Down-regulation of Notch-1 Inhibits Invasion by Inactivation of Nuclear Factor-κB, Vascular Endothelial Growth Factor, and Matrix Metalloproteinase-9 in Pancreatic Cancer Cells

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Abstract

Notch signaling plays a critical role in the pathogenesis and progression of human malignancies but the precise role and mechanism of Notch-1 for tumor invasion remains unclear. In our earlier report, we showed that down-regulation of Notch-1 reduced nuclear factor-κB (NF-κB) DNA-binding activity and matrix metalloproteinase-9 (MMP-9) expression. Because NF-κB, VEGF, and MMPs are critically involved in the processes of tumor cell invasion and metastasis, we investigated the role and mechanism(s) by which Notch-1 down-regulation (using molecular approaches) may lead to the down-regulation of NF-κB, vascular endothelial growth factor (VEGF), and MMP-9, thereby inhibiting invasion of pancreatic cancer cells through Matrigel. We found that the down-regulation of Notch-1 by small interfering RNA decreased cell invasion, whereas Notch-1 overexpression by cDNA transfection led to increased tumor cell invasion. Consistent with these results, we found that the down-regulation of Notch-1 reduced NF-κB DNA-binding activity and VEGF expression. Down-regulation of Notch-1 also decreased not only MMP-9 mRNA and its protein expression but also inactivated the pro-MMP-9 protein to its active form. Taken together, we conclude that the down-regulation of Notch-1 could be an effective approach for the down-regulation and inactivation of NF-κB and its target genes, such as *MMP-9* and *VEGF* expression, resulting in the inhibition of invasion and metastasis. (Cancer Res 2006; 66(5): 2778-84)

Introduction

The mammalian Notch gene family has four members: *Notch 1* to *Notch 4*. Notch genes are involved in cell proliferation and apoptosis, which affect the development and function of many organs (1, 2). Notch genes encode proteins that can be activated by interacting with a family of its ligands (3, 4). Upon activation, Notch is cleaved, releasing intracellular Notch (ICN), which translocates into the nucleus. The ICN associates with transcriptional factors, regulating the expression of target genes and thus plays important roles in development and cell growth (5, 6). Because Notch signaling plays important roles in the cellular developmental pathway, including proliferation and apoptosis, alterations in Notch signaling are associated with tumorigenesis (7). These observations suggest that dysfunction of ICN prevents differentiation, ultimately guiding undifferentiated cells toward malignant transformation (8). The Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands in various human cancers (9–11).

Notch-1 has been reported to cross-talk with another major cell growth and apoptotic regulatory pathway, namely nuclear factor-κB (NF-κB). Specifically, Notch-1 has been reported to strongly induce NF-κB promoter activity and induce expression of several NF-κB subunits (12). Levels of basal and stimulation-induced NF-κB activity were significantly decreased in mice with reduced Notch levels (13, 14). Constitutive levels of Notch activity are essential to maintain NF-κB activity in various cell types and NF-κB is known to play critical roles in the processes of tumor cell invasion and metastasis (15–19). Therefore, inactivation of Notch-1-mediated cell invasion could be partly mediated via inactivation of NF-κB activity.

In our earlier report, we showed that down-regulation of Notch-1 reduced NF-κB DNA-binding activity and MMP-9 expression (20). NF-κB and matrix metalloproteinases (MMP) are critically involved in the processes of tumor cell invasion and metastasis (15–19). Tumor metastasis occurs by a series of steps, including cell invasion, degradation of basement membranes, and the stromal extracellular matrix, ultimately leading to tumor cell invasion and metastasis. The MMPs are a family of related enzymes that degrade extracellular matrix, which are considered to be important factors in facilitating tumor invasion. Among these MMPs, MMP-9 has been considered to be an important factor in facilitating invasion and metastases in pancreatic cancer (18). Moreover, vascular endothelial growth factor (VEGF), the most well-characterized angiogenic factor, is known to play a vital role in tumor-associated microvascular invasion (21, 22). In human pancreatic cancer, VEGF has been found to be overexpressed (22). Therefore, in this study, we investigated the role and mechanism(s) by which Notch-1 down-regulation may lead to the down-regulation of NF-κB, VEGF, and MMP-9, thereby inhibiting invasion of pancreatic cancer cells through Matrigel. Our results suggest that down-regulation of Notch-1 causes decrease in the expression of MMP-9 and VEGF, resulting in the inhibition of pancreatic cancer cell invasion mediated through the inactivation of the DNA-binding activity of NF-κB.

Materials and Methods

Cell culture and experimental reagents. Human pancreatic cancer cell line BxPC-3 (American Type Culture Collection, Manassas, VA) was cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. Primary antibodies for Notch-1, VEGF, MMP-9 were

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purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Pierce (Rockford, IL). Notch-1 small interfering RNA (siRNA), VEGF siRNA, MMP-9 siRNA, and siRNA control were obtained from Santa Cruz Biotechnology. LipofectAMINE 2000 was purchased from Invitrogen. Chemiluminescence detection of proteins was done with the use of a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ). Protease inhibitor cocktail and all other chemicals were obtained from Sigma (St. Louis, MO).

Plasmids and transfections. The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain was a kind gift of L. Miele (Department of Biopharmaceutical Sciences and Cancer Center, University of Illinois at Chicago, Chicago, IL; ref. 8). BxPC-3 cells were transfected with Notch-1 siRNA and siRNA control, respectively, using LipofectAMINE 2000. BxPC-3 cells were stably transfected with human Notch-1 ICN or vector alone (pcDNA3) and maintained under neomycin selection.

Invasion assay. The invasive activity of the ICN or Notch-1 siRNA–transfected BxPC-3 cells was tested by using BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) according to the protocol of the manufacturer with minor modification. Briefly, transfected BxPC-3 cells (5×10^4) with serum-free medium were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium. After 48 hours of incubation, the cells in the upper chamber were removed, and the cells, which invaded through Matrigel matrix membrane, were stained with 4 μg/mL Calcein AM in Hanks buffered saline at 37°C for 1 hour. Then, fluorescence of the invaded cells was read in ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

Preparation of nuclear extract. The ICN or Notch-1 siRNA–transfected BxPC-3 cells were washed with cold PBS and suspended in 0.15 mL lysis buffer [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine]. The cells were allowed to swell on ice for 20 minutes and then 4.8 μL of 10% NP40 was added. The tubes were then vigorously mixed on a vortex mixer for a few seconds and centrifuged for 120 seconds in a microfuge. The nuclear pellet was resuspended in 30 μL of ice-cold nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF, 2 μg/mL leupeptin, 2 μg/mL aprotinin, and 0.5 mg/mL benzamidine] and incubated on ice with intermittent mixing. The tubes were then centrifuged for 5 minutes in a microfuge at 4°C and the supernatant (nuclear extract) was collected in a cold Eppendorf tube and stored at −70°C for later use. The protein content was measured by BCA method.

Electrophoretic mobility shift assay for measuring NF-κB activity. Electrophoretic mobility shift assay (EMSA) was done by incubating 10 μg of nuclear protein extract with IRDye 700-labeled NF-κB oligonucleotide (LI-COR, Lincoln, NE). The incubation mixture included 2 μg of poly(deoxyinosinic-deoxycytidylic acid) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotide on 8.0% native polyacralyamide gel using buffer containing 50 mmol/L Tris, 200 mmol/L glycine (pH 8.5), and 1 mmol/L EDTA and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1 (LI-COR). Supershift assay using NF-κB p65 antibody was also conducted to confirm the specificity of NF-κB DNA-binding activity. For loading control, 10 μg of nuclear proteins from each sample were subjected to Western blot analysis for retinoblastoma protein, which showed no alternation after transfection.

Real-time reverse transcription-PCR analysis for gene expression. The total RNA from ICN or Notch-1 siRNA–transfected BxPC-3 cells was isolated by Trizol (Invitrogen) and purified by RNeasy Mini kit and RNase-free DNase Set (Qiagen, Valencia, CA) according to the protocols of the manufacturer. One microgram of total RNA from each sample was subjected to first-strand cDNA synthesis using TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA) in a total volume of 50 μL, including 6.25 units MultiScribe reverse transcriptase and 25 pmol random hexamers. Reverse transcription reaction was done at 25°C for 10 minutes followed by 48°C for 30 minutes and 95°C for 5 minutes.

The primers used in the PCR reaction are as follows: MMP-9, forward primer (5′-CGGAGTGAGTTGAACCAG-3′) and reverse primer (5′-GTCCCAGTGGGGATTTAC-3′); VEGF, forward primer (5′-GCCTTGCCTTGCTGCTCTAC-3′) and reverse primer (5′-TTCTGCCCTCCTCCTTCTGC-3′); β-actin, forward primer (5′-CCACACTGTGCCCATCTACG-3′) and reverse primer (5′-AGGATCTTCATGAGGTAGTCAGTCAG-3′). The primers were checked by running a virtual PCR and primer concentration was optimized to avoid primer dimer formation. Also, dissociation curves were checked to avoid nonspecific amplification. Real-time PCR amplifications were undertaken in Mx4000 Multiplex QPCR System (Stratagene, La Jolla, CA) using 2× SYBR Green PCR Master Mix (Applied Biosystems). One microliter of reverse transcription reaction was used for a total volume of 25 μL quantitative PCR reactions. The thermal profile for SYBR real-time PCR was 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analyzed according to the comparative C_t method and were normalized by β-actin expression in each sample.

Western blot analysis. Cells were lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.5% Triton X-100, 2.5 mmol/L sodium orthovanadate, 10 μL/mL protease inhibitor cocktail, and 1 mmol/L PMSF] by incubating for 20 minutes at 4°C. The protein concentration was determined using the Bio-Rad assay system (Bio-Rad, Hercules, CA). Total proteins were fractionated using SDS-PAGE and transferred onto nitrocellulose membrane. The membranes were blocked with 5% nonfat dried milk or bovine serum albumin in 1× TBS buffer containing 0.1% Tween 20 and then incubated with appropriate primary antibodies. Horseradish peroxidase–conjugated anti-rabbit or anti-goat IgG was used as the secondary antibody and the protein bands were detected using the enhanced chemiluminesence detection system (Amersham Pharmacia Biotech). Quantification of Western blots was done using laser densitometry and the results are presented as the mean of three independent experiments with error bars representing SD. For reprobing, membranes were incubated for 30 minutes at 50°C in buffer containing 2% SDS, 62.5 mmol/L Tris (pH 6.7), and 100 mmol/L 2-mercaptoethanol, washed and incubated with desired primary antibody.

VEGF assay. The culture medium of the ICN or Notch-1 siRNA–transfected BxPC-3 cells grown in six-well plates was collected. After collection, the medium was spun at 800 × *g* for 3 minutes at 4°C to remove cell debris. The supernatant was either frozen at −20°C for VEGF assay later or assayed immediately using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN).

Reporter gene constructs and transfection. Matrix metalloproteinase-9-chloramphenicol acetyltransferase (MMP-9-CAT) containing a NF-κB binding site in the sequence of MMP-9 promoter was generously provided by Dr. Douglas Boyd (MD Anderson Cancer Center, Houston, TX). The MMP-9-CAT or empty vector was transiently cotransfected with cytomegalovirus-β-galactosidase into ICN or Notch-1 siRNA–transfected BxPC-3 cells using the LipofectAMINE 2000 method (Invitrogen). After incubation for 5 hours, the transfected cells were washed and incubated with 10% FBS for 48 hours. Subsequently, the CAT activities in the samples were measured by using CAT ELISA system (Roche, Palo Alto, CA) and β-Galactosidase Enzyme Assay System (Promega, Madison, WI) using an ULTRA Multifunctional Microplate Reader (TECAN).

MMP-9 activity assay. The ICN or Notch-1 siRNA–transfected BxPC-3 cells were seeded in six-well plates and incubated at 37°C. After 24 hours, the complete medium was removed and the cells were washed with serum-free medium. The cells were then incubated in serum-free medium for 48 hours. MMP-9 activity in the medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay kit (R&D Systems) according to the protocol of the manufacturer.

Densitometric and statistical analysis. The bidimensional optical densities of Notch-1 and β-actin proteins on the films were quantified and analyzed with Molecular Analyst software (Bio-Rad). The ratios of Notch-1 against β-actin were calculated. Statistical analysis was done using GraphPad InStat 3.0 (GraphPad Software, Inc., San Diego, CA). Statistical significance was assumed if $P \leq 0.05$.

Results

Down-regulation of Notch-1 decreased cancer cell invasion. To examine the role of Notch-1 on the invasion of human pancreatic cancer cells, the BxPC-3 cells were transfected with human Notch-1 siRNA or control siRNA. Down-regulation of Notch-1 by siRNA transfection showed low-expression of Notch-1 protein as confirmed by Western blot analysis (Fig. 1*A*). To evaluate whether the Notch-1 contributes to invasion of BxPC-3 cells, we used Matrigel invasion chamber assay to examine the invasive potential of Notch-1 siRNA–transfected BxPC-3 cells. As illustrated in Fig. 1*B*, Notch-1 siRNA–transfected cells showed a low level of penetration through the Matrigel-coated membrane compared with the control siRNA–transfected cells. The value of fluorescence from the invaded BxPC-3 cells was decreased ~4-fold compared with that of control siRNA (Fig. 1*B*).

To further confirm the role of Notch-1 in BxPC-3 cell invasion, the BxPC-3 cells were stably transfected with human Notch-1 ICN or vector alone (pcDNA3) and maintained under neomycin selection. We used Western blot analysis to confirm the overexpression of Notch-1 in Notch-1 ICN-transfected cells and we found a higher level of Notch-1 expression (Fig. 1*C*), which resulted in higher penetration of cells through the Matrigel-coated membrane compared with the control-transfected cells (Fig. 1*D*). Notch-1 cDNA–transfected cells induced a 3.5-fold increase in cells migrated through Matrigel-coated filters, indicating that overexpression of Notch-1 can enhance tumor invasion of BxPC-3 pancreatic cancer cells (Fig. 1*D*).

Down-regulation of Notch-1 decreased NF-κB DNA-binding activity. The NF-κB signaling pathway is involved in cancer cell invasion processes. Therefore, we measured the NF-κB DNA-binding activity in transfected BxPC-3 cells. Nuclear extracts from control and ICN or Notch-1 siRNA–transfected BxPC-3 cells were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. It was found that down-regulation of Notch-1 by Notch-1 siRNA transfection decreased NF-κB DNA-binding activity. However, Notch-1 overexpression by Notch-1 ICN transfection significantly induced NF-κB DNA-binding activity in stably transfected BxPC-3 cells compared with the control (Fig. 2). The specificity of NF-κB DNA binding to the DNA consensus sequence was confirmed by supershift. These results indicated that Notch-1 overexpression increased NF-κB DNA-binding activity in BxPC-3 cancer cells. The expression of MMP-9 and VEGF are regulated by NF-κB and has been reported to play an important role in tumor invasion (15–19, 21, 22). We therefore investigated whether MMP-9 and VEGF were involved in invasion induced by Notch-1.

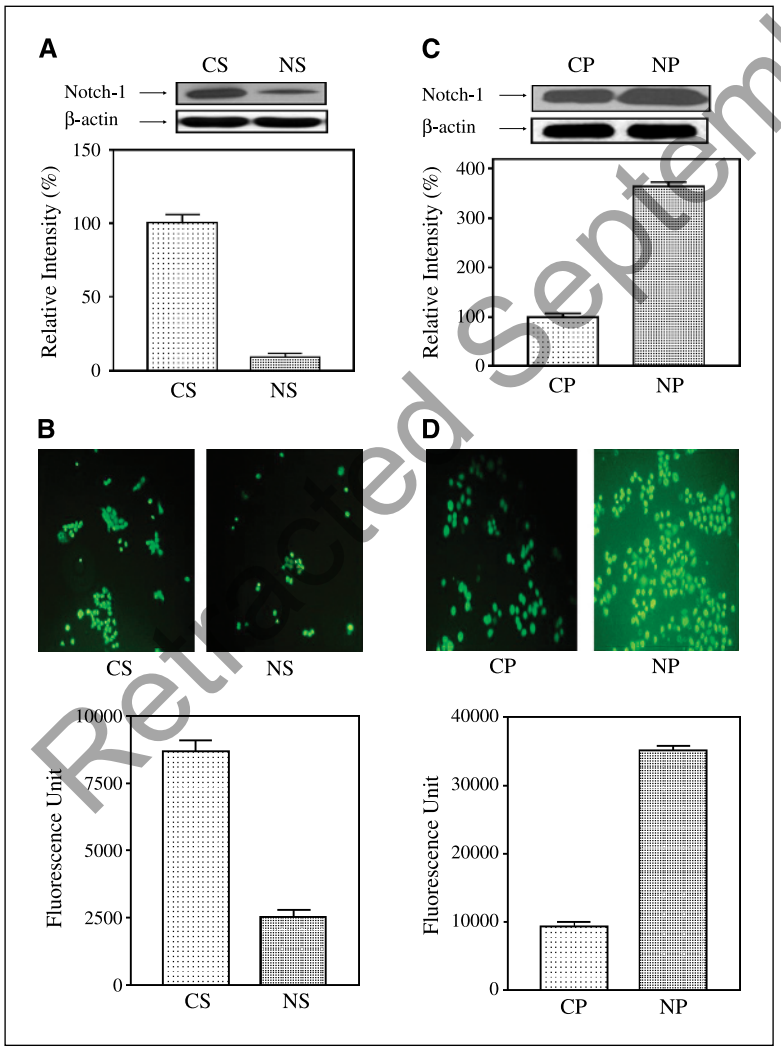

Figure 1. Down-regulation of Notch-1 decreased cancer cell invasion. *CS,* control siRNA; *NS,* Notch-1 siRNA; *CP,* control plasmid; *NP,* Notch-1 plasmid. *A,* Notch-1 was down-regulated in Notch-1 siRNA–transfected BxPC-3 cells, compared with siRNA control-transfected cells. *B, top,* invasion assay showing that Notch-1 siRNA–transfected cells resulted in low penetration of cells through the Matrigel-coated membrane, compared with control siRNA–transfected cells. *Bottom,* value of fluorescence from the invaded BxPC-3 cells. The value indicated the comparative amount of invaded BxPC-3 cells. *C,* overexpression of Notch-1 by cDNA transfection showed overexpression of Notch-1 protein as confirmed by Western blot analysis. *D, top,* Notch-1 cDNA–transfected cells showed a high level of penetration compared with control cells. *Bottom,* the value of fluorescence from the invaded BxPC-3 cells was increased ~3.5-fold compared with that of control plasmid.

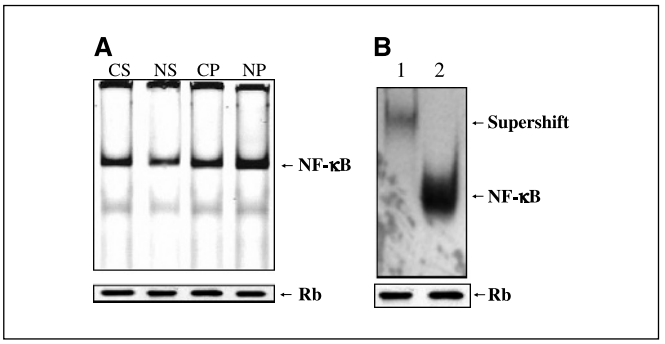

Figure 2. Notch-1 induced NF-κB DNA-binding activity. *A,* EMSA analysis was done for BxPC3 cells. Nuclear extracts were prepared from control and transfected cells and subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. Retinoblastoma protein level served as nuclear protein loading control. *B,* supershift assay showed that NF-κB band was shifted because of the formation of bigger complex after addition of anti-NF-κB p65 antibody. This assay confirmed the specificity of NF-κB binding to the DNA consensus sequence.

Down-regulation of Notch-1 decreased *MMP-9* gene transcription and its activity. To explore whether the invasiveness of transfected cells was associated with MMP-9 induction, real-time reverse transcription-PCR (RT-PCR) and Western blotting were conducted to detect the alteration in the expression of MMP-9. We found that both MMP-9 mRNA and protein levels were dramatically decreased in the Notch-1 siRNA–transfected cells (Fig. 3*A* and *B*). Next, we examined whether the down-regulation of Notch-1 could lead to a decrease in MMP-9 activity. There was a 4-fold decrease in the activity of MMP-9 (Fig. 3*C*). Furthermore, we found that overexpression of Notch-1 increased the expression and activity of MMP-9 (Fig. 3*A-C*). Our gene transfection experiments also showed that CAT activity of the MMP-9 promoter decreased in Notch-1 siRNA–transfected cells and these results are consistent with our results on the down-regulation of NF-κB resulting in the transcriptional down-regulation of MMP-9 and its activity in Notch-1 siRNA–transfected cells (Fig. 3*D*).

Down-regulation of Notch-1 reduced VEGF gene transcription and its activity. We further investigated whether Notch-1 expression has any effect on VEGF induction, whose expression is transcriptionally regulated by NF-κB. Real-time RT-PCR and Western blotting were done to detect the expression of VEGF. We found that both VEGF mRNA and protein levels were dramatically reduced in the Notch-1 siRNA–transfected cells (Fig. 4*A* and *B*). Most importantly, we also found that the down-regulation of Notch-1 could lead to a decrease in VEGF level (Fig. 4*C*). Our results also showed that overexpression of Notch-1 increased the expression and the level of VEGF, which are consistent with the regulation of the DNA-binding activity of NF-κB by Notch-1.

Down-regulation of MMP-9 and VEGF decreased cancer cell invasion. To further confirm the role of MMP-9 and VEGF in BxPC-3 cell invasion, the ICN-transfected BxPC-3 cells (stable transfectant) were transfected with human MMP-9 siRNA or VEGF siRNA. Down-regulation of MMP-9 or VEGF by siRNA transfection showed low expression of MMP-9 or VEGF protein as confirmed by Western blot analysis, respectively (Fig. 5*A*). To further test whether the Notch-1-induced invasion of BxPC-3 cells is mediated through MMP-9 and VEGF, we used Matrigel invasion chamber assay to examine the invasive potential of MMP-9 siRNA or VEGF siRNA–transfected BxPC-3 cells that were stably transfected with ICN. As illustrated in Fig. 5*B*, both MMP-9 siRNA and VEGF siRNA–transfected cells showed a low level of penetration through the Matrigel-coated membrane compared with the control siRNA–transfected cells. The value of fluorescence was decreased ~5.5-fold and 2.5-fold in the invaded BxPC-3 cells transfected with MMP-9 siRNA and VEGF siRNA compared with that of control siRNA, respectively (Fig. 5*C*).

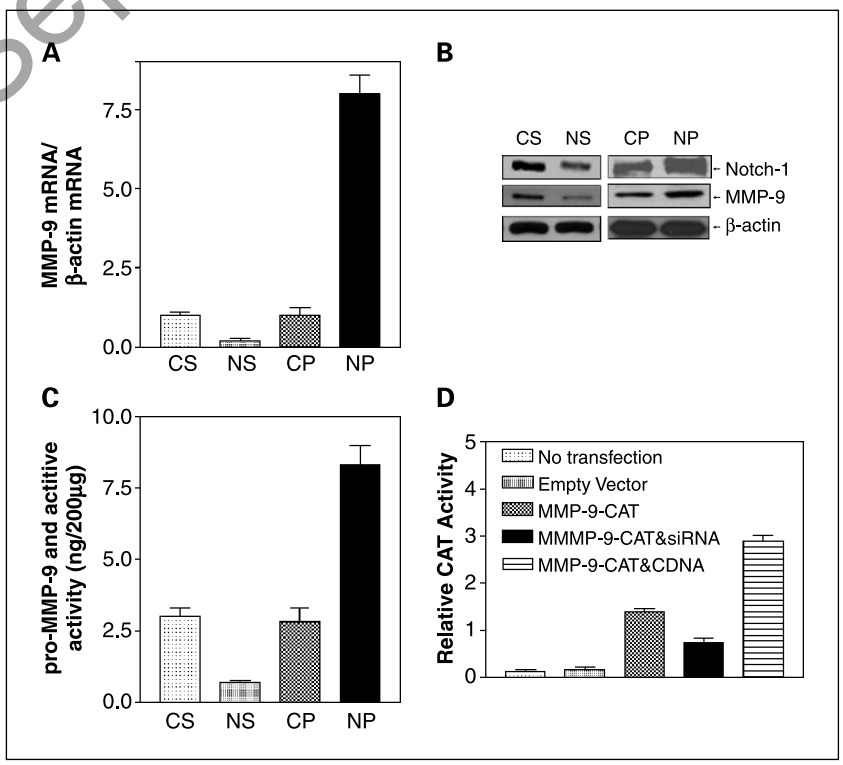

Figure 3. MMP-9 expression was up-regulated by Notch-1 ICN cDNA transfection and down-regulated by Notch-1 siRNA transfection. *A,* real-time RT-PCR analysis of MMP-9 mRNA expression in transfected BxPC-3 cells. *B,* Western blot analysis of MMP-9 protein expression in transfected BxPC-3 cells. *C,* MMP-9 activity assay showing that MMP-9 was up-regulated by ICN cDNA transfection and down-regulated by Notch-1 siRNA transfection. *D,* MMP-9 promoter transfection and CAT assay showing that the activity of MMP-9 promoter was inhibited by Notch-1 siRNA transfection and promoted by ICN cDNA transfection.

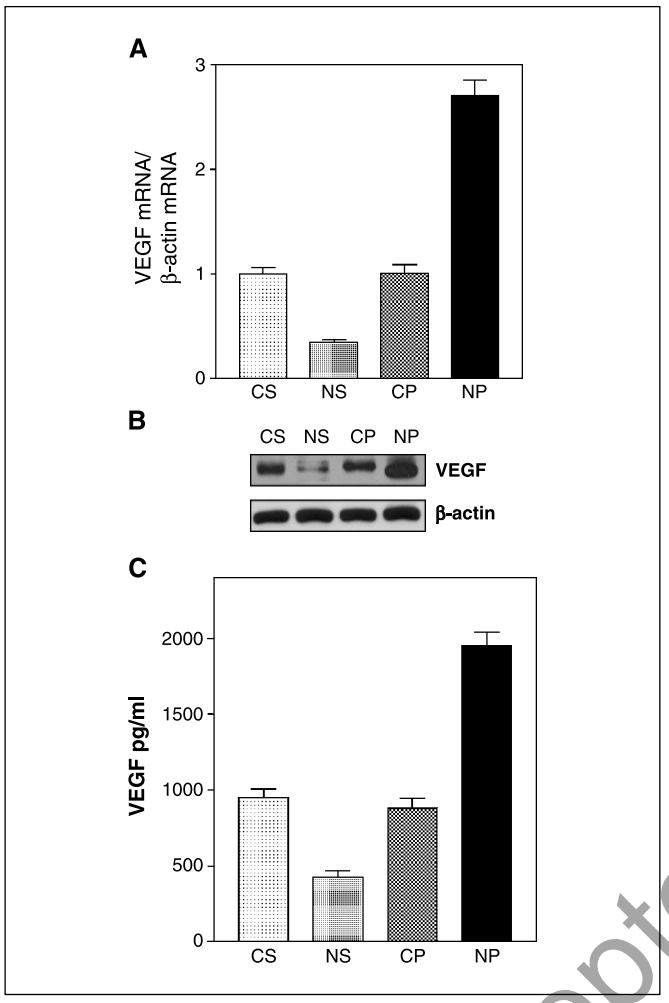

Figure 4. VEGF expression was up-regulated by cDNA transfection and down-regulated by Notch-1 siRNA transfection. *A,* real-time RT-PCR analysis of VEGF mRNA expression in transfected BxPC-3 cells. *B,* Western blot analysis of VEGF protein expression in transfected BxPC-3 cells. *C,* VEGF assay showing that VEGF level in culture medium was up-regulated by cDNA transfection and down-regulated by Notch-1 siRNA transfection.

Discussion

Notch signaling is an important cell signaling pathway, which is critically needed for the maintenance and regulation of the balance between cell proliferation, differentiation, and apoptosis. The *Notch* gene is known to suppress apoptosis and promote cell proliferation through a growth factor–mediated survival pathway (23). However, the precise role and mechanism of Notch for tumor invasion remains unclear. In this study, we have shown the following: (*a*) down-regulation of Notch-1 by siRNA decreased cell invasion but Notch-1 overexpression by cDNA transfection increased tumor cell invasion; (*b*) down-regulation of Notch-1 reduced NF-κB DNA-binding activity and VEGF expression; (*c*) down-regulation of Notch-1 also decreased not only MMP-9 mRNA and its protein expression, but also inactivated the pro-MMP-9 protein to its active form. Taken together, these results further support that the down-regulation of Notch-1 could be an effective approach for the inactivation of NF-κB and down-regulation of its target genes, such as *MMP-9* and *VEGF* expression, resulting in the inhibition of invasion and metastasis.

NF-κB activation has also been reported to be associated with metastatic phenotype and to regulate the expression of a variety of important genes in some cellular responses, including metastasis-related genes such as *VEGF* and *MMP-9* (15–19). Because NF-κB plays important roles in many cellular processes, studies on the interaction of NF-κB activation with other cell signal transduction pathways, including the Notch pathway, has received increased attention in recent years. Notch-1 has also been reported to cross-talk with the NF-κB pathway. Notch-1 strongly induces NF-κB promoter activity and induces the expression of several NF-κB subunits and NF-κB DNA-binding activity (12, 24). Shin et al. (25) recently reported that Notch-1 maintains NF-κB activity by direct interaction with p50 in the nucleus. This interaction retains active NF-κB complexes in the nucleus, leading to sustained NF-κB activity over time and in the activation of NF-κB–regulated genes. Activation of NF-κB leads to up-regulation of several downstream target genes, including *MMP-9* and *VEGF*. Thus, the down-regulation of Notch-1 results in lower NF-κB activity and its downstream targets. Therefore, it is possible that Notch-1-induced cell invasion is partly due to activation of the NF-κB pathway. In the present study, we showed that down-regulation of Notch-1 reduced NF-κB DNA-binding activity and concomitantly inhibited

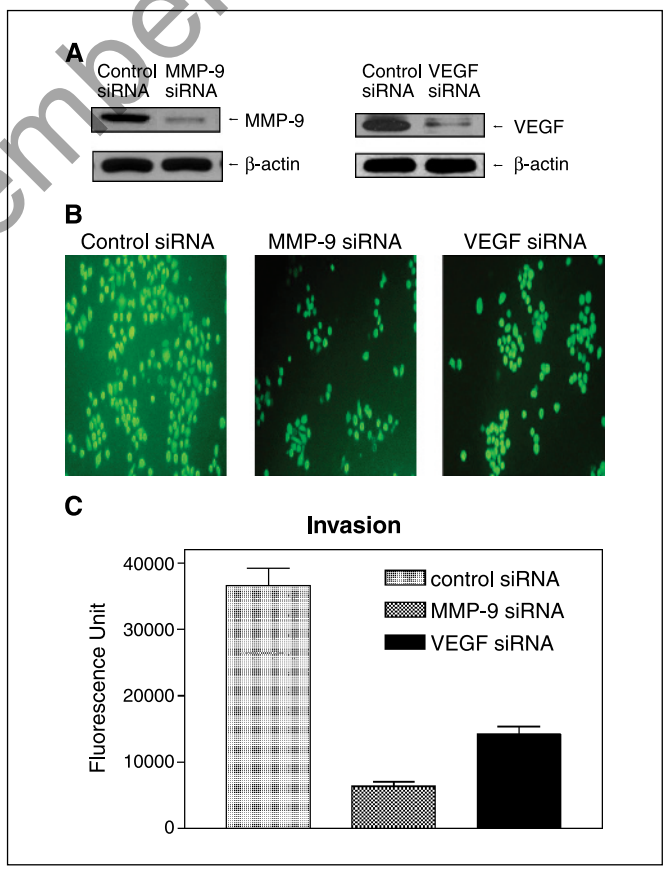

Figure 5. Down-regulation of MMP-9 or VEGF by siRNA transfection decreased tumor cell invasion. *A,* down-regulation of MMP-9 or VEGF by siRNA transfection showed low-expression of MMP-9 or VEGF protein in ICN-transfected BxPC-3 cells as confirmed by Western blot analysis. *B,* invasion assay showing that MMP-9 or VEGF siRNA–transfected cells resulted low penetration of cells through the Matrigel-coated membrane, compared with control siRNA–transfected cells. *C,* value of fluorescence from the invaded BxPC-3 cells. The value indicated the comparative amount of invaded BxPC-3 cells.

the expression and activation of MMP-9. MMPs are critically involved in the processes of tumor cell invasion and metastasis. MMP-9 has been implicated in metastasis because of its role in degradation of basement membrane collagen (15–19). Here, we showed that the inhibition of MMP-9 expression by down-regulation of Notch-1 may be mediated by the inhibition of NF-κB, whose binding site has been found in the promoter of MMP-9. Thus, these results suggest that down-regulation of Notch-1 could potentiate the antitumor and antimetastasis activities partly through the down-regulation of MMP expression. Because we observed that down-regulation of Notch-1 inhibited MMP-9 expression, we tested the effects of Notch-1 on the invasion of BxPC-3 pancreatic cancer cells. We found that down-regulation of Notch-1 inhibited the cell invasion of BxPC-3 cells. These results were consistent with MMP-9 data, showing that down-regulation of Notch-1 could inhibit cancer cell invasion partly through down-regulation of MMP-9.

Another important molecule involved in tumor cell invasion and metastasis is VEGF. Investigations by other laboratories have shown that VEGF promotes migration and invasion of pancreatic cancer cells. There was a trend toward an association between expression of VEGF and distant metastasis (26, 27). In this study, consistent with our invasion data, we found a significant reduction on the expression of VEGF by down-regulation of Notch-1. It is well accepted that the expression of MMP-9, VEGF, survivin, and cyclooxygenase-2 (COX-2) is regulated by NF-κB (27–31). In our early report, we showed that down-regulation of Notch-1 reduced survivin and COX-2 expression (20). Recent findings have shown that both COX-2 and survivin promote cancer cell invasion in pancreatic cancer cell (28, 29, 32–35). Down-regulation of Notch-1 inhibited NF-κB reporter gene and gene products, such as those involved in cell invasion. Based on our results, we speculate that one possible mechanism by which Notch-1 induces invasion is due to activation of NF-κB DNA-binding activity, which leads to up-regulation of NF-κB target genes, such as *MMP-9, VEGF, survivin*, and *COX-2*. On the basis of our results, we propose a hypothetical pathway by which Notch-1 may induce invasion of BxPC-3 cells, partly through NF-κB signaling pathway (Fig. 6). However, further in-depth studies are needed to investigate the precise molecular mechanism regarding the cause and effect relationship between Notch-1 and NF-κB during Notch-1 induced invasion of pancreatic cancer cells.

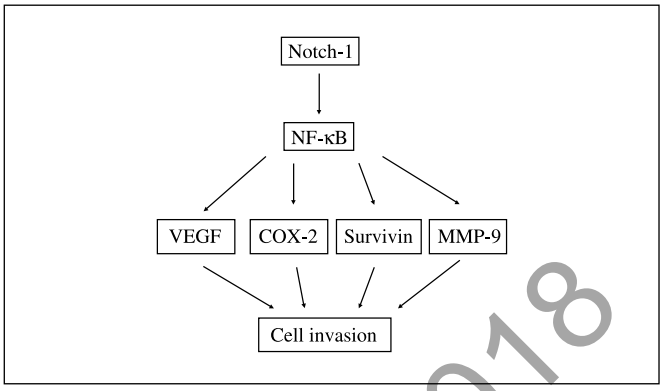

Figure 6. The schematic presentation of our proposed mechanism of how Notch-1 promotes pancreatic cancer cell invasion.

In summary, we presented experimental evidence that strongly supports the antitumor and antimetastasis effects of down-regulation of Notch-1 in BxPC-3 pancreatic cancer cells. Down-regulation of Notch-1 could be an effective approach for the inactivation of NF-κB and its target genes, such as *MMP-9* and *VEGF* expression, resulting in the inhibition of invasion and metastasis, which could be useful for devising novel preventive and therapeutic strategies for pancreatic cancer.

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Cancer Research

Retraction: Down-regulation of Notch-1 Inhibits Invasion by Inactivation of Nuclear Factor-κB, Vascular Endothelial Growth Factor, and Matrix Metalloproteinase-9 in Pancreatic Cancer Cells

This article (1) has been retracted at the request of the editors. Following an institutional review by Wayne State University (Detroit, MI), the primary affiliation for several of the authors, it was determined that the article (1) included falsification and/or fabrication of Western blot bands in multiple panels of Figs. 1–5. As a result of these findings, the institution recommended retraction and, upon internal review, the editors agree with this recommendation.

A copy of this Retraction Notice was sent to the last known email addresses for five of the six authors. Five authors (Z. Wang, S. Banerjee, Y. Li, K.M.W. Rahman, and F.H. Sarkar) did not respond; the remaining author (Y. Zhang) could not be located.

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1. Wang Z, Banerjee S, Li Y, Rahman KMW, Zhang Y, Sarkar FH. Down-regulation of Notch-1 inhibits invasion by inactivation of nuclear factor-κB, vascular endothelial growth factor, and matrix metalloproteinase-9 in pancreatic cancer cells. Cancer Res 2006;66:2778–84.

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