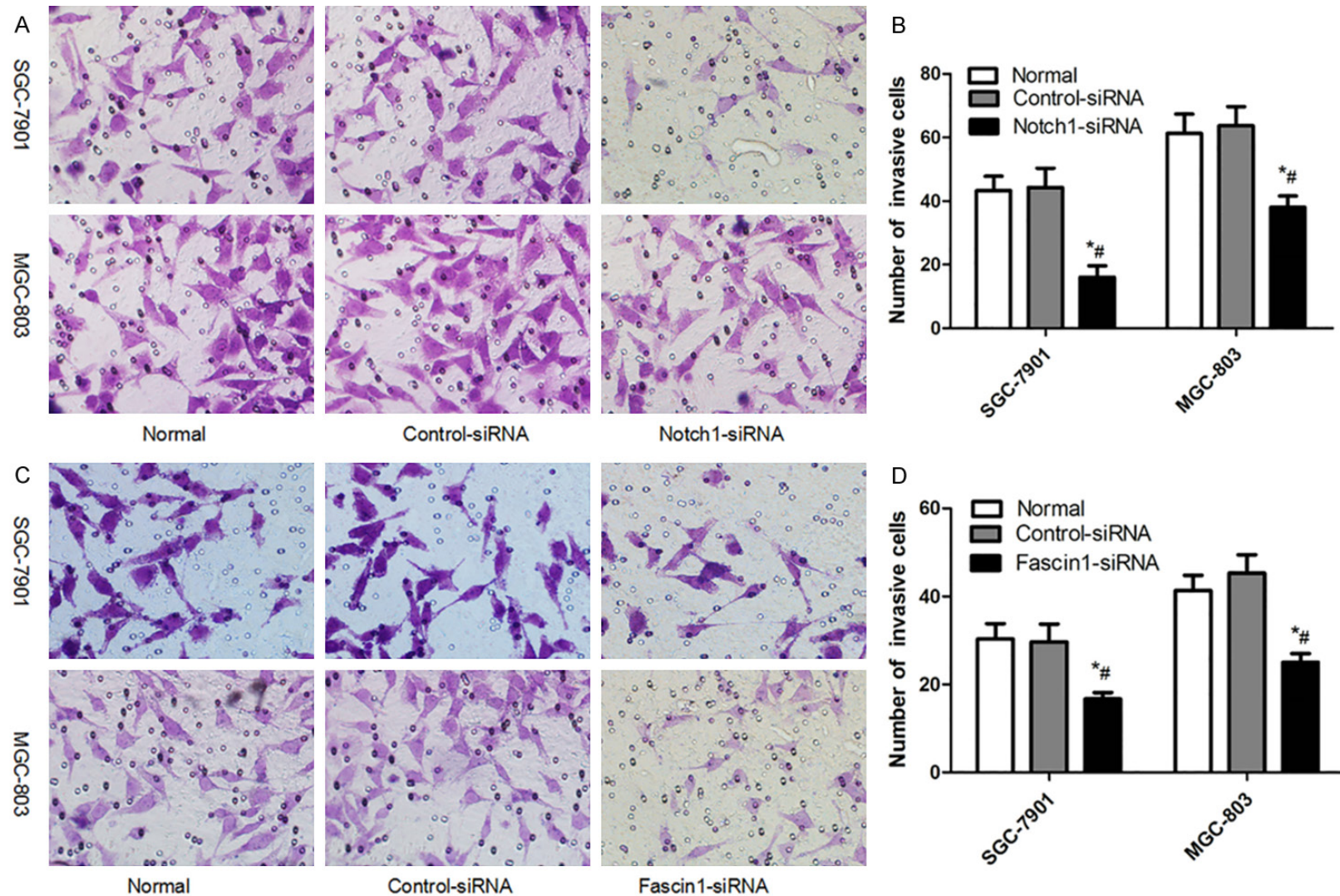
**Figure 2.** Knockdown of Notch1 or Fascin1 knockdown inhibited gastric cancer cells invasion. A and B. knockdown of Notch1 inhibited invasion of SGC-7901 and MGC-803 cells. C and D. knockdown of Fascin1 inhibited invasion of SGC-7901 and MGC-803 cells. *: Compared with Normal group, $P < 0.05$; #: Compared with Control-siRNA group, $P < 0.05$.

**Figure 3.** Knockdown of Notch1 or Fascin1 knockdown inhibited gastric cancer cells proliferation. A. SGC-7901 cells were transfected with Fascin1-siRNA and Control-siRNA. B, C. Knockdown of Fascin1 or Notch1 inhibited proliferation of SGC-7901 cells. D. MGC-803 cells were transfected with Fascin1-siRNA and Control-siRNA. E, F. Knockdown of Fascin1 or Notch1 inhibited proliferation of MGC-803 cells. *: Compared with Normal group, $P < 0.05$; #: Compared with Control-siRNA group, $P < 0.05$.
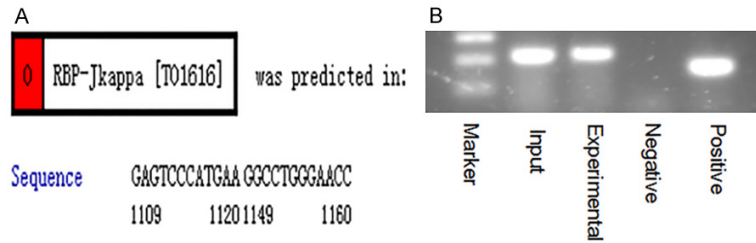
**Figure 4.** Notch1 regulates Fascin1 expression at transcriptional level. A. Potential RBP-JK binding sites in the Fascin1 promote predicted using PROMO. B. ChIP assay of the binding of RBP-JK to Fascin1 promoter using MGC-803 cells. Bands in the experimental group, input group and positive control group, but not in the negative control group.

*Knockdown of Notch1 inhibits gastric cancer cell proliferation and invasion*

MGC-803 and SGC-7901 cells were transfected with Notch1-siRNA and Control-siRNA and cultured for 24 h. Thereafter, transwell and CCK8 assays were performed to examine cell invasion and proliferation. We found that the invasion ability of the Notch1-siRNA group was significantly lower than those of the Control-siRNA and Normal groups for both gastric cancer cell lines (**Figure 2A**, **2B**). Furthermore, measurements of cell proliferation at 24 h, 48 h, and 72 h revealed significantly lower proliferation of cells transfected with Notch1-siRNA than of the other two groups for both cell lines (**Figure 3C**, **3F**). Thus, Notch1 knockdown significantly reduced gastric cancer cell proliferation and invasion.

*Knockdown of Fascin1 inhibits gastric cancer cell proliferation and invasion*

To test the role of Fascin1 in proliferation and invasion, we transfected MGC-803 and SGC-7901 gastric cancer cells with Fascin1-siRNA and Control-siRNA and cultured them for 24 h. The results of the transwell assay showed that the invasion ability of cells transfected with Fascin1-siRNA was significantly lower than for those transfected with Control-siRNA or no siRNA in both gastric cancer cell lines (**Figure 2C**, **2D**). In addition, the CCK8 assay results showed that the cell proliferation of the Fascin1-siRNA group was significantly lower than that of the other two groups in both cell lines (**Figure 3B**, **3E**). Thus, as for Notch1, Fascin1 knockdown significantly reduced the proliferation and invasion ability of MGC-803 and SGC-7901 cells.

*Notch1 regulates Fascin1 expression at transcriptional level*

To determine whether Notch1 regulates Fascin1 expression at the transcriptional level, we bioinformatically predicted two binding sites for the Notch1 signaling pathway transcription factor RBP-JK on the Fascin1 promoter (GAGTCCCATGAA and GGCC-TGGGAACC) (**Figure 4A**). We tested this prediction using ChIP. After agarose gel electrophoresis, we identified amplified bands in the experimental group but not in the negative control group; bands were also visible in the input group and the positive control group (**Figure 4B**). These results demonstrated that RBP-JK could directly bind the promoter region of Fascin1 in cells and suggested that Notch1 regulates Fascin1 expression at the transcriptional level.

*Expression of Notch1 and Fascin1 in cancerous tissues and adjacent normal tissues*

Both Notch1 and Fascin1 were mainly expressed in the cytoplasm (**Figure 5A**, **5B**). The positive expression rates of Notch1 in cancerous tissues and adjacent normal tissues were 62.5% and 20%, respectively ($P < 0.05$). The positive expression rate of Fascin1 in cancerous tissues was 30%, but there was no measurable expression in adjacent normal tissues ($P < 0.05$).
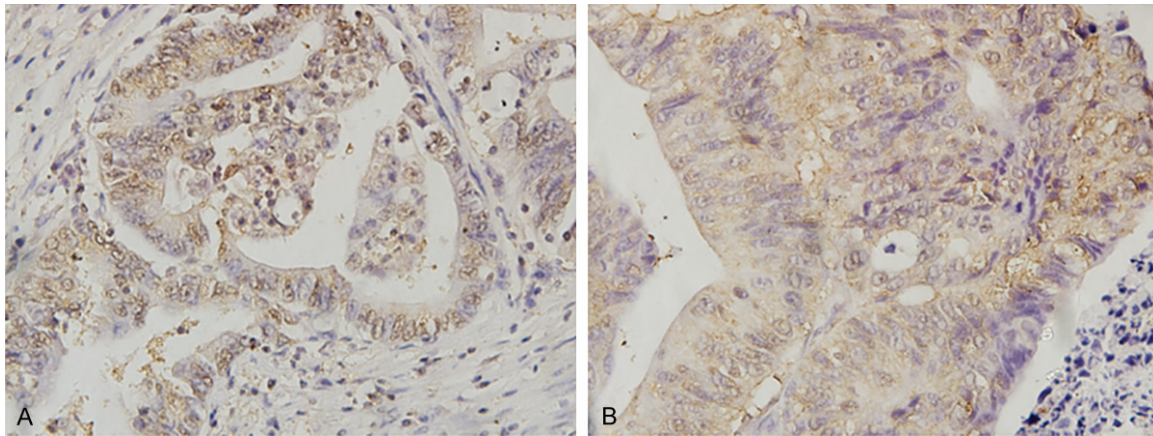
*Correlation of Notch1 and Fascin1 expression in gastric cancer tissues*

There were 11 cases with positive expression of both Notch1 and Fascin1 and 14 with negative expression of both. In addition, 14 cases exhibited positive Notch1 expression and negative Fascin1 expression, and one case showed negative Notch1 expression and positive Fascin1 expression. In gastric cancer, the expression of Notch1 and Fascin1 was positively correlated ($r = 0.394$, $P = 0.012$).

**Discussion**

As an evolutionarily conserved signaling pathway, Notch signaling is found in organisms from

**Figure 5.** Immunohistochemistry was done to analysis the different expression of Notch1/Fascin1 in cancerous tissues and adjacent normal tissues. The positive expression of Notch1 (A) and Fascin1 (B) in gastric cancer tissues (400×).

fruit flies to mammals and plays a very important role in cell development, differentiation, proliferation, apoptosis, and regulation of tissue homeostasis and neoplastic processes [13]. There are four kinds of Notch receptors (Notch1-4) and five kinds of Notch ligands (Jagged1, 2 and Delta-like 1, 3, 4) in mammals, and Notch signaling pathways are classified as Notch1, 2, 3, or 4 depending on the receptor involved [14]. Recent studies found that Notch signaling was involved in the occurrence and development of many tumors. Although most research has focused on Notch1 instead of the other Notch receptors and ligands, the role of Notch1 in the occurrence, progression, and prognosis of human malignant tumors remains of great interest [3, 15, 16].

Our results herein support the previous finding that Notch1 is highly expressed in gastric carcinoma [17]. Some scholars found that siRNA-mediated Notch1 silencing inhibited the proliferation and invasion of SGC-7901 cells [8]. In gastric cancer cell line BGC-823, down-regulation of Notch1 expression inhibited proliferation and reduced migration and invasion, suggesting that Notch1 might participate in carcinogenesis and metastasis of gastric cancer cells [18]. These results indicate that Notch1 is involved in the occurrence and development of gastric cancer and promotes the proliferation and invasion of gastric cancer cells.

Fascin1 is an actin-binding protein associated with tumor progression and tumor cell motility. In gastric carcinoma tissues, Fascin1 expression is often dramatically up-regulated, whereas it is usually undetectable in normal gastric

mucosa tissues [19]. High expression of Fascin1 in gastric carcinoma can promote gastric cancer cell proliferation, invasion, and metastasis [12, 19, 20]. Our experimental results supported this conclusion. Thus, Fascin1 is an oncogene in gastric carcinoma that promotes the invasion and proliferation of tumor cells.

In our study, knockdown of Notch1 or Fascin1 knockdown inhibited gastric cancer cell proliferation and invasion, and Notch1 expression was positively correlated with Fascin1 expression in human gastric cancer tissues. Furthermore, we showed that Notch1 knockdown downregulated Fascin1 expression at both the mRNA and protein levels. To explore the mechanism by which Notch1 regulates Fascin1 expression, we predicted two potential RBP-JK binding sites (GAGTCCCATGAA and GGCC-TGGGAACC) in the Fascin1 promoter and verified direct binding of RBP-JK to the Fascin1 gene by ChIP. Therefore, our results suggest that the Notch1 regulates Fascin1 expression at the transcriptional level.

In conclusion, Notch1 knockdown can down-regulate Fascin1 mRNA and protein expression, and Notch1 signaling regulates Fascin1 transcription through the transcription factor RBP-JK. Notch1 knockdown or Fascin1 knockdown decreased the proliferation and invasion of gastric cancer cells, suggesting that Notch1 regulates proliferation and invasion in gastric cancer cells by targeting Fascin1. Our results suggest that the Notch1/RBP-JK/Fascin1 axis could provide a molecular target for gastric cancer therapy.

## Disclosure of conflict of interest

None.

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