Down-regulation of Notch-1 contributes to cell growth inhibition and apoptosis in pancreatic cancer cells

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Abstract

Pancreatic cancer remains the fourth most common cause of cancer-related death in the United States. Notch signaling plays a critical role in maintaining the balance among cell proliferation, differentiation, and apoptosis, and thereby may contribute to the development of pancreatic cancer. To characterize Notch pathway function in pancreatic cancer cells, we explored the consequences of down-regulation of Notch-1 in BxPC-3, HPAC, and PANC-1 pancreatic cancer cells. Using multiple cellular and molecular approaches such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, apoptosis assay, flow cytometry, gene transfection, real-time reverse transcription-PCR (RT-PCR), Western blotting, and electrophoretic mobility shift assay for measuring DNA binding activity of nuclear factor κB (NF-κB), we found that down-regulation of Notch-1 inhibited cell growth and induced apoptosis in pancreatic cancer cells. Notch-1 down-regulation also increased cell population in the G_0-G_1 phase. Compared with control, small interfering RNA–transfected cells decreased expression of cyclin A, cyclin D1, and cyclin-dependent kinase 2. We found up-regulation of p21 and p27, which was correlated with the cell cycle changes. In addition, Notch-1 down-regulation also induced apoptosis, which could be due to decreased Bcl-2 and Bcl-X_L protein expression in pancreatic cancer cells. Because Notch-1 is known to cross-talk with another major cell growth and apoptotic regulatory pathway (i.e., NF-κB), we found that NF-κB is a downstream target of Notch because down-regulation of Notch reduced NF-κB activity. We also found that genistein, a prominent isoflavone, could be an active agent for the down-regulation of the Notch pathway. These findings suggest that Notch-1 down-regulation, especially by genistein, could be a novel therapeutic approach for the treatment of pancreatic cancer. [Mol Cancer Ther 2006;5(3):483–93]

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Introduction

Pancreatic cancer has the worst prognosis among all major cancers and remains the fourth most common cause of cancer related death in the United States and throughout the world (1). This could be due to the fact that no effective methods of early diagnosis are currently available as well as the lack of effective therapies, resulting in high mortality of patients diagnosed with pancreatic cancer. This disappointing outcome strongly suggests that innovative research is needed to control this deadly disease.

Notch signaling is involved in cell proliferation and apoptosis, which affect the development and function of many organs (2, 3). *Notch* genes encode proteins which can be activated by interacting with a family of its ligands (4, 5). On activation, Notch is cleaved, releasing intracellular Notch which translocates into the nucleus. The intracellular Notch associates with transcriptional factors, which regulate the expression of target genes, and thus plays important roles in development and cell growth (6, 7). To date, four vertebrate *Notch* genes have been identified: *Notch-1, Notch-2, Notch-3*, and *Notch-4*. In addition, five ligands, Dll-1, Dll-3, Dll-4, Jagged-1, and Jagged-2, have been found in mammals (8, 9).

Because Notch signaling plays important roles in the cellular developmental pathway including proliferation and apoptosis (10), alterations in Notch signaling are associated with tumorigenesis. These observations suggest that dysfunction of intracellular Notch prevents differentiation, ultimately guiding undifferentiated cells toward malignant transformation (11). It has been reported that the Notch signaling network is frequently deregulated in human malignancies with up-regulated expression of Notch receptors and their ligands in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas, and pancreatic cancer (12–14). It has also been reported that Notch-1 expression inhibits apoptosis (15–17), suggesting a possible role of *Notch* as an oncogene in many cancers.

Notch-1 has been reported to cross-talk with another major cell growth and apoptotic regulatory pathway [i.e., nuclear factor κB (NF-κB)]. Specifically, Notch-1 has been reported to strongly induce NF-κB2 promoter activity in reporter assays (18) and expression of several NF-κB subunits (19). Notch ligands activate NF-κB in human

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keratinocytes, and down-regulation of Notch-1 results in lower NF-κB activity. Levels of basal and stimulation-induced NF-κB activity were significantly decreased in mice with reduced Notch levels (20, 21). Constitutive levels of Notch activity are essential in maintaining NF-κB activity in various cell types. Notch and NF-κB pathways are key regulators of numerous cellular events such as proliferation, differentiation, and apoptosis. Therefore, inactivation of Notch-1-mediated cell growth inhibition and induction of apoptosis could be partly mediated via inactivation of NF-κB activity.

However, the mechanisms by which Notch-1 inhibits apoptosis in pancreatic cancer cells are still unclear. Because Notch-1 down-regulation showed antineoplastic effects *in vivo* and *in vitro* (15–17, 22), the potential for treating certain cancers could be achievable by inhibiting Notch signal transduction. Genistein, a natural isoflavonoid found in soybean products, consumed in a diet, has been associated with lower incidences of endometrial, breast, prostate, and pancreatic cancers and is believed to be a chemopreventive agent (23). Studies from our laboratory and others have shown that genistein can inhibit cell growth and induce apoptosis in various cancer cell lines (24–26). However, little is known about the *Notch-1* gene alteration in pancreatic cancer cells after genistein treatment. Therefore, we hypothesized that genistein may inhibit Notch-1 activation in pancreatic cancer cells leading to apoptotic cell death. Therefore, in this report, we tested our hypothesis on whether down-regulation of *Notch-1* gene expression, either by small interfering RNA (siRNA) or by genistein, could inhibit cell growth and induce apoptosis, which are mechanistically associated with the down-regulation of NF-κB in pancreatic cancer cells. Our data show that down-regulation of Notch-1 inhibits cell growth with concomitant induction of apoptosis. Our data also show that genistein down-regulated the expression of Notch-1 and its downstream molecules, suggesting that Notch-1 down-regulation, especially by genistein, could be a novel therapeutic approach for the treatment of pancreatic cancer.

Materials and Methods

Cell Culture and Experimental Reagents

Human pancreatic cancer cell lines BxPC-3, HPAC, and PANC-1 (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in a 5% CO_2 atmosphere at 37°C. Cell Death Detection ELISA Kit was obtained from Roche (Indianapolis, IN). Primary antibodies for Notch-1, Hes-1, cyclin D1, cyclin A, p21, p27, Bcl-2, and Bcl-X_L were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Pierce (Rockford, IL). Notch-1 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). Genistein was obtained from Toronto Research Chemicals (North York, Ontario, Canada). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO).

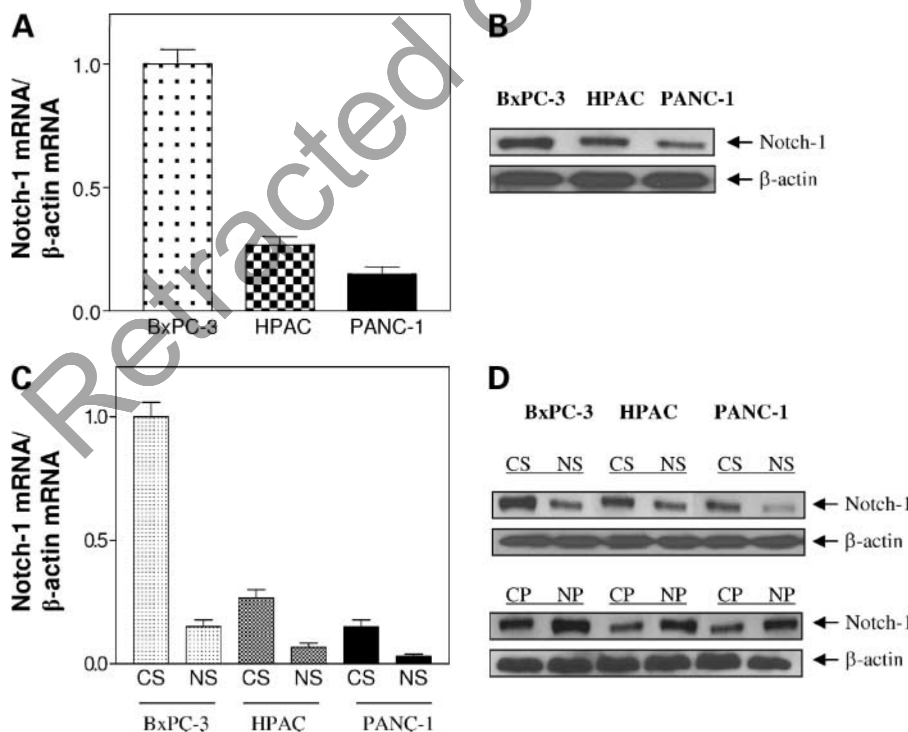

Figure 1. Constitutive expression and down-regulation of Notch-1 by siRNA in pancreatic cancer cell lines BxPC-3, HPAC, and PANC-1. *CS*, control siRNA; *NS*, Notch-1 siRNA; *CP*, control plasmid; *NP*, Notch-1 plasmid. **A** and **B,** Notch-1 mRNA and protein levels were measured by real-time RT-PCR and Western blotting. **C,** Notch-1 mRNA levels were down-regulated by Notch-1 siRNA. **D,** *top,* Notch-1 protein levels were down-regulated by siRNA in all three pancreatic cancer cells. *Bottom,* Notch-1 protein levels were overexpressed by cDNA transfection in different cell lines.

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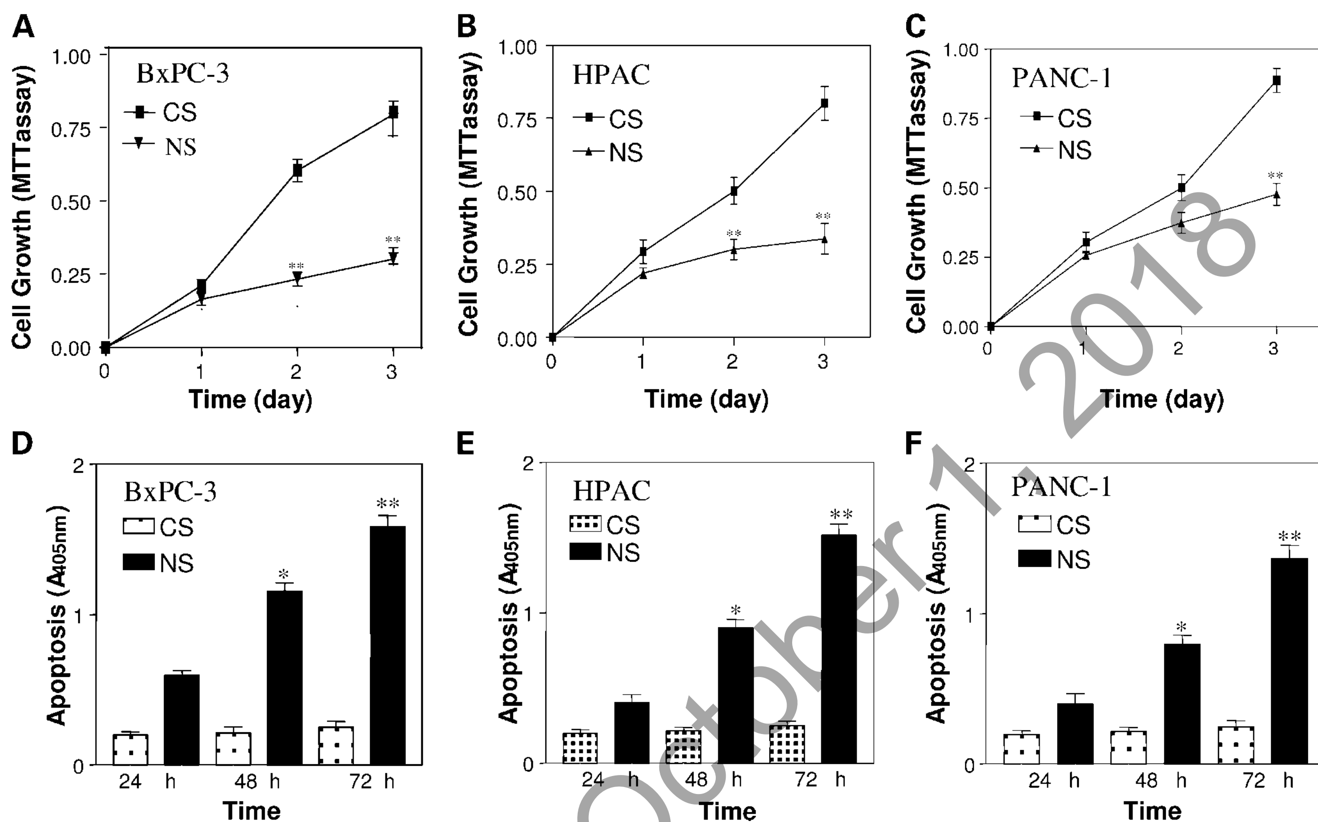

Figure 2. Effect of down-regulation of Notch-1 by siRNA on pancreatic cancer cell growth and apoptosis. **A** to **C**, inhibition of cancer cell growth tested by MTT assay. **D** to **F**, cell death assay for measuring apoptosis tested by ELISA. *, $P < 0.05$; **, $P < 0.01$, relative to control siRNA.

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Plasmids and Transfections

The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain and control plasmids were kind gifts from L. Miele (Department of Biopharmaceutical Sciences and Cancer Center, University of Illinois at Chicago, Chicago, IL; ref. 11). Cells were transfected with Notch-1 siRNA and siRNA control, respectively, using Lipofectamine 2000. Cells were stably transfected with human intracellular Notch or vector alone (pcDNA3) and maintained under neomycin selection.

Cell Growth Inhibition Studies by MTT Assay

The transfected cells (5×10^3) were seeded in a 96-well culture plate and subsequently incubated with MTT reagent (0.5 mg/mL) at 37°C for 2 hours and MTT assay was done as described earlier (27). Results were plotted as mean ± SD of three separate experiments having six determinations per experiment for each experimental condition.

Histone/DNA ELISA for Detection of Apoptosis

The Cell Death Detection ELISA Kit was used for assessing apoptosis in transfected cells according to the protocol of the manufacturer. Briefly, the cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Samples were then incubated with anti-DNA peroxidase followed by color development with ABTS substrate. The absorbances of the samples were determined with the Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 405 nm.

Flow Cytometry and Cell Cycle Analysis

The cells were synchronized in G_0 by serum starvation for 24 hours in phenol red–free RPMI with 0.1% serum. Subsequently, cells were released into complete medium containing 10% fetal bovine serum. The cell cycle was analyzed by flow cytometry. Briefly, 1×10^6 cells were harvested and washed in PBS, then fixed in 70% alcohol for 30 minutes at 4°C. After washing in cold PBS thrice, cells were resuspended in 1 mL of PBS solution with 40 μg of propidium iodide and 100 μg of RNase A for 30 minutes at 37°C. Samples were then analyzed for their DNA content by FACSCalibur (Becton Dickinson, Mountain View, CA).

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, 1 mmol/L phenylmethylsulfonyl fluoride] by incubating for 20 minutes at 4°C. The protein concentration was determined with the Bio-Rad assay system (Hercules, CA). Total proteins were fractionated by SDS-PAGE and transferred onto nitrocellulose membrane

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for Western blotting as described earlier (27). Quantification of blotting was done using laser densitometry and the results are presented as the mean of three independent experiments with error bars representing SD.

Real-time Reverse Transcription-PCR Analysis for Gene Expression Studies

The total RNA from transfected 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 (Applied Biosystems, Foster City, CA) in a total volume of 50 μL, including 6.25 units of MultiScribe reverse transcriptase and 25 pmol of 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 Notch-1 forward primer (5′-CACTGTGGCGGGTCC-3′) and reverse primer (5′-GTTGTATTGGTTCGGCACCAT-3′) and β-actin forward primer (5′-CCACACTGTGCCCATC-TACG-3′) and reverse primer (5′-AGGATCTTCATGAGG-TAGTCAGTCAG-3′). Real-time PCR amplications were done as described earlier (28).

Electrophoretic Mobility Shift Assay for Measuring NF-κB Activity

The transfected cells were washed with cold PBS and suspended in 0.15 mL of 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, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.5 mg/mL benzamidine]. The nuclear protein was prepared and subjected to DNA binding activity of NF-κB by electrophoretic mobility shift assay (EMSA) as described earlier (27).

Genistein Treatment of BxPC-3 Cells for Different Periods of Time

The transfected BxPC-3 cells were seeded in 100-mm dishes and allowed to attach for 24 hours, followed by the addition of different concentrations of genistein or 0.5 mmol/L Na_2CO_3 (solvent control) for different periods of time. The proteins were extracted and measured by Western blotting. In addition, the cell growth and apoptotic cell death in transfected cells with treatments were detected using MTT assay and Cell Death Detection ELISA Kit, respectively, following the procedure described earlier. NF-κB activity was measured by EMSA as discussed above.

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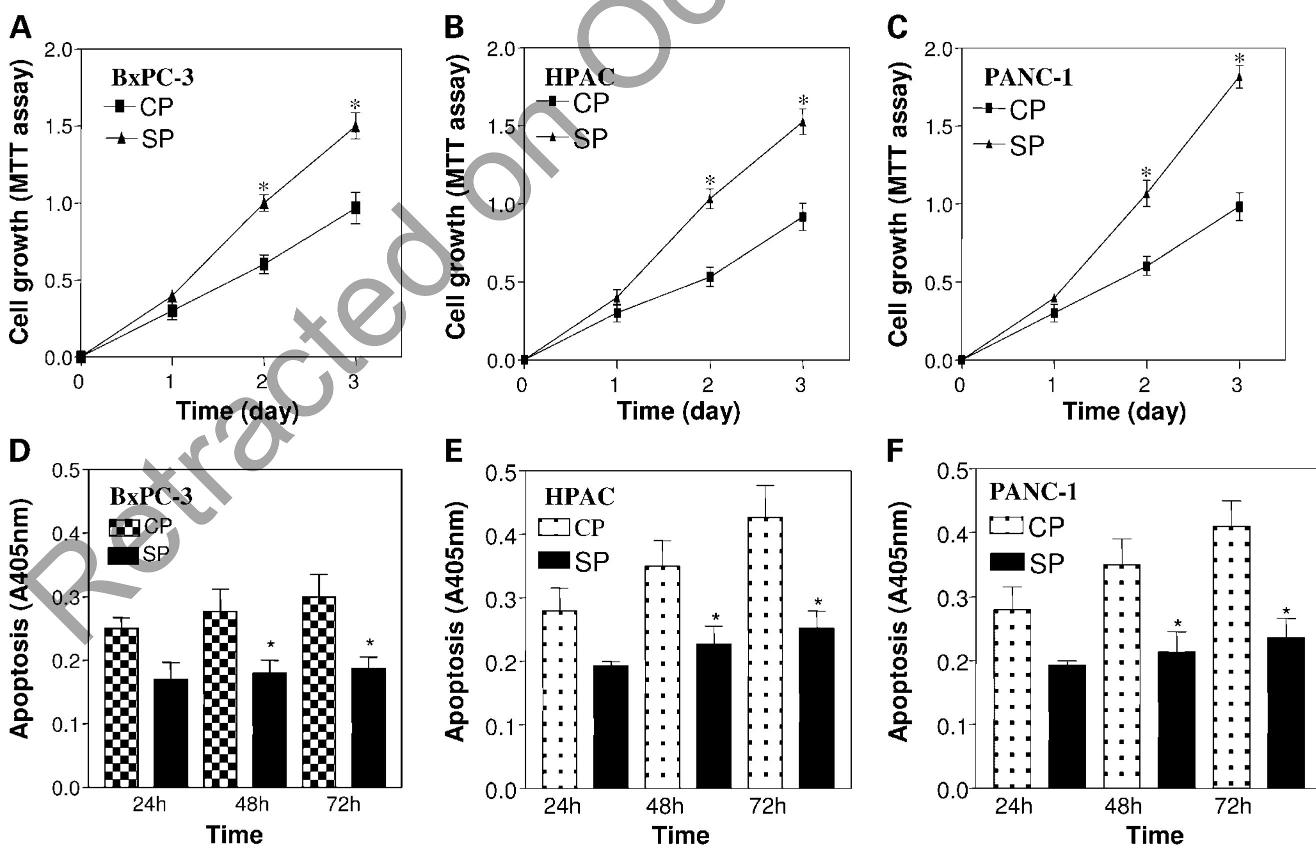

Figure 3. Effect of up-regulation of Notch-1 by cDNA plasmid on pancreatic cancer cell growth and apoptosis. **A** to **C**, promotion of cancer cell growth tested by MTT assay. **D** to **F**, cell death assay for measuring apoptosis tested by ELISA. *, $P < 0.05$; **, $P < 0.01$, relative to control plasmid.

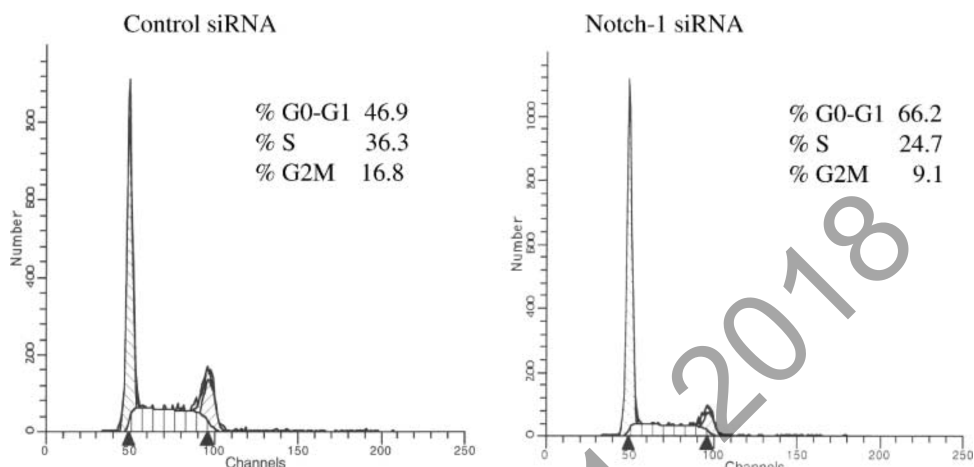

Figure 4. Effect of the down-regulation of Notch-1 on cell cycle distribution. HPAC cells were harvested for cell cycle analysis using propidium iodide staining. *X axis,* DNA content; *Y axis,* number of nuclei. Compared with the control, down-regulation of Notch-1 caused G_0-G_1 cell cycle arrest.

Densitometric and Statistical Analysis

The bidimensional absorbances 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. The cell growth inhibition by transfection or genistein treatment was statistically evaluated with GraphPad StatMate software (GraphPad Software, Inc., San Diego, CA). Comparisons were made between control and transfection or genistein treatment. $P < 0.05$ was used to indicate statistical significance.

Results

Down-Regulation of Notch-1 Expression by siRNA Inhibited Cell Growth and Induced Apoptosis

Initial studies were done to examine the relative levels of Notch-1 in three pancreatic cancer cells, BxPC-3, HPAC, and PANC-1, by real-time RT-PCR and Western blot analysis. All three cell lines expressed high levels of Notch-1 at both mRNA and protein levels (Fig. 1A and B). To determine whether Notch-1 could be an effective therapeutic target for pancreatic cancer, the effect of Notch-1 siRNA on cell growth of the pancreatic cancer cells was examined. The efficacy of Notch-1 siRNA for knockdown of Notch-1 mRNA and protein was confirmed through real-time RT-PCR and Western blotting. We observed that both Notch-1 mRNA and protein levels were barely detectable in Notch-1 siRNA–transfected cells compared with siRNA control–transfected cells (Fig. 1C and D). The cell viability was determined by MTT and the effect of Notch-1 siRNA on the growth of cancer cells was shown in Fig. 2A to C. We found that down-regulation of Notch-1 expression caused cell growth inhibition in all three pancreatic cancer cell lines.

To investigate whether the growth inhibitory effects of Notch-1 siRNA are partially related to the induction of apoptosis, the effect of Notch-1 siRNA on apoptotic cell death was examined using an ELISA-based assay. These results provided convincing data that down-regulation of Notch-1 induces apoptosis in all three pancreatic cancer cell lines (Fig. 2D–F). These data suggest that the growth inhibitory activity of Notch-1 down-regulation is partly attributed to an increase in cell death.

Overexpression of Notch-1 by cDNA Transfection Promoted Cell Growth and Inhibited Apoptosis

Pancreatic cancer cells were stably transfected with human intracellular Notch or empty vector alone (pcDNA3) and maintained under neomycin selection. The proteins were measured by Western blotting. The results showed that Notch-1 protein level was increased by intracellular Notch transfection (Fig. 1D). Intracellular Notch–transfected cells showed significant promotion of cell growth compared with empty vector–transfected control cells (Fig. 3A–C). We also found that overexpression of Notch-1 protected cells from apoptosis to a certain degree (Fig. 3D–F).

Down-Regulation of Notch-1 Induced Cell Cycle Arrest in G_0-G_1 Phase

To further investigate the growth inhibitory effect of Notch-1 knockdown in pancreatic cancer cells, we did cell

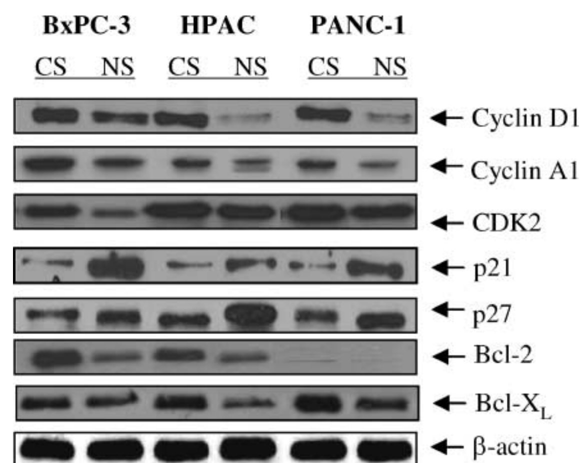

Figure 5. The level of expression of several known G_0-G_1 cell cycle regulatory factors as detected by Western blotting in all three pancreatic cancer cells.

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Table 1. Flow cytometric analysis of Notch-1-transfected cells

	G_0-G_1 (%)	S (%)	G_2-M (%)
BxPC-3			
CS	55.8 ± 0.7	30.6 ± 1.1	13.6 ± 0.9
NS	72.4 ± 1.4	22.1 ± 1.5	5.5 ± 0.6
CP	52.3 ± 3.5	29.2 ± 3.1	18.5 ± 0.4
NP	37.8 ± 1.2	42.4 ± 2.0	19.8 ± 3.1
HPAC			
CS	46.9 ± 2.4	36.3 ± 1.5	16.8 ± 1.2
NS	66.2 ± 1.8	24.7 ± 1.5	9.1 ± 1.1
CP	53.8 ± 2.0	34.4 ± 1.3	11.8 ± 2.1
NP	40.1 ± 1.9	36.8 ± 1.5	23.1 ± 1.6
PANC-1			
CS	40.5 ± 2.4	33.7 ± 1.8	25.8 ± 1.6
NS	67.4 ± 3.5	15.3 ± 1.2	17.3 ± 2.8
CP	46.1 ± 2.1	35.5 ± 1.5	18.4 ± 0.9
NP	29.8 ± 2.6	54.3 ± 1.2	15.9 ± 1.7

NOTE: Flow cytometry was done as described in Fig. 4. The mean values (± SE) represent the percentage of cells in the indicated phase of the cell cycle from three independent experiments.
Abbreviations: CS, control siRNA; NS, Notch-1 siRNA; CP, control plasmid; NP, Notch-1 plasmid.

cycle analysis using propidium iodide staining and flow cytometry. These results showed a typical G_0-G_1 arrest pattern in Notch-1 siRNA–transfected cells (Fig. 4). In contrast, synchronized intracellular Notch–transfected cells caused a greater drop in the fraction of cells at G_0-G_1 phase (Table 1).

To further characterize the G_0-G_1 arrest, we examined the level of expression of several known G_0-G_1 cell cycle regulatory factors. Consistent with cell cycle arrest, expression of cyclin A1, cyclin D1, and cyclin-dependent kinase (Cdk)-2 was found to be decreased whereas p21 and p27 expression was increased (Fig. 5), suggesting mechanistic roles of these molecules during Notch-1-induced cell cycle progression and cell cycle arrest by Notch-1 siRNA. We have also detected the expression of other proteins, such as cyclin B, Cdk4, and Cdk6, and did not find any change in the expression of those proteins (data not shown).

We also analyzed the expression of the apoptosis-related proteins Bcl-2 and Bcl-X_L. Our data showed that Bcl-2 and Bcl-X_L expression was down-regulated in siRNA-transfected cells (Fig. 5).

Down-regulation of *Notch-1* gene expression by Notch-1 siRNA inhibited NF-κB DNA-binding activity. We investigated whether the downstream effect of Notch-1 down-regulation was mechanistically associated with the NF-κB pathway. Nuclear proteins from transfected cells were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. The specificity of NF-κB DNA binding to the DNA consensus sequence was confirmed by supershift assay. The results showed that down-regulation of Notch-1 significantly inhibited NF-κB DNA-binding activity compared with control whereas Notch-1 cDNA transfection caused activation of NF-κB DNA-binding activity in all three cell lines tested (Fig. 6). These results provide evidence for a mechanistic cross-talk between Notch-1 and NF-κB in pancreatic cancer.

Down-Regulation of Notch-1 by Genistein Inhibited Cell Growth and Induced Apoptosis

Thus far, our results clearly show that Notch-1 down-regulation contributes to cell growth inhibition and apoptosis. However, the usefulness of siRNA approach for the inactivation of Notch-1 in a therapeutic setting is not available at the present time. Therefore, we have tested whether natural products could down-regulate the Notch signaling, and if so, then our approach could be useful therapeutically. Using various known chemopreventive

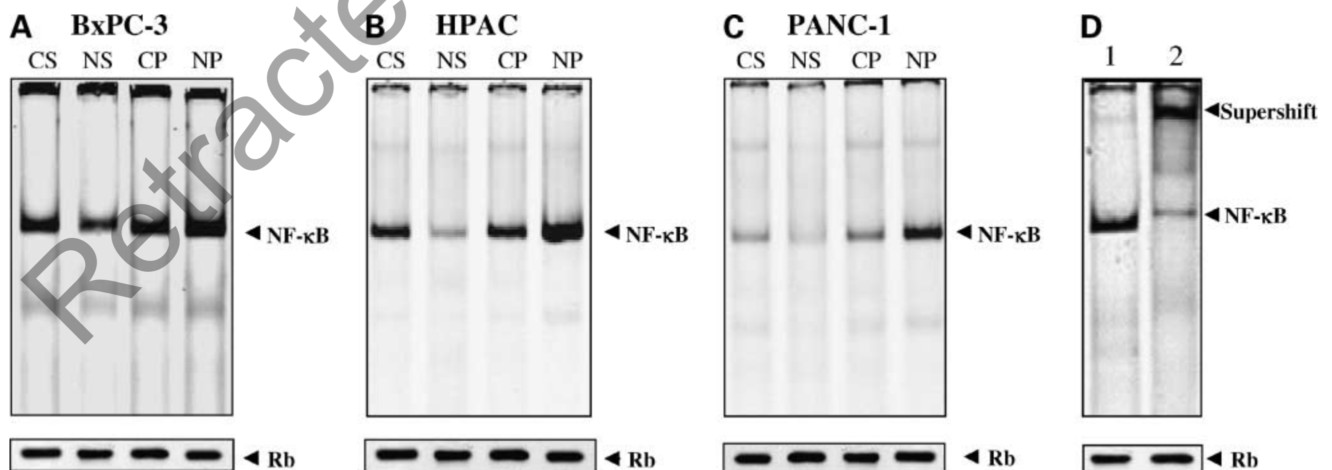

Figure 6. Down-Regulation of *Notch-1* gene expression by Notch-1 siRNA inhibited NF-κB DNA-binding activity. Nuclear proteins from siRNA- and cDNA-transfected cells were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. **A** to **C**, down-regulation of Notch-1 inhibited NF-κB DNA-binding activity compared with control whereas Notch-1 cDNA transfection caused activation of NF-κB DNA-binding activity in all three cell lines tested. **D,** NF-κB supershift analyses. EMSA experiments were done by additional 30-min incubations with polyclonal supershift antibodies against p65 before the addition of labeled probe. *1,* nonspecific antibody (anti–cyclin D1); *2,* p65 antibody.

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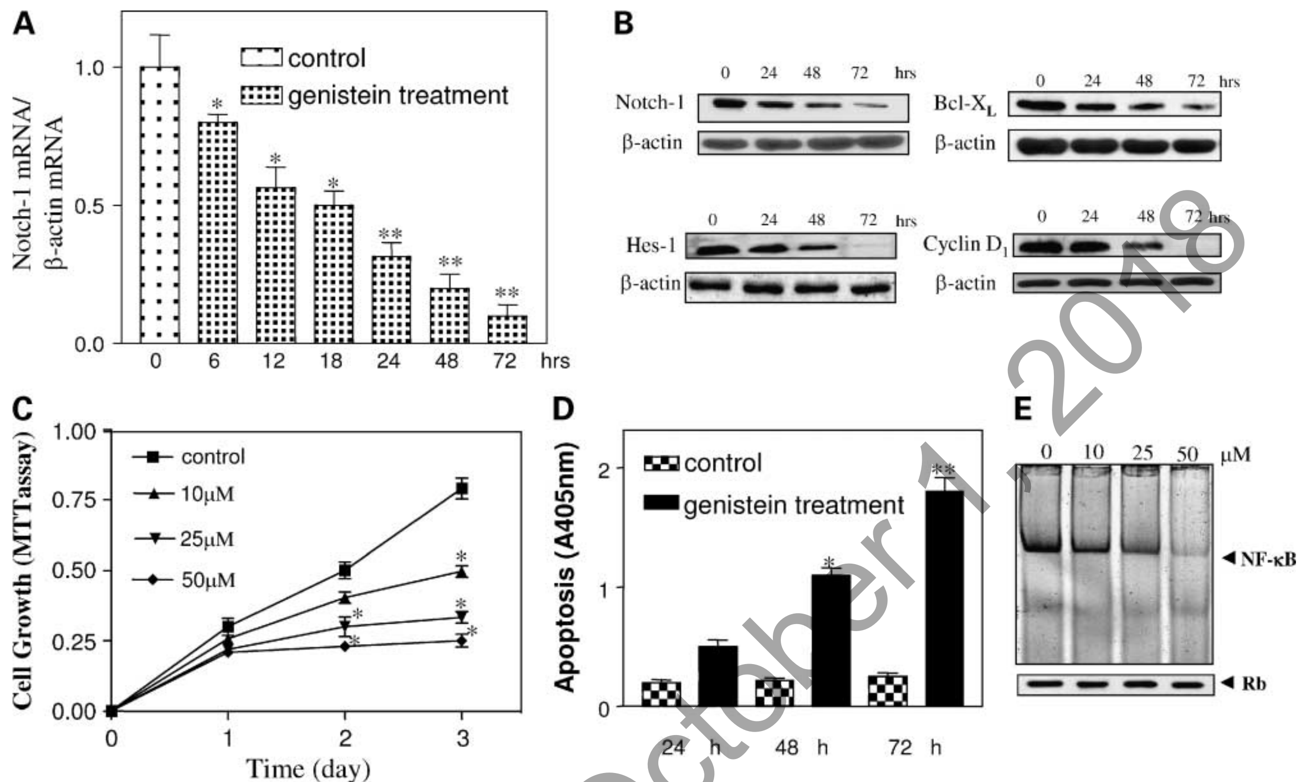

Figure 7. Down-regulation of Notch-1 by genistein inhibited cell growth and induced apoptosis. **A,** inhibition of Notch-1 mRNA after 6, 12, 18, 24, 48, and 72 h of treatment with 25 μmol/L genistein in BxPC-3 pancreatic cancer cells. The mRNA level in BxPC-3 pancreatic cancer cells treated with genistein was assessed by real-time RT-PCR. The expression of Notch-1 at the mRNA level was down-regulated after genistein treatment. *, $P < 0.05$; * *, $P < 0.01$, relative to solvent control. **B,** inhibition of Notch-1, Hes-1, cyclin D1, and Bcl-X$_L$ protein expression by 25 μmol/L genistein in BxPC-3 pancreatic cancer cells. Cells were treated with 25 μmol/L genistein for 24, 48, and 72 h. Western blot analysis showed that the protein levels of Notch-1, Hes-1, Bcl-X$_L$, and cyclin D1 were down-regulated in genistein-treated BxPC-3 pancreatic cancer cells in a time-dependent manner. **C,** inhibitory effect of genistein on the growth of BxPC-3 pancreatic cancer cells tested by MTT assay. BxPC-3 cells treated with 15, 25, and 50 μmol/L genistein for 3 d. The treatment of BxPC-3 pancreatic cancer cells with genistein resulted in cell growth inhibition. The inhibition of cell growth was dose and time dependent. *, $P < 0.01$, relative to solvent control. **D,** genistein-induced apoptosis in BxPC-3 pancreatic cancer cells measured by the histone/DNA fragment analysis using ELISA. Cells were treated with 25 μmol/L genistein for 24, 48, or 72 h. The induction of apoptosis was time dependent and was found to be more pronounced after 48 to 72 h of treatment. *, $P < 0.01$, relative to solvent control. **E,** NF-κB DNA binding activity was measured by EMSA. Genistein inhibits NF-κB DNA binding activity in BxPC-3 pancreatic cancer cells. Cells were treated with 10, 25, and 50 μmol/L genistein for 48 h. Nuclear extracts were prepared from control and genistein-treated cells and subjected to analysis for NF-κB DNA binding activity as measured by EMSA. Genistein significantly inhibited NF-κB DNA-binding activity whereas there was no change in Rb (used as a protein loading control).

agents such as curcumin, indole-3-carbinol, 3,3′-diindolylmethane, epigallocatechin gallate, genistein, and resveratrol, we found that genistein is the best agent tested thus far to down-regulate the expression of Notch-1 (data not shown). We used real-time RT-PCR and Western blotting to detect the Notch-1 level in BxPC-3 cells treated with genistein. The expression of *Notch-1* gene at the mRNA level was down-regulated after genistein treatment. The altered expression of *Notch-1* gene was observed as early as 6 hours after genistein treatment and was significantly more pronounced with longer treatment (Fig. 7A), suggesting transcriptional inactivation of *Notch-1* gene expression.

To verify whether the alternation of *Notch-1* gene at the level of transcription ultimately results in alternations at the level of translation, we conducted Western blotting for detection of Notch-1. Western blot analysis showed that the protein level of Notch-1 was down-regulated in genistein-treated BxPC-3 cells in a time-dependent manner (Fig. 7B). These results are in direct agreement with the RT-PCR data showing that genistein regulates the transcription and translation of *Notch-1* gene. In addition, we found that the expression of Notch-1 downstream target genes, including *Hes-1, Bcl-X$_L$*, and *cyclin D1*, were also down-regulated in genistein-treated cells (Fig. 7B).

We further investigated whether down-regulation of Notch-1 by genistein resulted in the inhibition of cell growth and induction of apoptosis in BxPC-3 cells. The treatment of BxPC-3 pancreatic cancer cells for 1 to 3 days with 10, 25, and 50 μmol/L of genistein resulted in cell growth inhibition in a dose- and time-dependent manner (Fig. 7C). BxPC-3 cells were treated with 25 μmol/L genistein for 24, 48, and 72 hours, respectively, to measure the degree of apoptosis. The induction of apoptosis was time dependent (Fig. 7D) and was found to be more pronounced after 48 to 72 hours of treatment. Our results

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also showed that genistein significantly inhibited NF-κB DNA-binding activity (Fig. 7E), which was in direct agreement with results previously published by our laboratory (29).

Down-Regulation of Notch-1 Expression by siRNA Promotes Genistein-Induced Cell Growth Inhibition and Apoptosis

Down-regulation of Notch-1 by siRNA transfection showed less expression of Notch-1 protein as confirmed by Western blotting (Fig. 8A). We have also found that the down-regulation of Notch-1 expression significantly inhibited cell growth induced by genistein (Fig. 8B). Notch-1 siRNA–transfected BxPC-3 cells were significantly more sensitive to spontaneous and genistein-induced apoptosis (Fig. 8C). Nuclear extracts from Notch-1 siRNA–transfected BxPC-3 cells with different treatments were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. The results showed that Notch-1 siRNA also inhibited NF-κB DNA-binding activity; however, genistein plus Notch-1 siRNA inhibited NF-κB activity to a greater degree compared with genistein alone (Fig. 8D).

Overexpression of Notch-1 by cDNA Transfection Reduced Genistein-Induced Cell Growth Inhibition and Apoptosis

Overexpression of Notch-1 by cDNA transfection showed overexpression of Notch-1 protein as confirmed by Western blot analysis (Fig. 9A), and this overexpression in Notch-1 rescued genistein-induced cell growth inhibition and abrogated genistein-induced apoptosis to a certain degree (Fig. 9B and C). Overexpression of Notch-1 by cDNA transfection partly abrogated inactivation of NF-κB DNA-binding activity by genistein (Fig. 9D). These results provide evidence for a potential cross-talk between Notch-1 and NF-κB signaling pathways during genistein-induced cell growth inhibition and apoptosis in BxPC-3 cells.

Discussion

Notch signaling plays important roles in maintaining the balance among cell proliferation, differentiation, and apoptosis (10). The *Notch* gene is abnormally activated in many human malignancies. It has been reported that the function of Notch signaling in tumorigenesis can be either oncogenic or antiproliferative, and the function is context dependent (15). In a limited number of tumor types, including human hepatocellular carcinoma and small lung cancer, Notch signaling is antiproliferative rather than oncogenic (30–32). However, most of the studies showed opposite function of Notch in many human cancers including pancreatic cancer (17, 33). Notch family members, some Notch ligands, and downstream molecules, such as Hes-1, have been found to be up-regulated in pancreatic cancer tissues (34). In the present study, we investigated the role of Notch-1 in cell proliferation and apoptosis in pancreatic cancer cell lines. In our study, down-regulation of Notch-1 elicited a dramatic effect on growth inhibition and induction of

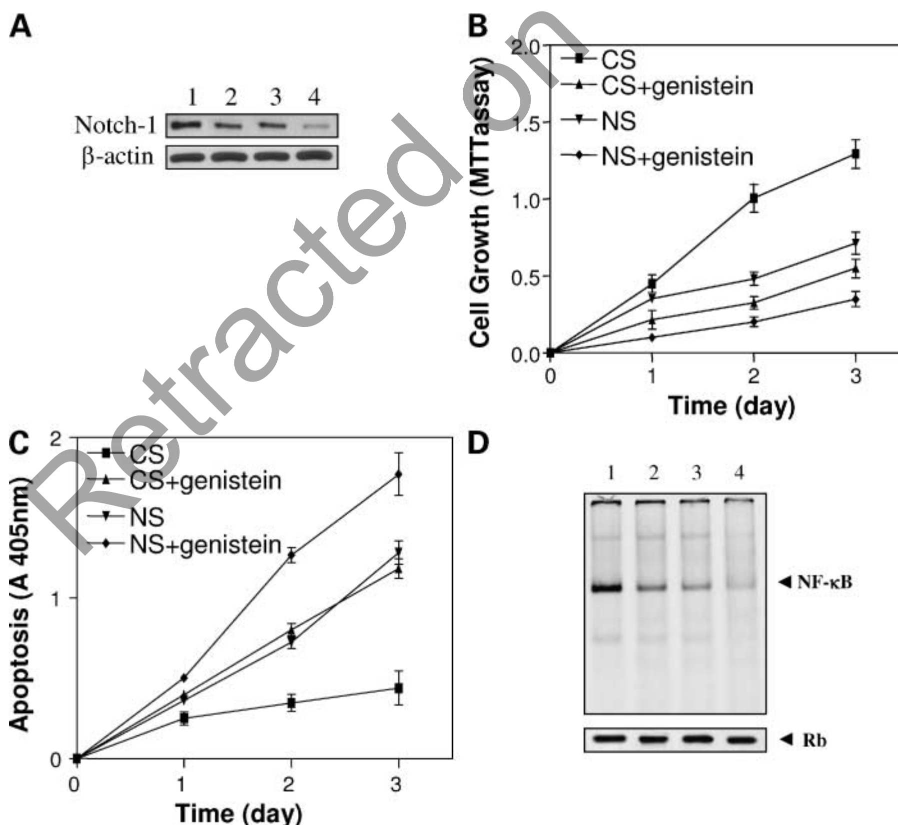

Figure 8. BxPC-3 pancreatic cancer cell growth inhibition and cell death induced by Notch-1 siRNA and genistein. **A,** Notch-1 expression was down-regulated by genistein and Notch-1 siRNA. Western blot analysis was used to detect the protein level of Notch-1. *1,* control; *2,* 25 μmol/L genistein; *3,* Notch-1 siRNA; and *4,* Notch-1 siRNA plus 25 μmol/L genistein. **B,** down-regulation of Notch-1 expression significantly inhibited cell growth. Genistein plus siRNA Notch-1 inhibited cell growth to a greater degree compared with genistein alone. **C,** BxPC-3 pancreatic cancer cell death induced by Notch-1 siRNA and genistein. Down-regulation of Notch-1 expression significantly increased apoptosis induced by genistein. Notch-1 siRNA–transfected BxPC-3 cells were significantly more sensitive to spontaneous and genistein-induced apoptosis. **D,** NF-κB DNA binding activity was measured by EMSA. Genistein and Notch-1 siRNA significantly inhibited NF-κB DNA-binding activity. Genistein plus Notch-1 siRNA inhibited NF-κB DNA binding activity to a greater degree compared with genistein alone. Rb level served as nuclear protein loading control. Control, cells treated with DMSO; *1,* control; *2,* 25 μmol/L genistein; *3,* Notch-1 siRNA; and *4,* Notch-1 siRNA plus 25 μmol/L genistein.

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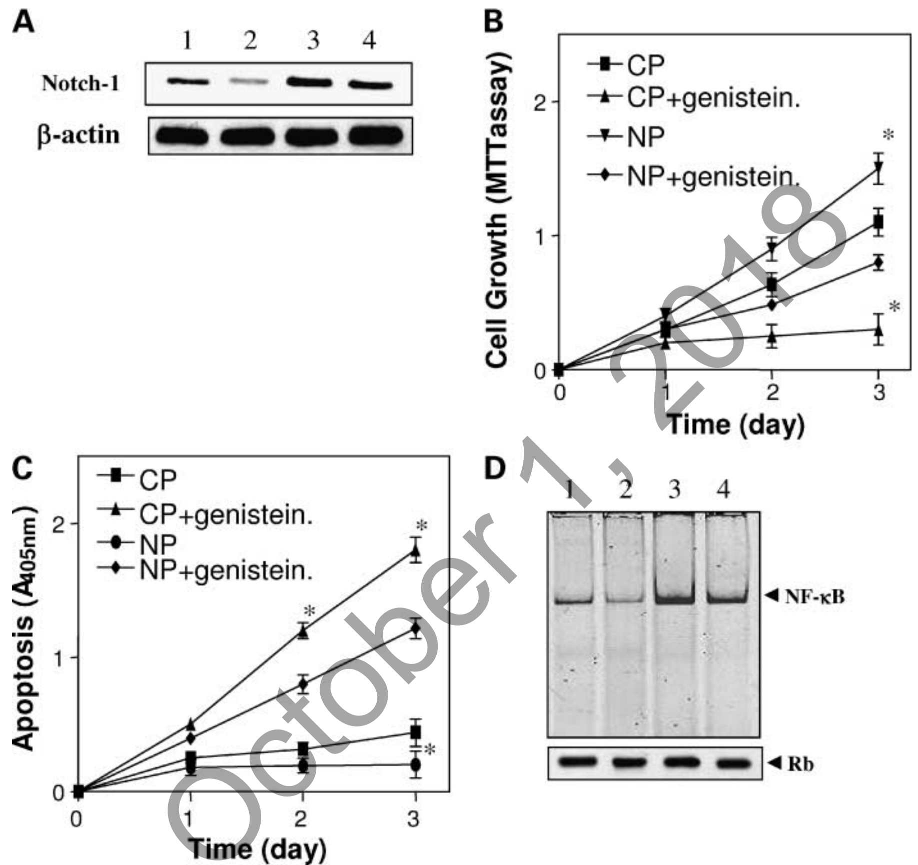

Figure 9. Overexpression of Notch-1 by cDNA transfection reduced genistein-induced cell growth inhibition and apoptosis. **A,** the efficacy of Notch-1 cDNA for overexpression of Notch-1 protein was tested by Western blot analysis. **B** and **C,** overexpression of Notch-1 by cDNA transfection rescued genistein-induced cell growth inhibition and abrogated genistein-induced apoptosis to a certain degree. **D,** overexpression of Notch-1 by cDNA transfection partly abrogated inactivation of NF-κB DNA-binding activity by genistein in BxPC-3 cells whereas there was no change in the levels of Rb (used as protein loading control). Control, cells treated with DMSO; *1,* control plasmid; *2,* control plasmid plus 25 μmol/L genistein; *3,* Notch-1 cDNA; and *4,* Notch-1 cDNA plus 25 μmol/L genistein.

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apoptotic processes in pancreatic cancer cells, as shown by MTT assay and DNA/histone fragmentation analysis, respectively. In contrast, up-regulation of Notch-1 expression caused cell growth promotion and protected cells from apoptosis to a certain degree. Thus, our results provide *in vitro* evidence in support of the role of *Notch-1* as an oncogene rather than a tumor suppressor gene in pancreatic cancer cells.

Because down-regulation of Notch-1 by siRNA reduced cell growth, we wondered if cell cycle arrest was related to the cell growth inhibition. Indeed, we found that Notch-1 down-regulation increased cell population in G_0-G_1 phase. In contrast, up-regulation by overexpression of Notch-1 cDNA reduced the cell number in G_0-G_1 phase. Cell proliferation is tightly regulated by expression and activation of cell cycle-dependent cyclins, Cdks, and Cdk inhibitors. Cdk inhibitors have negative effects on cell cycle machinery by binding to various cyclin-Cdk complexes and inhibiting their activities. There are two classes of Cdk inhibitors, the INK4 family and the KIP/CIP family. The KIP/CIP family, including $p21^{CIP}$, $p27^{KIP1}$, and $p57^{KIP2}$, interact with cyclin A-Cdk2, cyclin E-Cdk2, cyclin D-Cdk4, and cyclin D-Cdk6 complexes and inhibit their activities (35–38). Progression of a cell through the cell cycle is promoted by a number of cyclin-dependent kinases (Cdk) that, when complexed with specific regulatory proteins called cyclins, drive the cells forward through the cell cycle. Cyclin D interacts with Cdk2, Cdk4, and Cdk6, resulting in cell cycle progression through G_1 phase. To explore the mechanism involved in down-regulation of Notch-1-induced cell growth arrest, the expression of cell cycle proteins was examined. We observed a marked reduction in cyclin A, cyclin D1, and Cdk2 expression and a dramatic increase in $p21^{CIP}$ and $p27^{KIP1}$ expression in Notch-1 siRNA–transfected cells. In our study, the decrease in cyclin D1, cyclin A, and Cdk2 and the increase in Cdk inhibitor proteins, including $p21^{CIP}$ and $p27^{KIP1}$, were strongly correlated with the altered cell cycle distribution phenotype and growth suppression. These results suggest that Notch-1 affects pancreatic cancer cell cycle by regulating the expression levels of some cyclins (cyclin D1 and cyclin A) and Cdk inhibitors ($p21^{CIP}$ and $p27^{KIP1}$).

Recent reports have shown that Notch-1 expression regulates cell death through both apoptosis and cell cycle pathways in erythroleukemia cells with regulation of c-Jun NH_2-terminal kinase, Bcl-X_L, $p21^{cip1}$, $p27^{kip1}$, NF-κB, and the retinoblastoma protein Rb (19). Besides the role of Notch on proliferation, Notch may also play a role in apoptosis. In the present study, we clearly showed that down-regulation of Notch-1 induced apoptosis in pancreatic cancer cells, as assessed by the cell death ELISA assay. To explore the molecular mechanism by which down-regulation of Notch-1 results in the induction of apoptosis in pancreatic cancer cells, we examined the expression of

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antiapoptotic proteins Bcl-2 and Bcl-X_L in siRNA-transfected cells. We observed that down-regulation of Notch-1 reduced Bcl-2 and Bcl-X_L protein expression level. Because Bcl-2 and Bcl-X_L protect cells from apoptosis, our findings suggest that decreased Bcl-2 and Bcl-X_L expression may participate in apoptosis induced by down-regulation of Notch-1 in human pancreatic cancer cells. Thus, the inhibition of cell growth observed in pancreatic cancer cells treated with siRNA may be partly due to the increase in apoptosis.

Because NF-κB plays important roles in many cellular processes including transcriptional regulation of Bcl-2 and Bcl-X_L, research 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 been reported to cross-talk with NF-κB pathway (18–20). Constitutive levels of Notch activity are essential in maintaining NF-κB activity in various cell types. Levels of basal and stimulation-induced NF-κB activity were significantly decreased in mice with reduced Notch levels (21). We observed that down-regulation of Notch-1 reduced NF-κB activity. In contrast, overexpression of wild-type Notch-1 cDNA enhanced NF-κB activity. Because NF-κB pathways are key regulators of numerous cellular processes such as proliferation, differentiation, and apoptosis, our results clearly provide molecular evidence for a potential cross-talk between Notch and NF-κB pathways and suggest that cell growth inhibition and apoptosis induced by the down-regulation of Notch-1 may be partly mediated by the NF-κB pathway. Although we have shown that down-regulation of Notch-1 is feasible by Notch-1 siRNA, these approaches are not yet practically useful in the therapeutic arena. For that reason, we have used genistein in the current study, primarily because genistein was found to be a potent agent in the down-regulation of NF-κB pathway and also because of the known cross-talk between Notch and NF-κB pathways. We found that genistein down-regulated the transcription and translation of Notch-1 and its downstream genes, *Hes-1*, *cyclin D1*, *Bcl-X_L*, and *NF-κB*. In addition, genistein elicited a dramatic effect on growth inhibition and induction of apoptotic processes in BxPC-3 cells. Overexpression of Notch-1 by Notch-1 cDNA transfection abrogated genistein-induced apoptosis to a certain degree. Therefore, we strongly believe that down-regulation of Notch-1 by genistein is mechanistically linked to cell proliferation and apoptotic processes. The molecular mechanism(s) by which genistein exerts its inhibitory effects on BxPC-3 pancreatic cells, as revealed in the present study, has opened up exciting avenues for devising novel therapeutic strategies. Therefore, the down-regulation of Notch-1 and NF-κB by genistein could be a useful strategy in the schema of therapeutic approaches for the treatment of pancreatic cancer.

In summary, we found that Notch-1 plays a role in pancreatic cancer cell growth and apoptosis. Our data support the potential oncogenic role of Notch-1 in pancreatic cancer. Down-regulation of Notch-1 induced G_0-G_1 phase cell cycle arrest, with reduced levels of cyclin D1 expression and increased $p21^{CIP}$ and $p27^{KIP1}$ expression. In addition, Notch-1 down-regulation also induced apoptosis, which was partly due to decreased Bcl-2 and Bcl-X_L protein expression in pancreatic cancer cells. It seems that NF-κB is downstream of Notch-1 signaling because down-regulation of Notch-1 reduced NF-κB activity. We also found that genistein could be an active agent for the down-regulation of Notch-1 and NF-κB pathways. From these results, we conclude that Notch-1 down-regulation by genistein could be a novel therapeutic approach in pancreatic cancer.

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