Int J Clin Exp Med 2019;12(8):10326-10333
www.ijcem.com /ISSN:1940-5901/IJCEM0092171

Original Article

Notch1 promotes tumor growth in colon cancer by targeting Fascin1

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Received February 1, 2019; Accepted June 10, 2019; Epub August 15, 2019; Published August 30, 2019

Abstract: Dysregulation of Notch1 expression is associated with tumor growth. However, the precise mechanism by which dysregulated Notch1 expression induces colon cancer growth remains unknown. In the present study, expression levels of Notch1 and Fascin1 were found to be higher in human colon cancer tissues than in the adjacent non-malignant tissues, and their expression patterns were positively correlated in colon cancer. Following Notch1 knockdown, expression levels of Fascin1 mRNA and protein were significantly decreased in the colon cancer cell lines, HCT-116 and SW-620. Inhibition of colon cancer cell proliferation *in vitro*, following Notch1 knockdown, was detected by the CCK-8 assay. However, the ability of the cells to proliferate improved in the Notch1-siRNA+Fascin1 plasmid group compared to that in the Notch1-siRNA group. Fascin1 could rescue the inhibited proliferation of colon cancer cells induced by the down-regulation of Notch1. Furthermore, knockdown of Notch1 significantly inhibited growth of implanted colon cancers in nude mice. Western blot analysis showed that Fascin1 expression positively correlated with Notch1 expression in the implanted tumor tissues of nude mice. These findings indicate that Notch1 plays a critical role in tumor growth in colon cancer, possibly by directly targeting Fascin1. The Notch1/Fascin1 axis may, therefore, act as a potential molecular target for colon cancer therapy.

Keywords: Notch1, Fascin1, colon cancer, proliferation, growth

Introduction

Colon cancer is the most common and universal malignant tumor of the digestive tract [1]. In China, it has a very high incidence and mortality rate [2]. According to WHO data, there have been 245,000 new cases of colorectal cancer and 139,000 colorectal cancer-related deaths in China in 2012 [3]. Owing to the lack of relevant preventive health care, most patients with colon cancer in China and other developing countries are diagnosed at stage III or stage IV. This reduces the probability of surgical interventions in these patients. Therefore, it is important to elucidate the mechanism underlying the proliferation of the colon cancer cells for the prevention and treatment of colon cancer.

Studies have shown that the Notch1 signaling pathway plays an important role in regulating cell differentiation, organogenesis, and development under physiological conditions [4, 5]. Further, studies have also shown that the Notch1 signaling pathway plays an important role in the proliferation, differentiation, invasion, metastasis, and apoptosis of cancer cells [6-8]. Notch1, an important oncogene in colon cancer, plays a central role in Notch signaling [9-12]. However, the underlying mechanism of Notch1-regulated proliferation in colon cancer cells remains unclear.

Fascin1 is an evolutionarily conserved actin-binding protein, located in the sub-plasmalemmal spines, filamentous pseudopods, and actin-based processes. Fascin1 has been correlated with the growth and metastasis of tumors [13-15]. It is highly expressed in colon cancer and promotes the proliferation inhibiting apoptosis [16-18]. It has been reported that Notch1 can positively regulate the expression of Fascin1 in gastric cancer cells and further regulate its proliferation [19]. Therefore, Notch1 may regulate the proliferation of colon cancer cells by regulating the expression of Fascin1. Cell lines and animal models of colon cancer were used to test this hypothesis.

Materials and methods

Antibodies and reagents

Human tissue microarrays of colon cancer were obtained from Xian Alina. Human colon cancer cell lines, HCT-116 and SW-620, were purchased from Shanghai Cell Bank of Chinese Academy of Sciences. Notch1 antibody and beta-actin antibody were from Cell Signaling Technology. Fascin1 antibody was from R&D Systems. Fascin1-siRNA and Control-siRNA were from Santa Cruz Biotechnology. Lipofectamine® 2000 Reagent was from Invitrogen. SP immunohistochemical kit was from ZSGB-BIO. Female BALB/c-Nude nude mice aged 4-6 weeks with an average mass of 18-20 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.

Immunohistochemistry and determination of results

This study was approved by the Institutional Ethics Committee of Taian Central Hospital. The colon cancer tissue microarrays contain both colon cancers tissues and their corresponding adjacent mucosal tissues, including 85 patients. None of the patients had undergone chemotherapy or radiotherapy before surgery. Expression of Notch1 and Fascin1 was detected using a SP kit according to the manufacturer's instructions. The results were analyzed by a two-level scoring method to obtain a combined scoring of the proportion of the stained cells and staining intensity [19].

Cell culture and transfection

Human colon cancer cell lines: HCT-116 and SW-620, were cultured in a constant temperature incubator with RPMI-1640 culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution. The cells were divided into four groups: Control-siRNA group (transfected with Control-siRNA), Notch1-siRNA group (transfected with Notch1-siRNA), Notch1-siRNA+Fascin1 plasmid group (co-transfected with Notch1-siRNA and Fascin1 plasmid), and Notch1-siRNA+vector group (co-transfected with Notch1-siRNA and Control plasmid). The cells were inoculated on a six-well plate and transfected when cell confluence reached 70% using Lipofectamine® 2000 Reagent, according to the manufacturer's instructions.

Real-time PCR

Total RNA was extracted using TRIzol reagent and the RNA concentration was determined. mRNA level was quantified with the real-time PCR. The primer sequences were as follows: Notch1 forward 5'-CGCCTTTGTGCTTCTGTTCTTCG-3'; and reverse 5'-TTCTTGGTCTCCAGGTCCTCGTC-3'; Fascin1 forward 5'-GCCAGGGTATGGACCTGTCTG-3' and reverse 5'-CACGCCACTCGATGTCAAAGTA-3'; β-actin forward 5'-AGCGAGCATCCCCCAAAGTT-3' and reverse 5'-GGGCACGAAGGCTCATCATT-3'.

Western blot analysis

Total cell protein from the HCT-116 and SW-620 cells was extracted 72 h post-transfection. Equal (30 μg per sample) amounts of protein were electrophoresed by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% skimmed milk powder for 1 hour at room temperature. The protein samples were incubated with the corresponding primary antibodies with gentle shaking at 4°C overnight. The next day, the primary antibodies were washed, and the protein was incubated with the secondary antibodies for 1 h at room temperature. Signals were detected using the ECL method and scanned using the Bio-Rad gel imaging analysis system.

Viability assay

The CCK-8 method was used. A single cell suspension was prepared using logarithmic growth phase cells of each group. The cells were inoculated into the 96-well plates. After culturing for 24, 48, or 72 hours, the culture medium was discarded and 100 μL complete culture medium was added to each well. Each hole was incubated for 2 hours at 37°C after adding 10 μL CCK-8 working fluid. The optical density at 450 nm was measured by the enzyme labeling instrument.

Model of subcutaneous implantation tumors in nude mice

Notch1-shRNA and Control-shRNA of Lentivirus packaging with interference vectors were syn-

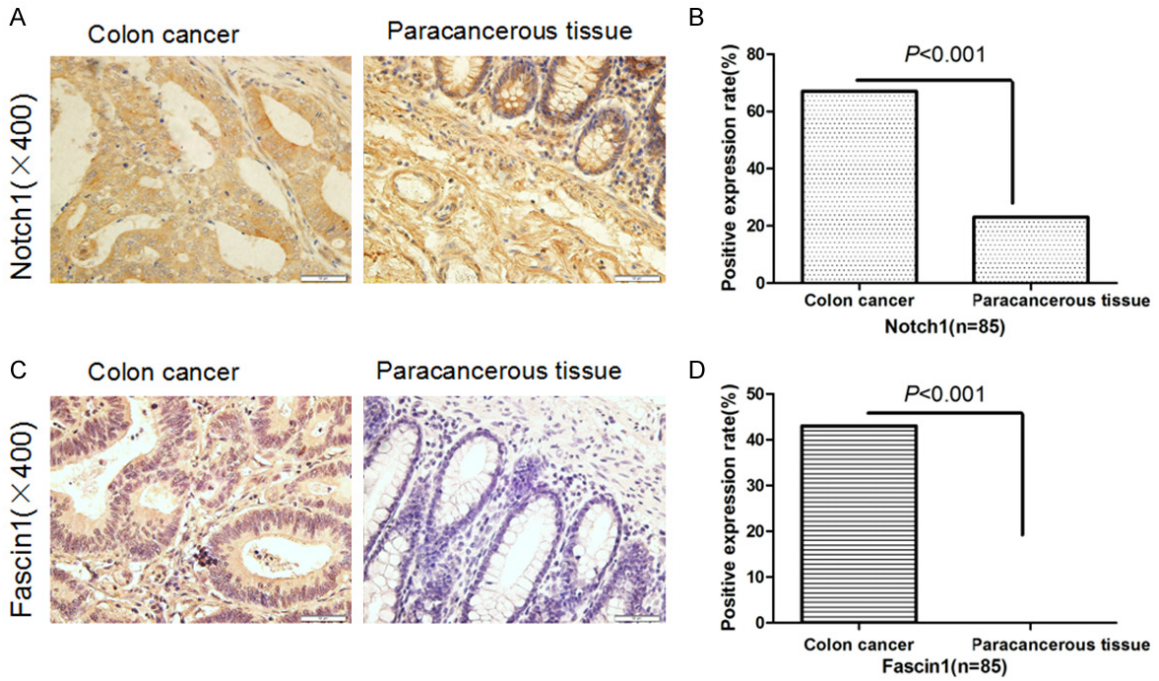

Figure 1. Immunohistochemistry analysis of the expression of Notch1 and Fascin1 in colon cancer and adjacent tissues (×400). A-D. Notch1 and Fascin1 expression were significantly higher in the colon cancer tissues than in adjacent tissues ($p < 0.001$).

thesized by Shanghai GenePharma Co., Ltd, transfected into HCT-116 cells, and screened and sub-cultured for three generations to form stable expression cell lines. To determine the proliferation capacity of Notch1-shRNA-HCT-116 (Notch1-shRNA group) and Control-shRNA-HCT-116 (Control-shRNA group) in vivo, a total of 1×10^8 cells was injected subcutaneously into nude mice. There were 4 nude mice in each group. The growth of tumors was observed, and the long axis and short axis of tumors were measured every week. Four weeks later, the nude mice were sacrificed by cervical dislocation. The transplanted tumors were removed, and the sizes of the subcutaneous implanted tumors in each group were measured. The tumors were weighed and the average value was calculated.

Statistical analysis

All experiments were repeated at least three times. All data was analyzed using SPSS 13.0. T-test was used to compare two group, and three groups were performed by one-way ANOVA. The counting data was analyzed by chi-square test. $p < 0.05$ was considered statistically significant.

Results

High expression of Notch1 and Fascin1 in colon cancer tissues compared with adjacent tissues

Notch1 was mainly expressed in the cytoplasm of tissues on the colon cancer microarray. The positive expression rates of Notch1 in colon cancer tissues and paracancerous tissues were 67.1% (57/85) and 23.5% (20/85), respectively ($P < 0.05$) (**Figure 1A**, **1B**). Fascin1 was mainly expressed in the cytoplasm, and its positive expression rate was 43.5% (37/85) in the colon cancer tissues. However, there was no Fascin1 expression in para-cancerous tissues($P < 0.05$) (**Figure 1C**, **1D**).

Positive correlation of expression of Notch1 and Fascin1 in colon cancer

Notch1 and Fascin1 were co-expressed in 32 samples with colon cancer; 25 samples were Notch1-positive and Fascin1-negative and 5 samples were Notch1-negative and Fascin1-positive. Notch1 and Fascin1 expression was not observed in 23 samples. Overall, in colon cancer, expression of Notch1 and Fascin1 were positively correlated ($r=0.363$, $p=0.001$).

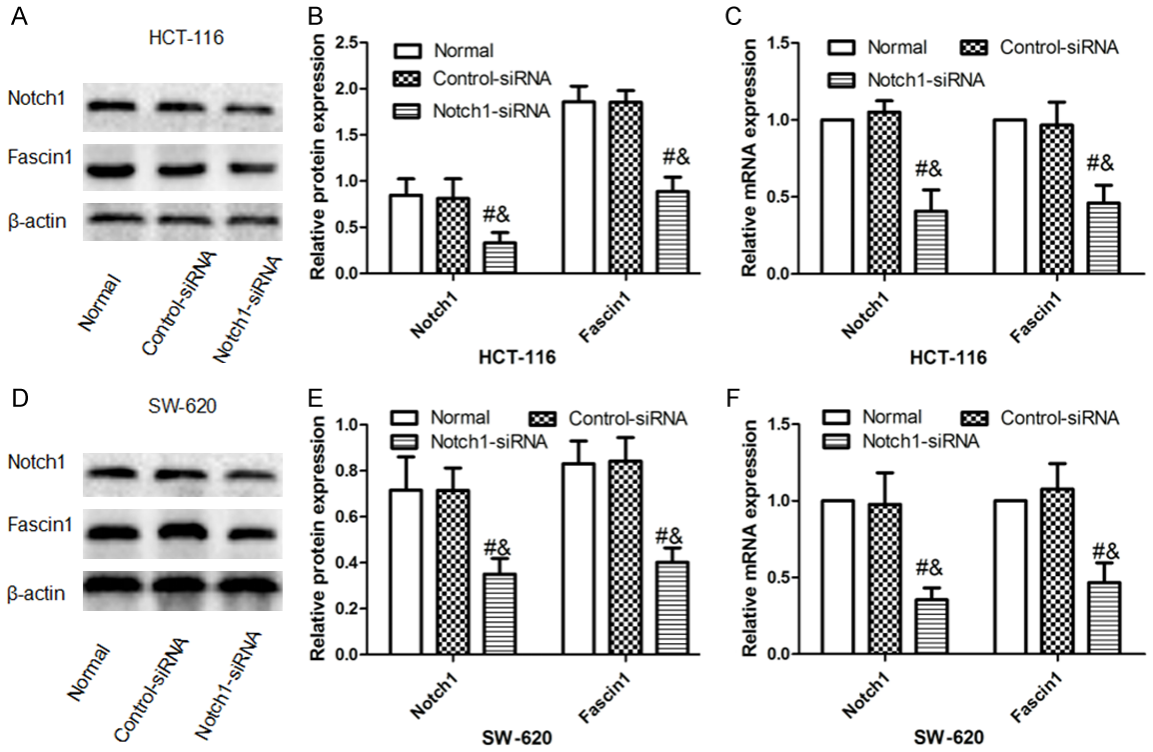

Figure 2. Notch1 regulates Fascin1 expression in colon cancer cells. A. HCT-116 cells were transfected with Notch1-siRNA and Control-siRNA; B, C. Notch1/Fascin1 protein and mRNA levels were detected by real-time PCR and Western blot analysis. D. SW-620 cells were transfected with Notch1-siRNA and Control-siRNA; E, F. Notch1/Fascin1 protein and mRNA 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, $p < 0.05$.

Notch1 directly promotes Fascin1 expression in colon cancer cells

To investigate whether Notch1 could regulate Fascin1, Notch1-siRNA and Control-siRNA were transfected into the colon cancer cell lines, HCT-116 and SW-620. mRNA and protein expression levels of Notch1 and Fascin1 were detected by real-time PCR and western blotting, respectively. Upon siRNA knockdown of Notch1, the mRNA and protein expression of Fascin1 was significantly decreased in both the cell lines (**Figure 2**). These results indicate that Notch1 could positively regulate the Fascin1 expression.

Notch1 promotes the proliferation of colon cancer cells by targeting Fascin1 in vitro

HCT-116 and SW-620 cells were transfected with either Control-siRNA, Notch1-siRNA, Notch1-siRNA+Fascin1 plasmid, or Notch1-siRNA+vector (Control plasmid). Western blotting was performed to check the expression level of Fascin1 (**Figure 3A**, **3C**). Thereafter, the CCK-8 assay was performed to examine cell proliferation ability at 0, 24, 48, and 72 hours. In HCT-116 and SW-620 cells, the proliferation ability of cells in the Notch1-siRNA group decreased significantly as compared to the Control-siRNA group. However, the proliferation ability of the cells improved in the Notch1-siRNA+Fascin1 plasmid group compared to the Notch1-siRNA group. There was no difference between the cell proliferations of the Notch1-siRNA and Notch1-siRNA+vector groups (**Figure 3B**, **3D**). This indicated that Fascin1 can rescue proliferation defects of colon cancer cells induced by the down-regulation of Notch1 expression. Collectively, these results suggest that Notch1 regulates the proliferation of colon cancer cells by targeting Fascin1 *in vitro*.

Notch1 may promote colon cancer cells growth in vivo by targeting Fascin1

Subcutaneous nodules were observed 7 days after inoculation of the implanted tumors in nude mice. The tumorigenesis rate was 100%. The results showed that the volumes of the

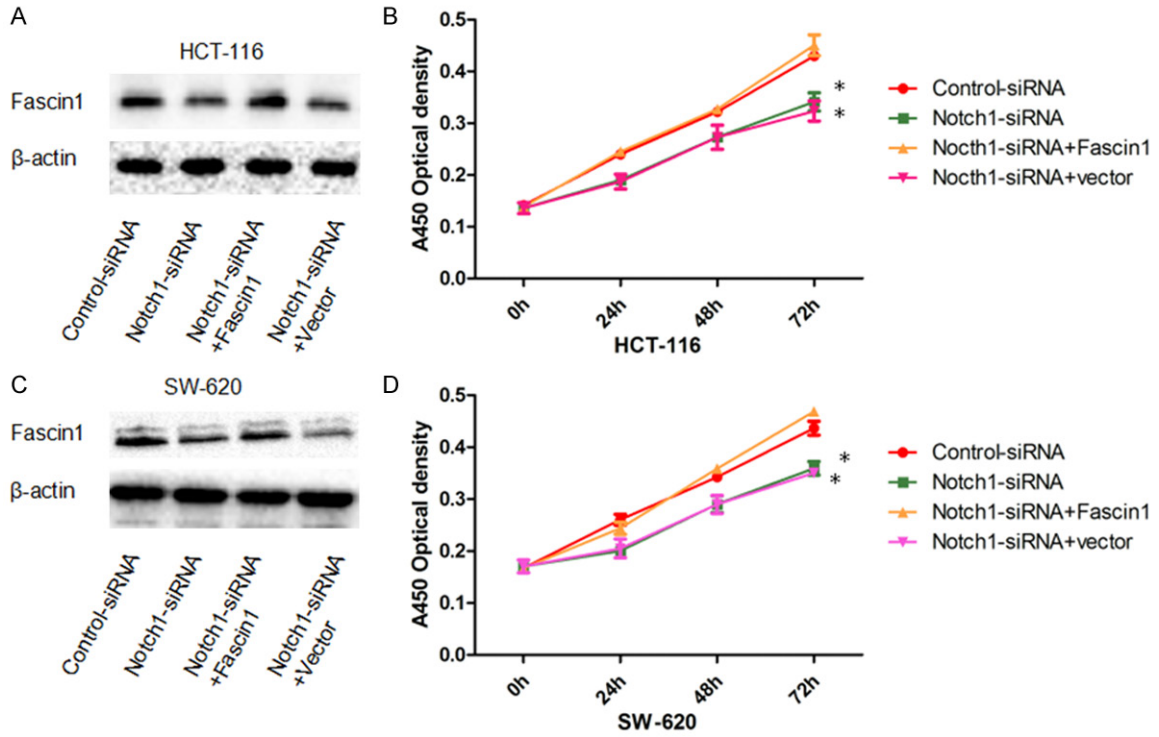

Figure 3. Notch1 regulates the proliferation of colon cancer cells by targeting Fascin1. A, C. HCT-116 and SW-620 cells were transfected with Control-siRNA, Notch1-siRNA, Notch1-siRNA+Fascin1 plasmid, and Notch1-siRNA+vector (Control plasmid). B. Knockdown of Notch1 inhibited proliferation of HCT-116 cells, but co-transfection of Notch1-siRNA+Fascin1 into HCT-116 cells significantly rescues proliferation of HCT-116 cells after suppression of Notch1-siRNA. D. Knockdown of Notch1 inhibited proliferation of SW-620 cells, but co-transfection of Notch1-siRNA+Fascin1 into SW-620 cells significantly rescues proliferation of SW-620 cells after suppression of Notch1-siRNA. *: $p < 0.05$.

subcutaneous implanted tumors in the Notch1-shRNA group was lower than that in the Control-shRNA group at 7, 14, 21, and 28 days (**Figure 4A**). The results showed that the sizes of the tumors in the Notch1-shRNA group was significantly lower than that in Control-shRNA group (**Figure 4B**, **4C**). In addition, Western blot analysis revealed that tumors from the Notch1-shRNA group had reduced Fascin1 levels compared with Control-shRNA group (**Figure 4D**). In summary, these results suggest that Notch1 may promote the growth of colon cancer *in vivo* by upregulating Fascin1 expression. These observations were consistent with the *in vitro* results.

Discussion

In the recent years, a large number of studies have found that the abnormalities in various signaling pathways play an important role in the occurrence and development of cancer [20-22]. Currently, it is clear that colon cancer is a complex process involving multi-step and multi-gene participation, which involves the interconnection of multiple signaling pathways [23, 24]. Among many signaling pathways, the Notch signaling pathway has become a hotspot in the field of colon cancer research. Notch1 is an oncogene and plays a central role in the Notch signaling pathway [10, 25].

The Notch signaling pathway is involved in many stages of intestinal development and intestinal epithelial renewal, and the abnormal activation of Notch1 signaling pathway is associated with the occurrence and development of colon cancer [26-28]. Notch1 expression in colon cancer tissues was significantly higher than that in para-cancerous tissue [25, 29]. Notch1 overexpression stimulates colon cancer cell outgrowth [12]. Consequently, Notch1 knockdown can significantly inhibit the proliferation of colon cancer cells [27]. In colon cancer tissues, Fascin1 expression is often markedly up-regulated, whereas it is usually undetectable in adjacent tissues [19]. The growth of tumor cells in colon cancer is associated with

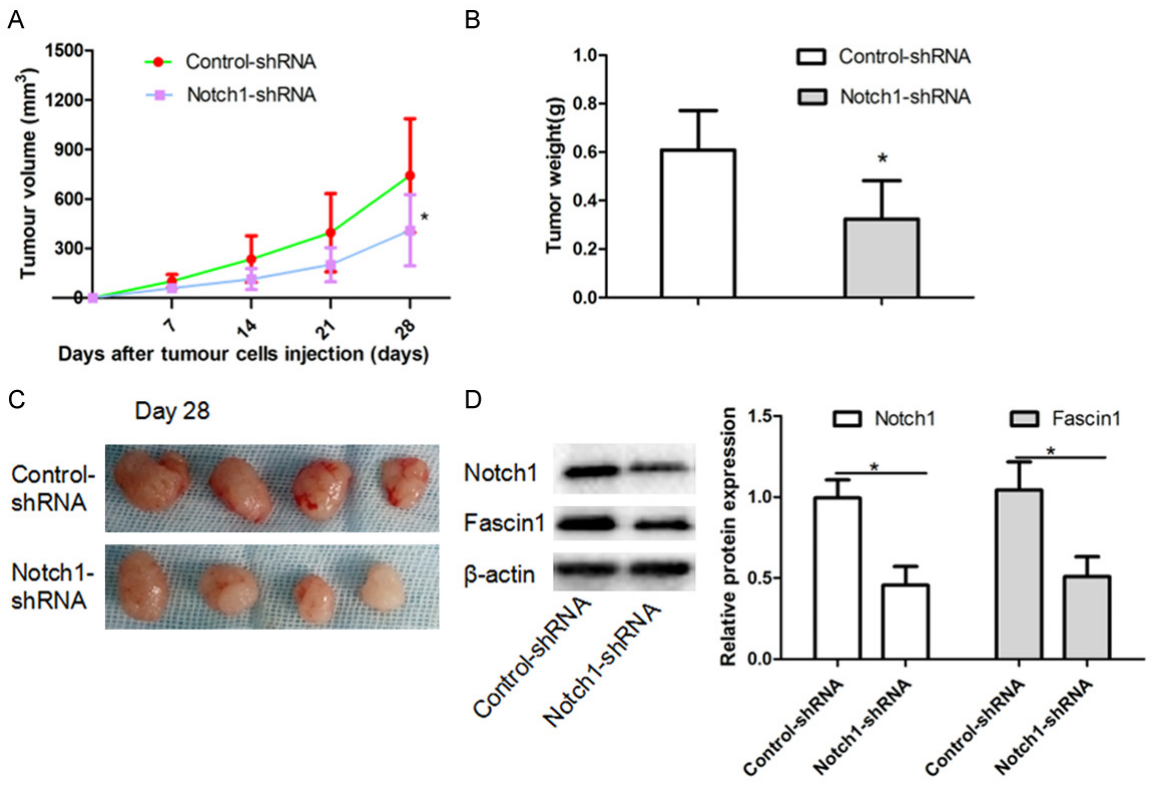

Figure 4. Knockdown of Notch1 inhibited colon cancer growth *in vivo*. A-C. Stable low-expression of Notch1 suppressed colon cancer growth in vivo. D. Notch1-shRNA group had reduced Fascin1 levels compared with Control-shRNA group by western blot analysis. *: $p < 0.05$.

an enhanced expression level of Fascin1 [16, 17, 30]. These results were also confirmed by our experiments, indicating that both Notch1 and Fascin1 are involved in colon cancer cell proliferation.

In gastric cancer, Notch1 can positively regulate the expression of Fascin1 and regulate the proliferation of cancer cells by targeting Fascin1 [19]. Therefore, a similar mechanism may exist in colon cancer. Expression levels of Notch1 and Fascin1 in human colon cancer were thus analyzed and Notch1 correlated positively with Fascin1 in colon cancer. This result supported that Notch1 targets to Fascin1. Furthermore, knock-down of Notch1 in HCT-116 and SW-620 cells showed that Fascin1 expression was significantly decreased. This further confirmed regulation of Fascin1 by Notch1. Notch1 may thus promote growth of colon cancer by regulating Fascin1.

In colon cancer cells HCT-116 and SW-620, Notch1 knockdown significantly reduced the proliferation of colon cancer cells. When Notch1-siRNA and Fascin1 plasmid were co-transfected into colon cancer cells, the proliferation of tumor cells significantly improved as compared to cells with only Notch1 knockdown. This shows that Fascin1 can improve the proliferation ability of the colon cancer cells by down-regulation of Notch1. These results suggest that Notch1 regulates the proliferation of colon cancer cells by regulating the Fascin1 expression *in vitro*. In the subcutaneous tumorigenesis experiment in nude mice, Notch1 knockdown led to a significant decrease in the tumor proliferation. Western blotting further confirmed that expression of Fascin1 in Notch1-shRNA group was significantly decreased, which further demonstrated the positive regulatory role of Notch1 on Fascin1. The *in vitro* and *in vivo* data suggests that Notch1 promotes growth of colon cancer by upregulating Fascin1 expression.

In conclusion, Notch1 was found to promote growth of colon cancer by functioning as an oncogene through direct induction of Fascin1. The Notch1/Fascin1 axis may therefore have potential therapeutic value in the treatment of colon cancer.

Acknowledgements

This work was supported by the Taian City Science and Technology Development Plan Project (2018NS0246) and the Shandong Province Medical and Health Science and Technology Development Plan Project (2018WS155).

Disclosure of conflict of interest

None.

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