

Silencing of Jagged1 inhibits cell growth and invasion in colorectal cancer

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Dysregulated Notch signaling has a critical role in the tumorigenesis. Jagged1, a Notch ligand, is overexpressed in various human cancers. Recent studies revealed the involvement of Jagged1 in colorectal cancer (CRC) development. These basic studies provide a promising potential for inhibition of the Notch pathway for the treatment of CRC. Herein, we aimed to investigate the consequences of targeting Jagged1 using shRNA on CRC both *in vitro* and *in vivo* to test their potential to inhibit this key element for CRC treatment. We found that downregulation of Jagged1 with lentiviral Jagged1-shRNA resulted in decreased colon cancer cell viability *in vitro*, most likely mediated through reduced cell proliferation. Importantly, Jagged1 knockdown induced G₀/G₁ phase cell cycle arrest, with reduced Cyclin D1, Cyclin E and c-Myc expression. Silencing of Jagged1 reduced the migration and invasive capacity of the colon cancer cells *in vitro*. Furthermore, colon cancer cells with knockdown of Jagged1 had much slower growth rate than control cells in a xenograft mouse model *in vivo*, with a marked downregulation of cell proliferation markers (PCNA, Ki-67, and c-Myc) and metastasis markers (MMP-2 and MMP-9). These findings rationalize a mechanistic approach to CRC treatment based on Jagged1-targeted therapeutic development.

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Subject Category: Cancer

Colorectal cancer (CRC) is the third most common malignancy in males and second most common in females worldwide.¹ The highest incidence of CRC is found in Australia, New Zealand and North America, and the morbidity is rapidly increasing in Eastern Asia and Eastern Europe.^{2,3} About 40–50% of all patients with CRC will present with metastasis either at the time of diagnosis or develop distant relapses after therapy, and the median overall survival of metastatic CRC is less than 2 years.⁴ Current therapeutic options for advanced CRC, such as chemotherapy and radiotherapy, only have limited efficacy and can barely improve patient survival. A better understanding of the molecular mechanisms involved in CRC development and progression is imperative for the improvement of therapeutic approaches that can benefit patients with CRC.

The Notch signaling has an important role in cell growth and differentiation, which affects the development and function of many organs.^{5,6} Notch receptors (Notch1–4 in humans) are a

group of transmembrane proteins in many cell types. Binding to their ligands (Jagged1, 2 and delta-like ligand (DLL) 1, 3, and 4) on the surface of neighboring cells leads to the cleavage of Notch receptor by γ -secretase and subsequent release of the Notch intracellular domain (NICD). As the constitutively active domain of the Notch receptor, NICD can translocate to the nucleus, where it binds to and forms a complex with the transcriptional regulator termed CBF-1-Su (H), and LAG-1 (CSL) or RBP-J ϵ , leading to the displacement of co-repressors previously bound to CSL and recruitment of co-activators. The co-activators then induce expression of the target genes, such as the hairy and enhancer of split (Hes) and Hes-related repressor protein (Hey) families. Accumulating evidences indicate that dysregulated Notch pathway has a critical role in the progression of several malignancies such as prostate cancer, breast cancer, glioma and head and neck cancers. Furthermore, high expression levels of several key members of the Notch cascade such as Notch1 and Jagged1

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Abbreviations: Apex, purinic-apyrimidinic endonuclease-1; CDK, cyclin-dependent kinase; CRC, colorectal cancer; DLL, delta-like ligand; Hes, hairy and enhancer of split; Hey, Hes-related repressor protein; L-Jagged1-shRNA, lentiviral Jagged1-shRNA; MMP, matrix metalloproteinase; NICD, notch intracellular domain; PCNA, proliferating cell nuclear antigen

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are associated with increased progression and metastatic potential, recurrence and a poorer overall survival.^{7–11} Recently, Lu *et al.*¹² showed endothelial cells could secrete Jagged1 and mediate the activation of Notch signaling in CRC, leading to increased expression of Hes1. Kim *et al.*¹³ demonstrated that Jagged1 was a mediator of a purinic–pyrimidinic endonuclease-1 (apex)-induced CRC progression.

The role of Notch signaling in the development of CRC has attracted wide attention over the past few years.¹⁴ Animal studies have shown that Notch is active in intestinal crypts and adenomas in APC^{Min/+} mice.¹⁵ Moreover, deletion of one Jagged1 allele is sufficient to significantly reduce the size of tumors in the APC mutant background concomitant with a reduction in the amount of active Notch1.¹⁶ It was shown that Notch signaling is strongly activated in primary human CRC and has an important role in the initiation and progression of CRC through the regulation of the main cellular functions associated with tumorigenesis, such as apoptosis, proliferation, angiogenesis, and cell migration.^{17–19} Thus, the Notch pathway has been regarded as an attractive target for CRC therapy.^{20,21} One approach currently being explored in clinical trials is blocking the cleaving process of Notch receptors with γ -secretase inhibitors. These agents have shown the therapeutic benefit for CRC.²² Although γ -secretase inhibitors are valuable tools for delineating the function of Notch cascade, they were found to be not specific for Notch signaling and were associated with various adverse effects such as gastrointestinal toxicity and liver injury.²³ Notch downregulation is fundamental in driving secretory cell differentiation in the normal intestinal mucosa^{15,24,25} thus, inactivation of the Notch signaling causes secretory cell metaplasia, and results in inflammatory response.²⁵ The efficacy of this class of compounds needs exploring, but the relative lack of target specificity suggests that new and more specific strategies targeting this pathway should be pursued. A recent study has demonstrated that inactivation of DLL1 and DLL4-mediated Notch signaling results in loss of intestinal proliferating progenitors due to conversion into postmitotic goblet cells, but inducible deletion of Jagged1 has no overt phenotype.²⁶ Moreover, expression of Jagged1 was restricted to enteroendocrine cells or undetectable in the mucosa of the human small and large intestine, respectively. In contrast, increased expression was found in human colon tumors.²⁰ These data suggest that Jagged1 may be a more specific target molecule for developing new therapy against CRC.

As most studies have focused on the effects of inhibiting the Notch receptor and its downstream signaling, specific inhibition of Jagged1 in CRC has not been fully explored. In this study, we aimed to determine the efficacy of specific targeting of Jagged1 as a therapeutic approach for CRC. *In vitro* and *in vivo* models were utilized to investigate the effects of Jagged1 knockdown on cell viability and invasion in CRC.

Results

High expression of Jagged1 in human CRC tissues was associated with activation of the Notch pathway. We first examined the expression of Jagged1 in 24 pairs of human colon cancer tissues and matched non-cancerous colonic mucosa by qPCR. As shown in Figure 1a, the majority

(20/24, or 83%) of cancer tissues (tumor) exhibited higher expression level of Jagged1 relative to their corresponding non-cancerous controls (normal). Figure 1b shows that the average expression of Jagged1 mRNA was ~6-fold higher in tumor tissues than in normal tissues ($P < 0.01$). Higher expression levels (>2.5-fold) of Jagged1 protein in colon cancer tissues were also confirmed by immunohistochemistry (Figures 1c and d). The increased Jagged1 expression in tumor tissues was associated with activation of Notch signaling, with an increased expression of Notch target gene Hes1 (Figure 1e). These data confirm that Jagged1 is frequently overexpressed in CRC and likely responsible for the constitutive activation of Notch signaling, implying that selectively, targeting this protein may present a novel therapeutic strategy in CRC. Therefore, we designed shRNA and packaged it into a lentiviral vector to generate lentiviral Jagged1-shRNA (L-Jagged1-shRNA), and the efficacy of this vector-mediated Jagged1 knockdown against CRC was tested in a series of *in vitro* and *in vivo* assays.

Knockdown of Jagged1 led to reduced Notch signaling activity in CRC cells. Protein expression of Jagged1, as measured by western blot, was prominent in human colon cancer cell lines HCT15, HT29, DLD1, HCT116 and SW480 (Figure 2a). When these colon cancer cells were infected with L-Jagged1-shRNA for 72 h, there was a marked reduction in the expression of endogenous Jagged1 (Figure 2b), confirming a successful gene knockdown. Importantly, silencing Jagged1 was sufficient to significantly decrease the expression of Hes1 at both mRNA and protein levels, as exemplified in HT29 cells (Figures 2c and d, respectively), suggesting that the expression of Jagged1 is a general characteristic of colon cancer cell lines, and that Jagged1 knockdown causes Notch signaling activity inhibition.

Downregulation of Jagged1 induced cell growth inhibition and G₀/G₁ cell cycle arrest in colon cancer cells. Given the increased expression of Jagged1 in the CRC tissues and colon cancer cell lines, we determined whether Jagged1 downregulation affected colon cancer cell proliferation *in vitro*. HCT15, HT29, DLD1 and HCT116 cells were transiently transfected with L-Jagged1-shRNA or scrambled-shRNA for 24 h, and cell viability was assessed by [³H]-thymidine incorporation assay 72 h later. As shown in Figure 3a, Jagged1 downregulation decreased cell viability by 39%, 58%, 47%, and 41% in HCT15, HT29, DLD1, and HCT116 cells, respectively. In addition, Jagged1 knockdown cells exhibited decreased anchorage-dependent growth property. The clonogenicity was reduced by 76%, 62%, and 85% in HCT15, HCT116, and SW480 cells, respectively (Figure 3b), indicating that downregulation of Jagged1 suppressed colon cancer cell proliferation.

To determine the mechanism by which Jagged1 downregulation may affect cell growth, cells were infected with L-Jagged1-shRNA or scrambled-shRNA, and cell cycle analysis was carried out. As shown in Figure 4b, knockdown of Jagged1 led to decrease in the percentage of SW480 cells in S phase by nearly 10% and increased the G₀/G₁ fraction

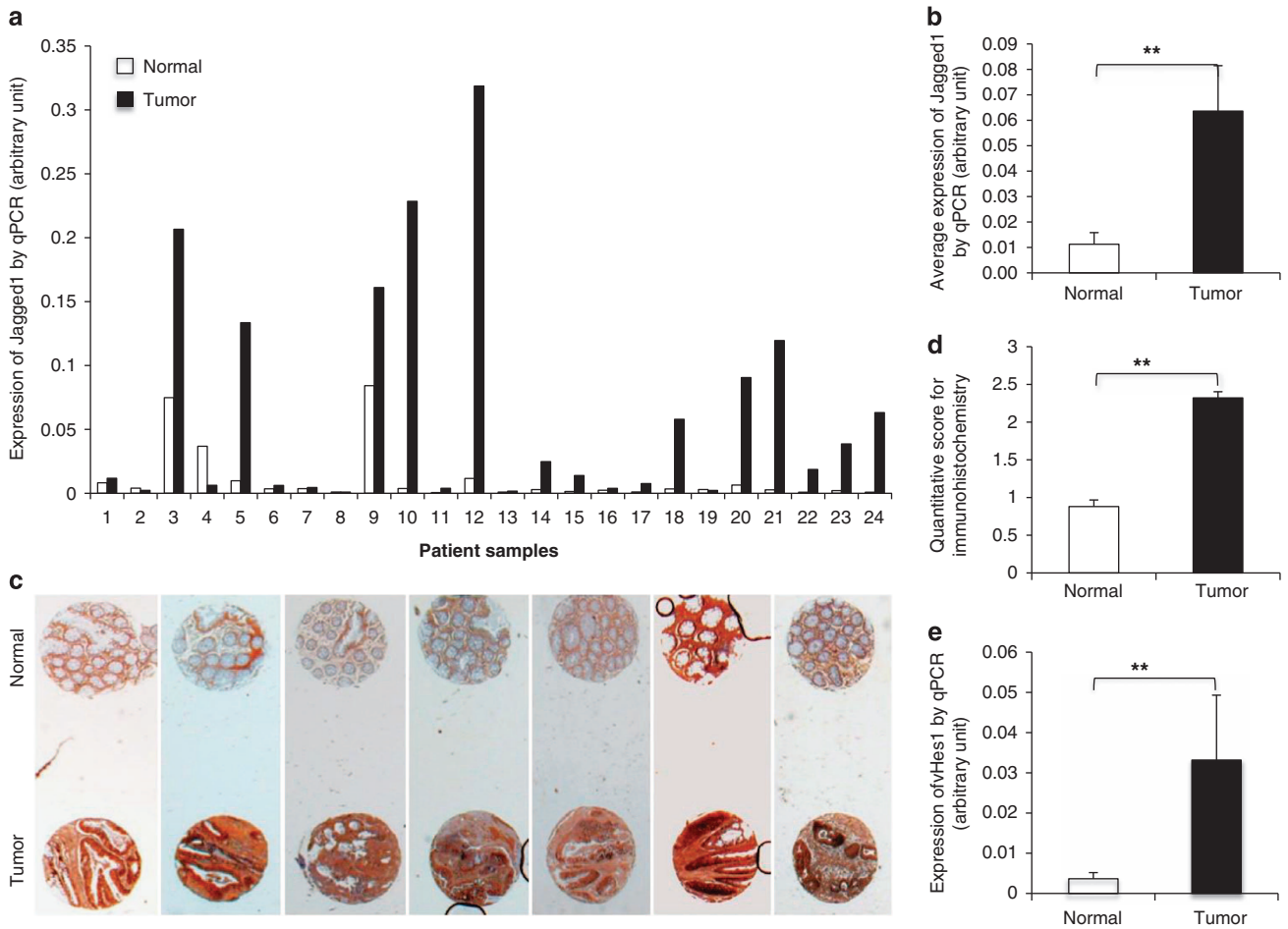


Figure 1 Expression of Jagged1 by qPCR in 24 pairs of colon cancer (tumor) and matched non-cancerous colonic tissues (normal) (a). On average, higher expression level of Jagged1 was found in tumor than in normal tissues ($n=24$, b). In seven selected cases, higher expression of Jagged1 in tumor tissues was confirmed by immunohistochemistry ($n=7$, c, d). Expression of Notch downstream target gene Hes1 was examined by qPCR in cancerous (tumor) and matched non-cancerous (normal) colon tissues (e). $**P<0.01$

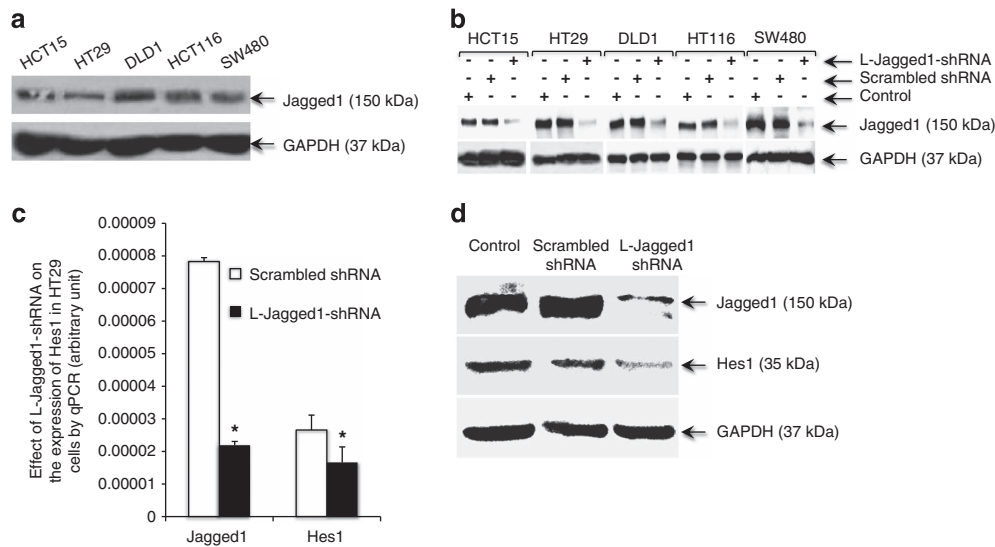


Figure 2 Expression of Jagged1 was determined by western blot in five colon cancer cell lines (a). L-Jagged1-shRNA successfully knocked down Jagged1 in these cells (b). Downregulation of Jagged1 was associated with a reduced expression of Notch downstream target Hes1 in HT29 cells at the mRNA (c) and protein levels (d). $*P<0.05$. The experiments were repeated at least three times

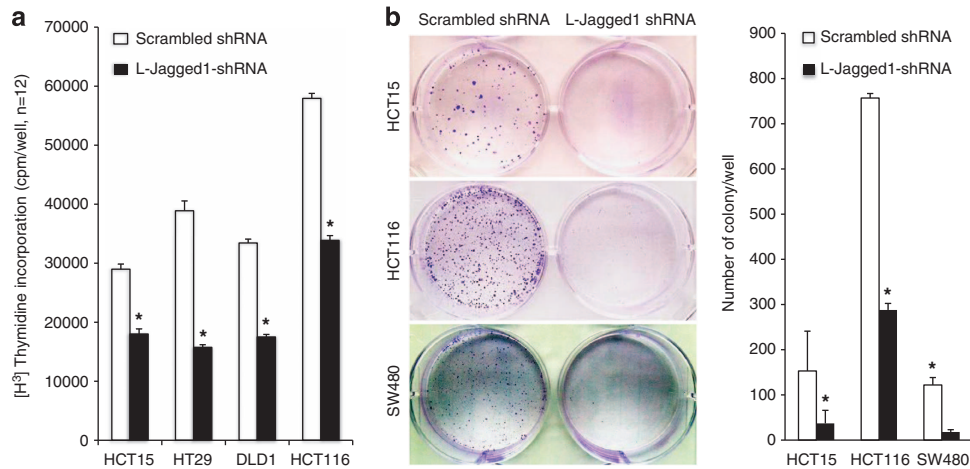


Figure 3 Knockdown of Jagged1 led to a significant inhibition of proliferation of colon cancer cells HCT15, HT29, DLD1, and HCT116 (a). Marked reduction of colony formation was observed in HCT15, HCT116, and SW480 cells transfected with L-Jagged1-shRNA (b). * $P < 0.05$. The experiments were repeated at least three times

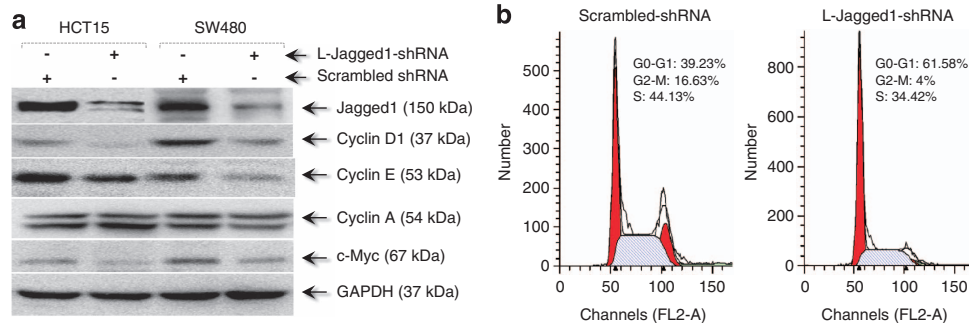


Figure 4 Expression of cell cycle-related proteins Cyclin D1, Cyclin E, Cyclin A, and c-Myc in human colon cancer cells HCT15 and SW480 after treatment with L-Jagged1-shRNA (a). Cell cycle analysis was examined by FACScan using SW480 cells as an example (b). The experiments were repeated at least three times

from 39.23% to 61.58%, indicating a G₀/G₁ cell cycle arrest. A similar result was found in HCT15 cells (data not shown). Furthermore, cell cycle-related protein expression was assessed by western blot. Consistent with the accumulation of cells in G₀/G₁ phase, Cyclin D1, Cyclin E, and c-Myc significantly decreased in L-Jagged1-shRNA-treated HCT15 and SW480 cells, whereas Cyclin A remained unchanged, compared with scrambled-shRNA-treated cells (Figure 4a). In addition, there was no significant increase in apoptosis upon Jagged1 knockdown in HCT15, HCT116, and SW480 cells as detected by AnnexinV/PI staining (data not shown). Together, these results indicated that Jagged1 silencing in colon cancer cells was associated with a block in cell cycle progression and not increased apoptosis.

Downregulation of Jagged1 decreased the migration and invasion capacity of colon cancer cells. Elevated cell migration and invasion are associated with increase in the metastatic potential of cancer cells. This may be independent of cell proliferation rates. Therefore, we studied the effect of Jagged1 downregulation on the migration and invasion of colon cancer cells. Cell migration was determined by wound-healing assay. As shown in Figure 5a, Jagged1 downregulation significantly suppressed the migration of HCT116 cells. The migration index of Jagged1 knockdown cells was

decreased by 49%, 47%, and 36% at 24, 48, and 72 h, respectively (Figure 5b). To examine the cell invasion activity *in vitro*, we used transwell inserts coated with matrigel matrix. After Jagged1 knockdown, the invasiveness of HCT116 cells was decreased by 43% as compared with the control cells (Figures 5c and d).

Silencing of Jagged1 suppressed the growth of xenograft tumors in nude mice. To explore the effects of Jagged1 knockdown *in vivo*, xenograft tumors were generated by injecting HCT15 cells stably infected with either control vector (scrambled-shRNA) or L-Jagged1-shRNA. The cells were injected s.c. into the flanks of nude mice. As shown in Figure 6a, tumors derived from HCT15 cells previously infected by scrambled-shRNA grew fast, particularly 13 days after injection. Compared with the control group, tumors derived from L-Jagged1-shRNA-infected cells grew much slower throughout the experiment, suggesting that knockdown of Jagged1 dramatically impaired the tumorigenic growth of HCT15 cells. At harvest, the tumors from L-Jagged1-shRNA-infected cells weighed much lighter than those from control cells (>8-fold) (Figure 6b). The average volume of tumors derived from Jagged1 knockdown cells showed ~10-fold reduction compared with control (Figures 6c and d).

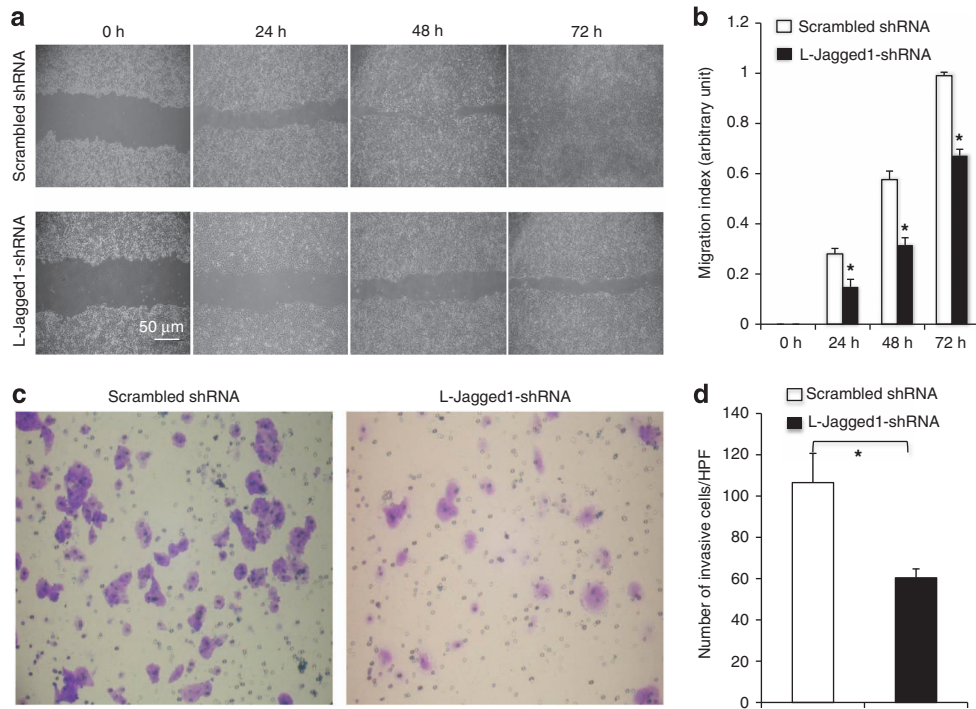


Figure 5 Knockdown of Jagged1 led to a significant inhibition of the migratory ability of HCT116 cells (a). Knockdown of Jagged1 led to a significantly slower migration at 24, 48, and 72 h after cells were infected with L-Jagged1-shRNA (b). * $P < 0.05$. Similarly, inhibition of Jagged1 led to a reduced invasive ability of HCT116 cells (c, d). * $P < 0.05$. The experiments were repeated at least three times

To further verify the antitumor effects of Jagged1 silencing, we next examined the expression of cell proliferation markers (Ki-67, PCNA, c-Myc), metastasis markers (MMP-9, MMP-2) and angiogenesis marker CD31 at the mRNA and protein levels in xenograft tumors. As shown in Figure 7a, there was a significant reduction in the mRNA expression of Ki-67, PCNA, c-Myc, MMP-9, and MMP-2 in tumor tissues. The mRNA level of CD31 in tumors was not altered upon Jagged1 knockdown. Immunohistochemical staining confirmed these findings, as exemplified by the reduced Ki-67 and MMP-9 expression in the xenograft tumors derived from HCT15 cells infected by L-Jagged1-shRNA (Figure 7b).

Discussion

In the current study, we found that Jagged1, one of the most important ligands for the Notch signaling, is overexpressed in ~80% of human CRC tissues, and is associated with a marked elevated expression of Notch target gene Hes1. Targeting Jagged1 induces growth inhibition in colon cancer cells both *in vitro* and *in vivo*, at least in part by causing G₀/G₁ phase cell cycle arrest. In addition, downregulation of Jagged1 reduces the migration and invasion of colon cancer cells, most likely involving decreased MMPs.

Notch signaling is critical for determination of cell fate within a wide variety of tissues by regulation of growth, differentiation, and apoptosis. Abnormal Notch signaling is associated with tumorigenesis⁷⁻¹¹ and cell invasion.^{11,27-29} Previous studies have shown aberrant expression of the Notch pathway in CRC.^{16,20,30} In particular, Jagged1 was found to be overexpressed in ~50% of human colon tumors, while it was

undetectable in the normal colonic mucosa.²⁰ Moreover, the levels of Jagged1 expression were correlated with tumor differentiation parameters and stages of CRC.^{30,31} It was found that high expression of Jagged1 is associated with poor clinical outcome in some malignancies such as breast cancer and prostate cancer, whereas another study demonstrated high Jagged1 levels were correlated with better prognosis of CRC.³¹ The association and prognostic value of Jagged1 in CRC remains to be investigated further. In this study, we confirmed that >80% of CRC tissues expressed higher level of Jagged1 compared with normal colonic tissues, and that this was associated with a marked elevated expression of the Notch target gene Hes1. These data suggested that Jagged1 is likely a major ligand responsible for Notch signaling activation in CRC, and that selective targeting of this protein may present a novel therapeutic strategy.

We demonstrated that lentiviral vectors carrying Jagged1-shRNA-mediated Jagged1 silencing effectively blocked the Notch signaling in colon cancer cells, as reflected by a strong inhibition of Notch target genes. Downregulation of Jagged1 expression led to a reduced cell proliferation, colony formation, migration, and invasion. *In vivo* studies showed that xenograft tumors derived from Jagged1 knockdown cells displayed a significant reduction of growth speed and tumor volume, suggesting that direct downregulation of Jagged1 has an antitumor effect in colon cancer.

Notch signaling influences cell proliferation depending on the cell type. For example, the Notch1 pathway has been found to have a tumor-suppressive effect on murine skin tumor and small cell lung cancer.^{32,33} However, Notch1 appears to function as an oncogene in several human cancers.⁷⁻¹¹ It has

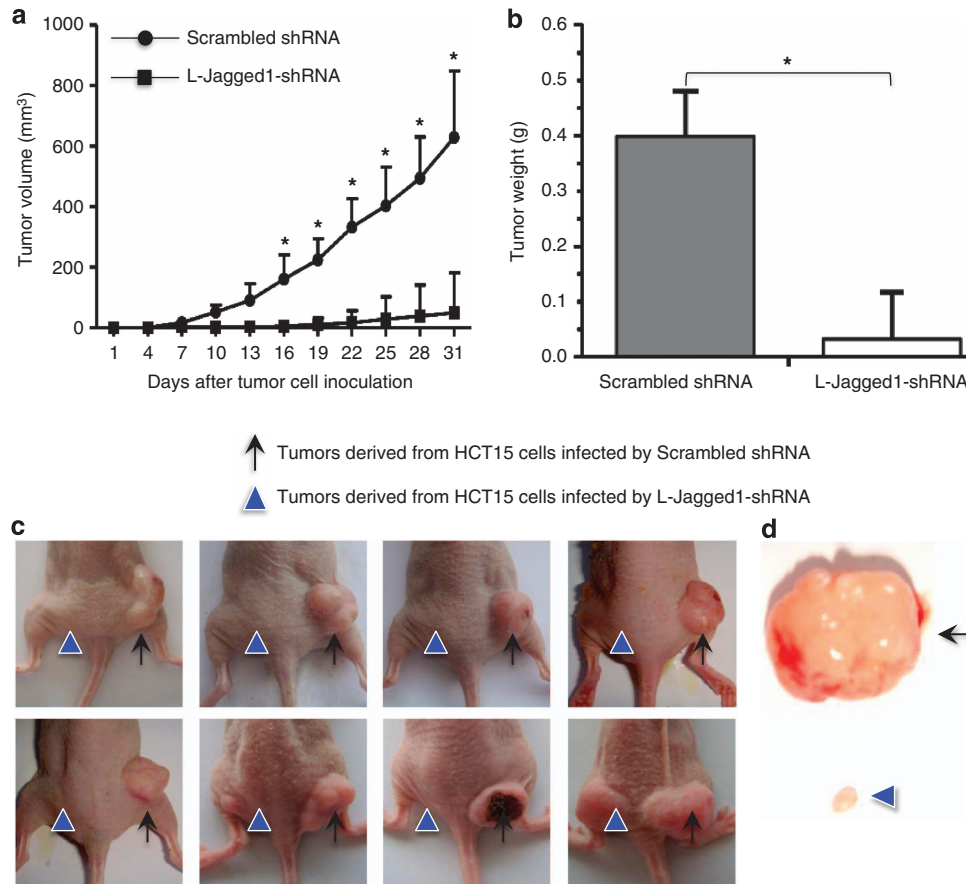


Figure 6 Effect of downregulation of Jagged1 by L-Jagged1-shRNA on the growth of xenograft tumors in nude mice. Mice ($n = 8$) were s.c. injected into the flanks with HCT15 cells stably infected with L-Jagged1-shRNA (left) or scrambled-shRNA (right). Tumor volumes were measured once every 3 days and tumor tissues were harvested 31 days after cell injection. The tumor growth curve was shown (a). The average tumor weight ($n = 8$) was measured after the mice were harvested (b). Representative tumor-bearing mice (c) and the harvested xenograft tumor tissues (d) are shown. $*P < 0.05$

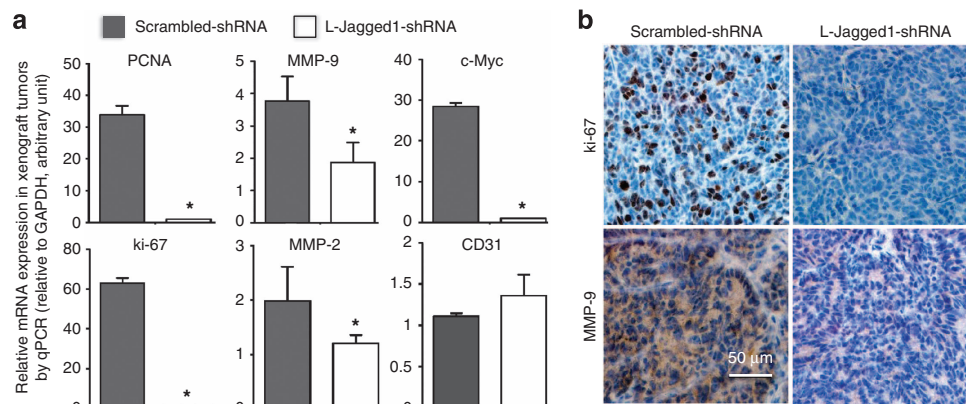


Figure 7 Expression of cell proliferation markers (PCNA, Ki-67, c-Myc) and metastatic markers (MMP-9, MMP-2), as well as the endothelial cell marker CD31 in xenograft tumors derived from HCT15 cells stably transfected with L-Jagged1-shRNA or scrambled-shRNA, was determined by qPCR (a). $*P < 0.05$. Expression of cell proliferation marker Ki-67 and metastatic marker MMP-9 in the xenograft tumors was determined by immunohistochemistry (b)

been reported that Jagged1 is processed in a fashion similar to Notch1, ultimately resulting in the release of a nuclear targeted intracellular domain.³⁴ Thus, Jagged1 is itself important in development and carcinogenesis. Here we found that downregulation of Jagged1 attenuated the growth of

colon cancer cells and that this was associated with retarded cell cycle progression. The *in vivo* results also showed that Jagged1 knockdown dramatically impaired the tumorigenic growth of HCT15 cells. In agreement with this, we found a reduction in the number of Ki-67-positive cells in the tumors

derived from Jagged1 knockdown cells compared with the ones from the control cells. Taken together, these observations suggested a positive role of Jagged1 in colon cancer cell growth and that Jagged1 may function as an oncogene in CRC.

The cell cycle is regulated by a series of checkpoints monitoring genomic integrity and ensuring that DNA replication proceeds in a coordinated manner. Aberrations in cell cycle progression occur in the majority of human malignancies. Different combinations of cyclin and CDK subunits operate at checkpoint controls during the cell cycle to integrate mitogenic and antiproliferative signals. Cyclin D1 and Cyclin E have a critical role in controlling G1/S transition.³⁵ The present study indicates that downregulation of Jagged1 causes G₀/G₁ phase cell cycle arrest via a reduction of Cyclin D1 and Cyclin E levels, which appears to be the underlying mechanism in colon cancer cell growth inhibition. c-Myc is also important in cell cycle regulation and tumorigenesis.^{36,37} Jagged1 knockdown reduced c-Myc expression, so it might also contribute to the G₀/G₁ phase arrest. It was shown that regulation of the cell cycle by Notch signaling involves the coordination of different, and sometimes antagonizing, pathways in a highly cell context-dependent manner. Our observation was in line with the previous study by others²⁰ that inhibition of Notch signaling by γ -secretase inhibitors resulted in G₁ phase arrest in colon cancer cells. However, downregulation of Jagged1 induced S phase arrest in prostate cancer cells with reduced CDK2 kinase activity and increased p27 expression.³⁸ On the other hand, Jagged1 knockdown results in an accumulation of ovarian cancer cells in the G₂-M phase,³⁹ suggesting the control of cell cycle progression by Jagged1 is cell-type specific.

Previous studies have shown that constitutively activated Notch signaling is involved in apoptosis resistance, and that abrogating Notch activation by the γ -secretase inhibitor, Notch1-siRNA, and DLL1-siRNA can induce apoptosis in human cancer cells.^{40,41} However, in the present study, a very mild apoptosis was detected in colon cancer cells upon Jagged1 knockdown both *in vitro* and *in vivo*. These findings are partially consistent with another report that Jagged1-shRNA leads to significant cell growth inhibition in ovarian cancer cells but to a minor extent of apoptosis.⁴² We speculate that several key members of the Notch cascade have different roles in apoptosis, and induction of apoptosis is not a major mechanism for targeting Jagged1-mediated Notch signaling activation in CRC.

The Notch pathway was found to participate in many aspects of metastasis, the epithelial to mesenchymal transition, cell migration, invasion, and angiogenesis.^{43,44} γ -Secretase inhibitors were shown to reduce cell migration in different cancer cell lines.⁴⁵ A recent report has shown that tumor-derived Jagged1 promotes bone metastasis of breast cancer.¹¹ It is noteworthy that, in our study, downregulation of Jagged1 significantly reduced the migration and invasion in colon cancer cells, and was associated with decreased expression of MMP-2 and MMP-9. MMP-2 and MMP-9 belong to the family of matrix metalloproteinases, and are present in invasive CRC.⁴⁶ The expression of these enzymes is known to be regulated by Notch signaling in cancer cells.

For instance, downregulation of Notch1 decreases MMP-9 levels in pancreatic cancer cells²⁷ and prostate cancer cells.²⁹ Here, we detected a decreased level of MMP-2 and MMP-9 upon Notch1 knockdown, suggesting that Jagged1 probably affects cell invasion via its action on the production of matrix-degrading enzymes. Although further studies are necessary to fully characterize the roles of Notch signaling in metastasis of CRC, the inhibitory effect of Jagged1 knockdown on colon cancer cell migration is of particular interest, and Jagged1 may be a useful target molecule for developing new therapy against CRC.

Previous studies have shown that Jagged1 may have its own signaling function that is important to tumorigenesis independent of the canonical Notch pathway.³⁹ Our study has demonstrated that Jagged1 targeting led to a marked reduction in the expression of Notch downstream target Hes1, suggesting that downregulation of Jagged1-mediated antitumor effect was at least partly through a Notch-dependent mechanism in colon cancer cells. Since systemic inhibition of Notch signaling by γ -secretase inhibitors is associated with adverse effects such as gastrointestinal toxicity and liver injury, Jagged1 may possibly constitute a promising target for CRC patients. It was noted that delivery of lentiviral vectors to patients may be practically impossible because protecting normal cells from being infected by the virus is a demanding task. Nanoparticles have been demonstrated to transport siRNA across cellular membranes and are biodegradable, biocompatible, and have low immunogenicity.^{47,48} Nanoparticles have been used to carry siRNAs against certain oncoproteins for the treatment of melanoma in a phase I clinical trial and showed no dose limiting cytotoxicity.⁴⁸ Moreover, targeting Jagged1 using this delivery system decreased cell viability and reversed taxane chemoresistance in ovarian cancer cells.³⁹ Thus, our results establish a foundation on which targeted therapy can be developed.

In conclusion, we have shown that targeting Jagged1 inhibits proliferation, migration, and invasion of colon cancer cells *in vitro*, as well as suppresses the tumor growth and decreases tumor load in nude mice. With the ability to identify subsets of cancer patients with Jagged1 overexpression, antagonism of this signaling molecule could ultimately provide a useful therapeutic strategy for CRC.

Materials and Methods

Chemicals, reagents, and cell lines. Human colon cancer cell lines HCT15, HCT116, HT29, DLD1, and SW480 were purchased from the American Type Culture Collection (Manassas, VA, USA). Trizol, PCR-related reagents, and all cell culture-related materials were purchased from Invitrogen (Carlsbad, CA, USA). All the primary antibodies except anti-c-Myc (BD Biosciences, San Jose, CA, USA), anti-GAPDH (Abcam, Cambridge, UK) and anti-Ki-67 (DAKO, Glostrup, Denmark), and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Detection of Jagged1 in human colon cancer tissues by tissue microarray (TMA). Tissue microarray and fresh human CRC tissues and matched non-cancerous colonic tissues were described in detail in our previous publication.⁴⁹ These cancer tissues were largely obtained from patients who underwent surgical operations for CRC. All cancer tissues, matched non-cancerous tissues, and TMAs were provided by M Ebert of the Technical University, Munich, Germany. In the current study, all 24 tissues were adenocarcinomas, and classified into well ($n=3$), moderately ($n=18$), and

poorly ($n=3$) differentiated. Patient ages ranged from 49 years to 77 years (mean, 64 years), consisting of 16 males and 8 females.

The expression of Jagged1 was detected by immunohistochemistry in TMA slides, as reported in our previous publications.⁴⁹ The slides were evaluated by a senior pathologist (KW Chan), and the quantitative score was generated as previously reported.^{49,50} The study was approved by the institutional human ethics committee of the relevant institutions.

Construction of recombinant lentiviral vectors. A 21-mer oligonucleotide sequence from nucleotide 1496 to 1516 of the Jagged1 human mRNA sequence was used. The hairpin sense and antisense sequences were as follows: 5'-CCGGTGGTATTTCAGGACCCAACTGTGCTCGAGCAGATTGGGCTCTGAATACCTTTTTG-3' and 5'-AATTCAAAAGGTATTTCAGGACCCAACTGTGCTCGAGCACAGTTGGGCTCCTGAATACCA-3'. The oligonucleotides were annealed and cloned into the *AgeI/EcoRI* sites of the shRNA vector pLKO.1-puro (Addgene, Cambridge, MA, USA). Expression of the shRNA was controlled by the human U6 promoter. To produce lentiviral particles, the constructed Jagged1-shRNA or scrambled-shRNA plasmid was co-transfected with psPAX2 packaging plasmid and pMD2.G envelope plasmid into 293T cells using X-tremeGENE HP DNA transfection Reagent (Roche, Basel, Switzerland). The titer of the purified virus was determined in 293T cells by the serial dilution method.

Cell culture and construction of Jagged1 knockdown cells. Human colon cancer cell lines HCT116, HCT15, HT29, DLD1, and SW480 were cultured in RPMI1640 medium supplemented with 10% FBS and 1% Pen-Strep at 37 °C with 5% CO₂. Cells were passaged in our laboratory for less than 6 months after resuscitation. Mycoplasma contamination was tested by PCR during culture, and no additional authentication was done as cells came from national repositories. Cells were grown to 70–80% confluence, and incubated with the constructed lentiviral vectors for 24 h in growth medium containing 8 µg/ml of polybrene (Santa Cruz). Seventy-two hours after infection, the medium was changed to fresh RPMI1640 containing 10 µg/ml puromycin (Santa Cruz). Puromycin-resistant colonies were used for subsequent studies.

Cell proliferation and colony formation assay. Cell proliferation was determined by [³H]-thymidine incorporation assay, as we previously reported.⁵¹ Colony formation assay was used to determine the anchorage-dependent growth property of the cells and was performed, as described previously.⁵² Briefly, Jagged1 knockdown and the control cells were plated in six-well plates at a density of 3000 cells/well. Plates were incubated for 7 days, and cell colonies were visualized by staining with crystal violet and macroscopically counted.

Cell cycle analysis. Cell cycle was detected by propidium iodide (PI) staining and flow cytometric analysis. Briefly, cells were washed with PBS, fixed with 70% ethanol at 4 °C overnight. After washing with PBS, cells were re-suspended in 50 µg/ml -of PI containing 0.5% Triton-X 100 and 100 µg/ml of RNase A and incubated in the dark at 37 °C for 30 min. The cells were then analyzed for DNA content using a FACS Calibur Cytometer (BD Biosciences). A minimum of 10 000 events per sample was acquired and subsequently analyzed with ModFit software (Verity Software House, Topsham, ME, USA).

Cell migration and invasion assay. Cell migration was analyzed by a conventional wound-healing assay, as described previously.⁵³ Briefly, Jagged1-shRNA or scrambled-shRNA stably infected cells were grown in six-well plates to complete confluence. Wound injury was made with the tip of a sterile micropipette, and detached cells were removed by washing with PBS. Cells were then incubated and allowed to migrate for up to 72 h. Photographs were taken and migration index was calculated as follows: migration index = [(initial wound width–width of wound at time point tested)/initial wound width] × 100%.

In vitro invasion assay was performed using a 24-well transwell insert (8 µm pore size) pre-coated with BD Matrigel matrix (BD Biosciences) according to the manufacturer's instruction. Cells transfected with Jagged1-shRNA or scrambled-shRNA were seeded into the upper chamber in serum-free medium at a density of 50 000 cells/well. The medium containing 10% FBS was placed in the lower chamber to act as a chemoattractant, and cells were further incubated for the indicated time. Non-invading cells were removed from the upper chamber and the

invaded cells remaining on the lower surface of the insert were fixed and stained with crystal violet. Cells were quantified as the average number of cells found in five random microscopic fields in three independent inserts.

Western blot and qPCR assays. Cells were lysed in RIPA buffer (Sigma, St. Louis, MO, USA) supplemented with 1% protease inhibitor cocktail. Proteins from the cell lysate were separated on a 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were probed with appropriate antibodies and detected with Enhanced Chemiluminescence Assay (Pierce Biotechnology, Rockford, IL, USA).

For the gene expression at the mRNA level, total RNA was extracted, quantified, and converted to cDNA, and qPCR was performed using SYBR Green (Applied Biosystems, Foster City, CA, USA) system. The sequences of primers used in qPCR were as follows: *JAGGED1* forward: 5'-TCGGGTCAGTTCGAGTTGGA-3' and reverse: 5'-CGTTCACGTTCTGCATGGAC-3'; *PCNA* forward: 5'-GCGTG AACCTACCAGTATGT-3' and reverse: 5'-TCTTCGGCCCTTAGTGTAAATGAT-3'; *Ki67* forward: 5'-ACGCCTGGTACTATCAAAGG-3' and reverse: 5'-CAGACC CATTACTTGTGTTGGA-3'; *c-MYC* forward: 5'-GGCTCCTGGCAAAGGTCA-3' and reverse: 5'-GTGCGTAGTTGTGCTGATGT-3'; *MMP2* forward: 5'-TACAGGAT CATTGGCTACACACC-3' and reverse: 5'-GGTCACATCGCTCCAGACT-3'; *MMP9* forward: 5'-TGTACCGCTATGGTTACTACTCG-3' and reverse: 5'-GGCAGGGAC AGTTGCTTCT-3'; *HES1* forward: 5'-ACGTGCGAGGGCGTTAATAC-3' and reverse: 5'-GGGGTAGTGCATGGCATTGA-3'; *CD31* forward: 5'-ACAGTGTGA CATGAAGAGCC-3' and reverse: 5'-TGTA AACAGCACGTCATCCTT-3'; *GAPDH* forward: 5'-CTGGGCTACTGAGCACC-3' and reverse: 5'-AAGTGGTCGTTG AGGGCAATG-3'. The relative quantification of mRNA was normalized to GAPDH.

Xenograft tumorigenesis in nude mice. Athymic nude mice (BALB/c nu/nu) (male, 6–8-week-old) were purchased from the Academy of Military Medical Science (Beijing, China), and were acclimated for 7 days in the laboratory before experimentation. To establish xenograft tumors, 1 × 10⁷ Jagged1 knockdown and the control HCT15 cells were injected subcutaneously into the dorsal flanks of the mice. Tumor volumes were calculated every 3 days according to the following formula: volume (mm³) = [(shortest diameter)² × longest diameter]/2. On the day of harvest the tumor tissues were excised, size was measured and weight was determined. Harvested tumor tissues were either snap frozen for RNA analysis or fixed in 10% neutral formalin for histology assay. All animal experiments were approved by the Committee on the Use of Live Animals in Teaching and Research of the Tianjin Medical University.

Statistical analysis. Data were expressed as mean ± S.D. Student's independent two-sample *t*-tests were used for comparisons. A *P* value of <0.05 was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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