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Journal of Cellular Biochemistry 109:726–736 (2010)

Journal of Cellular Biochemistry

Down-Regulation of Notch-1 and Jagged-1 Inhibits Prostate Cancer Cell Growth, Migration and Invasion, and Induces Apoptosis Via Inactivation of Akt, mTOR, and NF-κB Signaling Pathways

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ABSTRACT

Notch signaling is involved in a variety of cellular processes, such as cell fate specification, differentiation, proliferation, and survival. Notch-1 over-expression has been reported in prostate cancer metastases. Likewise, Notch ligand Jagged-1 was found to be over-expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostatic tissues, suggesting the biological significance of Notch signaling in prostate cancer progression. However, the mechanistic role of Notch signaling and the consequence of its down-regulation in prostate cancer have not been fully elucidated. Using multiple cellular and molecular approaches such as MTT assay, apoptosis assay, gene transfection, real-time RT-PCR, Western blotting, migration, invasion assay and ELISA, we found that down-regulation of Notch-1 or Jagged-1 was mechanistically associated with inhibition of cell growth, migration, invasion and induction of apoptosis in prostate cancer cells, which was mediated via inactivation of Akt, mTOR, and NF-κB signaling. Consistent with these results, we found that the down-regulation of Notch-1 or Jagged-1 led to decreased expression and the activity of NF-κB downstream genes such as MMP-9, VEGF, and uPA, contributing to the inhibition of cell migration and invasion. Taken together, we conclude that the down-regulation of Notch-1 or Jagged-1 mediated inhibition of cell growth, migration and invasion, and the induction of apoptosis was in part due to inactivation of Akt, mTOR, and NF-κB signaling pathways. Our results further suggest that inactivation of Notch signaling pathways by innovative strategies could be a potential targeted approach for the treatment of metastatic prostate cancer. J. Cell. Biochem. 109: 726–736, 2010.

KEY WORDS: NOTCH-1; JAGGED-1; PROSTATE CANCER; CELL GROWTH; INVASION

Prostate cancer has become a significant health problem because it is one of the most frequently diagnosed tumors in men and the second leading cause of cancer-related death in the United States (Jemal et al., 2009). Despite an initial efficacy of androgen-deprivation therapy, most patients with prostate cancer progress from androgen-dependent status to hormone-refractory prostate cancer also known as castrate resistant cancer for which there is no curative therapy. Therefore, development of novel strategies for the treatment of prostate cancer is highly desirable for improving the survival outcome of this deadly disease.

Among many molecular targets, Notch signaling is very attractive because it is involved in a variety of cellular processes, such as cell fate specification, differentiation, proliferation, and survival. Four Notch receptors (Notch 1–Notch 4) and five ligands (Jagged-1, Jagged-2, Delta-1, Delta-3, and Delta-4) have been described in mammals (Wang et al., 2008). Binding of ligand to its receptor induces metalloproteinase-mediated and gamma secretase-mediated cleavage of the Notch receptor. The Notch intracellular domain (ICN) is released from the plasma membrane and translocates into the nucleus, where it forms a complex with the

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Department of Defense Postdoctoral Training Award; Grant number: W81XWH-08-1-0196; Grant sponsor: National Cancer Institute, NIH; Grant numbers: 5R01CA083695, 1R01CA101870.

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Received 11 August 2009; Accepted 10 November 2009 • DOI 10.1002/jcb.22451 • © 2010 Wiley-Liss, Inc.
Published online 5 January 2010 in Wiley InterScience (www.interscience.wiley.com).

members of the CSL transcription factor family. This complex mediates the transcription of target genes such as Hes-1 (hairy enhancer of split), cyclin D, Hey-1, and others (Miele, 2006; Miele et al., 2006). Because Notch signaling plays important roles in the cellular developmental pathway including proliferation and apoptosis, activation of Notch signaling pathways are associated with tumorigenesis. 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 were found in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and Large-cell lymphomas and pancreatic cancer (Miele and Osborne, 1999; Miele, 2006; Miele et al., 2006; Rizzo et al., 2008; Villaronga et al., 2008; Kopan and Ilagan, 2009).

Moreover, emerging evidence suggest that Notch signaling pathways also play important role in prostate development and progression, especially because Notch signaling pathway was found to be over-expressed in prostate cancer cell lines (Shou et al., 2001; Zhang et al., 2006; Leong and Gao, 2008; Bin et al., 2009). In addition, Notch-1 over-expression has been reported in prostate cancer metastases. Specifically, bone metastases from prostate cancer patients expressed Notch-1 protein in osteoblastic prostate cancer metastatic cells (Zayzafoon et al., 2004). Likewise Notch ligand Jagged-1 was found to be highly expressed in metastatic prostate cancer compared to localized prostate cancer or benign prostate tissues, and high Jagged-1 expression in a subset of clinically localized tumors was found to be significantly associated with tumor recurrence (Santagata et al., 2004). Recently, Hafeez et al. reported that silencing of Notch-1 inhibited invasion of prostate cancer cells by inhibiting the expression of MMP-9 and uPA (Bin et al., 2009). However, it is not clear whether Jagged-1 plays a role in prostate cancer progression. Moreover, the precise molecular mechanism by which activation of Notch signaling pathway leads to prostate cancer cell growth and invasion, and the mechanistic consequence of the down-regulation of Notch signaling in prostate cancer has not been fully understood.

In the present study, we sought to gain molecular evidence in support of the mechanistic consequence of Notch-1 and Jagged-1 down-regulation in cell growth, migration, invasion and apoptosis using human prostate cancer cells. Our results show that down-regulation of Notch-1 and Jagged-1 could be an effective approach for inhibiting cell growth, migration and invasion, and inducing apoptotic cell death, which was associated with inactivation of Akt, mTOR (mammalian Target of Rapamycin) and NF-κB, and the expression and activity of NF-κB target genes such as, MMP-9, uPA. Our results suggest that inactivation of Notch signaling pathways by innovative strategies could be a potential targeted approach for the treatment of metastatic prostate cancer.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL REAGENTS

Human PC cell lines, including PC-3, DU145, LNCaP, and C4-2B cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 units/ml penicillin, and 2.5 mM glutamine in a humidified incubator with 5% CO_2 and 95% air at 37°C. Primary mouse embryo fibroblasts (MEFs) were harvested from E13.5 embryos as described previously (Skeen et al., 2006). *Akt* WT, $Akt1^{-/-}$ (Akt1 KO), $Akt1/2^{-/-}$ (Akt DKO) MEF were cultured in DMEM with 10% FBS. Primary antibodies for full-length Notch-1 (H-130), cleaved Notch-1 (C-20), Jagged-1, uPA, uPAR, VEGF, and MMP-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against mTOR, phospho-mTOR (Ser^{2448}), p70S6K, phospho-p70S6K (Thr^{389}), 4E-BP1 (eukaryotic translation initiation factor 4E–binding protein 1), phospho-4E-BP1 (Thr^{37}/Thr^{46}), Akt, and phospho-Akt (Ser^{473}) were purchased from Cell Signaling Technology. All secondary antibodies were obtained from Pierce (Rockford, IL). The monoclonal antibody to β-actin, Gamma secretase inhibitor and PI3K inhibitor, LY294002, were purchased from Sigma–Aldrich. Gamma secretase inhibitor L-685,458 and DAPT were obtained from Calbiochem (San Diego, CA). 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, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO).

PLASMIDS AND TRANSFECTION PROCEDURES

Notch-1, Jagged-1 and control siRNA were obtained from Santa Cruz Biotechnology. PC-3 cells were transfected with Notch-1 siRNA, Jagged-1 siRNA and siRNA control, respectively, using Lipofectamine 2000 as described earlier (Wang et al., 2006a). The Notch-1 cDNA plasmid encoding the Notch-1 intracellular domain (NICD) was a kind gift of L. Miele {Department of Medicine and Pharmacology, University of Mississippi Cancer Institute, Jackson, MS). PC-3 cells were transfected with human Notch-1 ICN, Akt, p65 or vector alone (pcDNA3), respectively.

LUCIFERASE REPORTER ASSAY

The Notch-1 siRNA and Jagged-1 siRNA transfected PC-3 cells were transiently transfected with CBF-1 luciferase constructs. The Notch-CBF-1 reporter, 4× wild-type CBF-1 Luc, which contains four tandem repeats of the consensus CBF-1 DNA binding sequence, GTGGGAA and N-terminally tagged FLAG CBF-1, was generous gifts from Dr. Diane Hayward (Johns Hopkins University School of Medicine, USA). Wild-type (4× WT CBF1 luc) CBF-1 luciferase reporter plasmid was co-transfected with β-galactosidase using Lipofectamine (Invitrogen). CMV-β-gal reporter construct transfection was used for normalization of transfection efficiency. Luciferase and β-galactosidase assay (Promega) were done in accordance with the manufacturer's instructions. Luciferase activity was expressed relative to β-galactosidase activity.

CELL GROWTH INHIBITION STUDIES BY 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

The transfected prostate cancer 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 h and MTT assay was performed as described earlier (Wang et al., 2006b). In addition to the above assay, we have also done clonogenic assay for assessing the effects of treatment as shown below.

CLONOGENIC ASSAY

To test the survival of transfected cells, siRNA transfected PC-3 cells were trypsinized, and the viable cells were counted (trypan blue exclusion) and plated in 100 mm Petri dishes in a range of 100–1,000 cells to determine the plating efficiency as well as for assessing the effects of transfection on clonogenic survival. The cells were then incubated for 10–12 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator. The colonies were stained with 2% crystal violet and counted. The surviving fraction was normalized to untreated control cells with respect to clonogenic efficiency.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS

The Cell Death Detection ELISA Kit was used for assessing apoptosis according to the manufacturer's protocol. Briefly, transfected cells were lysed and the cell lysates were overlaid and incubated in microtiter plate modules coated with anti-histone antibody for detection of apoptosis as described earlier (Wang et al., 2006b).

REAL-TIME REVERSE TRANSCRIPTION-PCR ANALYSIS FOR GENE EXPRESSION STUDIES

The total RNA from transfected cells was isolated by Trizol (Invitrogen) according to the manufacturer's protocols. 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). RT reaction was performed at 25°C for 10 min, followed by 48°C for 30 min and 95°C for 5 min. The primers used in the PCR reaction for Notch-1, Jagged-1, MMP-9, VEGF, uPA, and β-actin were described before (Wang et al., 2006a, 2007). Real-time PCR amplications were performed as described earlier (Wang et al., 2006b).

PREPARATION OF NUCLEAR LYSATES

Cells were lysed in lysis buffer (0.08 M KCl, 35 mM HEPES, pH 7.4, 5 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 25 mM $CaCl_2$, 0.15 M sucrose, 2 mM phenylmethylsulfonyl fluoride, 8 mM dithiothreitol) and frozen at −80°C overnight. The cell suspension was thawed and passed through a 28-gauge needle three times. A small aliquot of the cells were checked for cell membrane breakage using trypan blue. Then the cell suspension was centrifuged, and the pellet was suspended in lysis buffer, and the nuclei were lysed by sonication. After centrifugation, supernatant was saved as nuclear lysate.

WESTERN BLOT ANALYSIS

Whole cell lysate of the cells was prepared by sonicating the cells lysed in 62 mM Tris–HCl and 2% SDS. In another set of experiments, cytoplasmic and nuclear proteins were also extracted. The protein concentration was measured by the BCA protein assay (Pierce). Total proteins were fractionated using SDS–PAGE and transferred onto a nitrocellulose membrane for Western blotting as described before (Wang et al., 2006a).

IMMUNOFLUORESCENCE STAINING

The transfected cells were plated on cover slips in each well of an 8-well chamber for 48 h. Cells were then fixed with paraformaldehyde for 15 min, rinsed with PBS, and incubated with 5% goat serum for 30 min. The cells were then incubated with anti-Notch-1, anti-Jagged-1, and anti-pAkt antibody for 2 h, respectively. After washing with PBS, the cells were incubated with FITC-conjugated secondary antibody for 45 min and washed with PBS. Cell images were observed under a fluorescent microscope.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts were prepared from treated samples and electrophoretic mobility shift assay was done by incubating 10 μg nuclear extract with IRDye-700-labeled NF-κB oligonucleotide as described earlier (Wang et al., 2006a). The DNA-protein complex formed was visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

UROKINASE PLASMINOGEN ACTIVATOR (uPA) ACTIVITY ASSAY

The culture medium of the transfected cells grown in 6-well plates was collected. After collection, the medium was spun at 800*g* for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for uPA assay later or assayed immediately using commercially available ELISA kits (American Diagnostica, Inc., Stamford, CT).

MMP-9 ACTIVITY ASSAY

The transfected cells were seeded in 6 well plates and incubated at 37°C. After 24 h, 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 h. MMP-9 activity in the medium and cell lysate was detected by using Fluorokine E Human MMP-9 Activity Assay Kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's protocol.

VEGF ASSAY

The transfected cells were seeded in 6-well plates (1.0×10^5 cells per well) and incubated at 37°C. After 24 h, the cell culture supernatant was harvested and cell count was performed after trypsinization. After collection, the medium was spun at 800*g* for 3 min at 4°C to remove cell debris. The supernatant was either frozen at −20°C for later VEGF assay or assayed immediately using commercially available ELISA kits (R&D Systems, Inc., Minneapolis, MN).

CELL MIGRATION AND INVASION ASSAY

Cell migration was assessed using 24-well inserts (BD Biosciences, Bedford, MA) with 8 μm pores according to the manufacturer's protocol. The invasive activity of the siRNA transfected cells was tested using the BD BioCoat Tumor Invasion Assay System (BD Biosciences) as described before. Briefly, transfected PC-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 24 h 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 h. 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.

DENSITOMETRIC AND STATISTICAL ANALYSIS

The statistical significance of differential findings between experimental groups and control was determined by Student's *t*-test. *P* values <0.05 were considered statistically significant.

RESULTS

NOTCH SIGNALING PATHWAY IN PROSTATE CANCER CELLS

The baseline expression of the Notch signaling was determined in a panel of human prostate cancer cell lines that included PC-3, DU145, LNCaP, and C4-2B. The assays also included the expression of Notch activated downstream signaling proteins (phosphorylated Akt and mTOR). The results showed that the Notch signaling pathway was frequently but differentially dysregulated among different human prostate cancer cell lines. Specifically, the expression of Jagged-1 was highest in PC-3 cell line (Supplemental Fig. 1A,B). The higher expression of Notch-1 (full size and cleaved size) was found in LNCaP and C4-2B cell lines (Supplemental Fig. 1A,B). It is important to note that we focused our studies on the cleaved Notch-1 because it is the active functional form of Notch. Therefore, Notch-1 in our all figure legends means active cleaved Notch-1, except Supplementary Figure 1B, which has been labeled appropriately. We also found that high expression of pAkt and its downstream signaling mTOR pathway in LNCaP and C4-2B cells, suggesting that Notch signaling seems to regulate the Akt and mTOR pathways.

In the present study, we choose the PC-3 cell line to detect whether Notch can regulate Akt and mTOR pathway especially because PC-3 cells showed higher expression of Jagged-1 and moderate levels of Notch-1. In addition, the efficacy of Notch-1 siRNA for knockdown of Notch-1 was better in PC-3 cells compared to LNCaP and C4-2B cells, which was in part due to higher levels of Notch-1 expression and the siRNA approach was not adequate. Moreover, LNCaP and C4-2B are androgen receptor (AR) expressing cell lines and since androgen can regulate the Notch expression especially Notch-2 and Jagged-1 were found to be up-regulated in LNCaP cells after androgen exposure (Martin et al., 2004) and we also found that the expression of Notch-1 and its target genes (Hey-1 and Bcl-2) was up-regulated in PC-3 AR stable transfected cells (Supplemental Fig. 1D). Furthermore, in DU-145 the expression of pAkt and mTOR signaling was barely detectable and, thus, this cell line was not useful for our purposes for investigation of the cross-talk between Notch and Akt. All these results prompted use to use PC-3 cells for the current study; however, further in-depth studies would be needed to ascertain the precise molecular regulation of Notch and AR and their cross-talks in the future for elucidating the role of Notch in cell growth, invasion and angiogenesis of AR expression cell lines, which is being planned for our future studies.

DOWN-REGULATION OF NOTCH-1 AND JAGGED-1 EXPRESSION BY siRNA INHIBITED CELL GROWTH AND INDUCED APOPTOSIS

We have previously reported that Notch-1 and Jagged-1 are highly expressed in PC-3 prostate cancer cell line (Zhang et al., 2006). To determine whether Notch signaling pathway could be an effective therapeutic target for prostate cancer, the biological effect of down-regulation of Notch signaling pathway using Notch-1 and Jagged-1 siRNAs was examined by assessing cell growth of the prostate cancer cells. The effect of siRNA for knocking down Notch-1 and Jagged-1 at mRNA and protein levels was confirmed by real-time RT-PCR and Western blotting. We found that both Notch-1 and Jagged-1 at mRNA level and protein levels were barely detectable in Notch-1 siRNA and Jagged-1 siRNA transfected cells, respectively, compared to siRNA control transfected cells (Fig. 1A). We also found that the expression of Notch-1 downstream gene Hes-1 and Hey-1 was down-regulated by Notch-1 siRNA or Jagged-1 siRNA (Fig. 1A). Although Notch-1 siRNA and Jagged-1 siRNA transfection resulted in a decrease in the cleaved form of Notch-1 by Western blotting, we

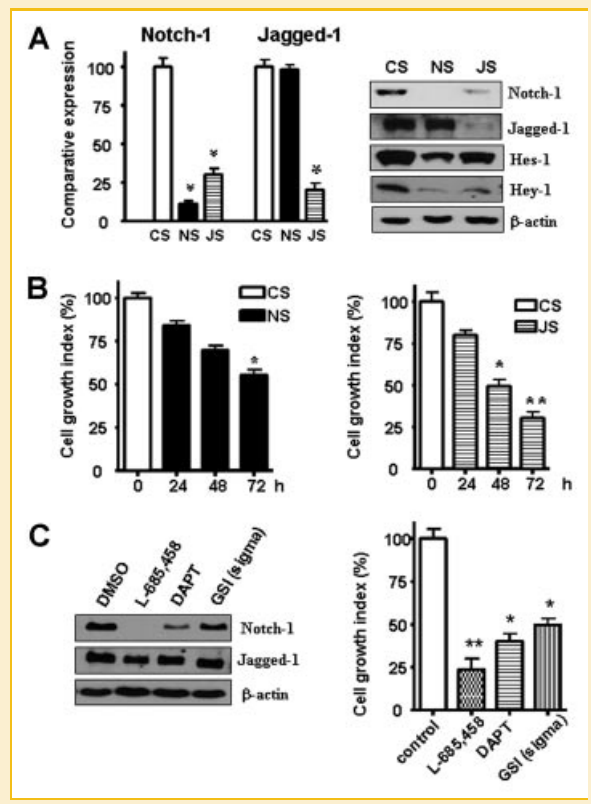

Fig. 1. Effects of down-regulation of Notch-1 expression on PC-3 prostate cancer cell growth. CS: Control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. $^{*}P<0.05$, $^{**}P<0.01$ relative to control. A: Left, Real-time RT-PCR was done to detect the Notch-1 and Jagged-1 mRNA level. We found that Notch-1 and Jagged-1 mRNA levels were down-regulated by Notch-1 and Jagged-1 siRNA, respectively. The results are presented as the mean of three independent experiments with error bars representing standard deviation (SD). Right, Notch-1, Jagged-1, Hes-1 and Hey-1 protein levels in Notch-1 and Jagged-1 siRNA transfected PC-3 cells were determined using Western Blot analysis. We found that these proteins were down-regulated upon Nocth-1 and Jagged-1 siRNA transfection, respectively. B: Inhibition of cancer cell growth by Notch-1 siRNA and Jagged-1 siRNA tested by MTT assay. The results were plotted as means ± SD of three separate experiments having six determinations per experiment for each experimental condition. C: L-685,458 and DAPT were γ-secretase inhibitors, which prevent the cleavage of the Notch receptor, blocking Notch signal transduction. Left, Western blotting assay showing that GSI inhibited the Notch-1 expression. Right, Inhibition of cancer cell growth by GSI for 72 h as assessed by MTT assay.

wanted to determine if this protein was functional. Thus, we carried out a standard CBF-1 binding luciferase reporter assay. NICD binds with CBF-1 and other proteins to form a DNA binding complex. This complex activates the transcription of target genes. As expected, we found that Notch-1 siRNA and Jagged-1 siRNA transfected PC-3 cells co-transfected with the luciferase construct resulted in a significantly decrease in relative luciferase activity, respectively, indicating that the decrease in CBF-1 binding was due to the inhibition of NICD (Supplementary Fig. 2A). The cell viability was further determined by MTT assay as shown by Figure 1B. We found that down-regulation of Notch-1 or Jagged-1 expression by siRNAs caused cell growth inhibition of PC-3 prostate cancer cell line. In order to further confirm the role of Notch on cell growth, the PC-3 cells were treated with gamma secretase inhibitors (GSI). We found that GSI inhibited the Notch-1 expression and consequently GSI had a strong effect in inhibiting the growth of PC-3 cell line (Fig. 1C). In addition, we have also tested the effects of down-regulation of Notch-1 or Jagged-1 on cell viability by clonogenic assay as shown below.

INHIBITION OF CELL GROWTH/SURVIVAL BY CLONOGENIC ASSAY

To determine the effect of Notch signaling on cell growth, cells were transfected with Notch-1 siRNA or Jagged-1 siRNA and assessed for cell viability by clonogenic assay. Both Notch-1 siRNA and Jagged-1 siRNA transfection resulted in a significant inhibition of colony formation of PC-3 cells when compared to control (Fig. 2A). Overall, the results from clonogenic assay was consistent with the MTT data as shown in Figure 1B, suggesting that down-regulation of Notch-1 and Jagged-1 inhibited cell growth of PC-3 prostate cancer cells. Next, we examined whether the inhibition of cell growth was also accompanied by the induction of apoptosis in Notch-1 siRNA or Jagged-1 siRNA transfected cells.

DOWN-REGULATION OF NOTCH-1 AND JAGGED-1 EXPRESSION BY siRNA INDUCED APOPTOSIS

We investigated whether the overall growth inhibitory effects of Notch-1 siRNA or Jagged-1 siRNA are in part due to induction of apoptosis, which was examined by using an ELISA-based assay. These results provided convincing data that down-regulation of Notch-1 or Jegged-1 by siRNAs induced apoptosis in PC-3 prostate cancer cell line (Fig. 2B). These data suggest that the overall growth inhibitory activity of Notch-1 or Jagged-1 down-regulation was in part contributed by increased cell death.

DOWN-REGULATION OF NOTCH-1 INHIBITED AKT AND mTOR PATHWAY IN PC-3 CELLS

The cross-talk between Notch and Akt pathway has been previously reported in human cancer cell lines (Gutierrez and Look, 2007; Palomero et al., 2007; Bedogni et al., 2008). Therefore, we investigated whether Notch inactivation would reduce Akt function in PC-3 prostate cancer cell lines. We found that down-regulation of Notch-1 by siRNA or GSI led to decreased Akt phosphorylation and its downstream genes phosphorylation, such as mTOR, 4EBP-1, and S6K (Fig. 3A). To further confirm our results, we also did

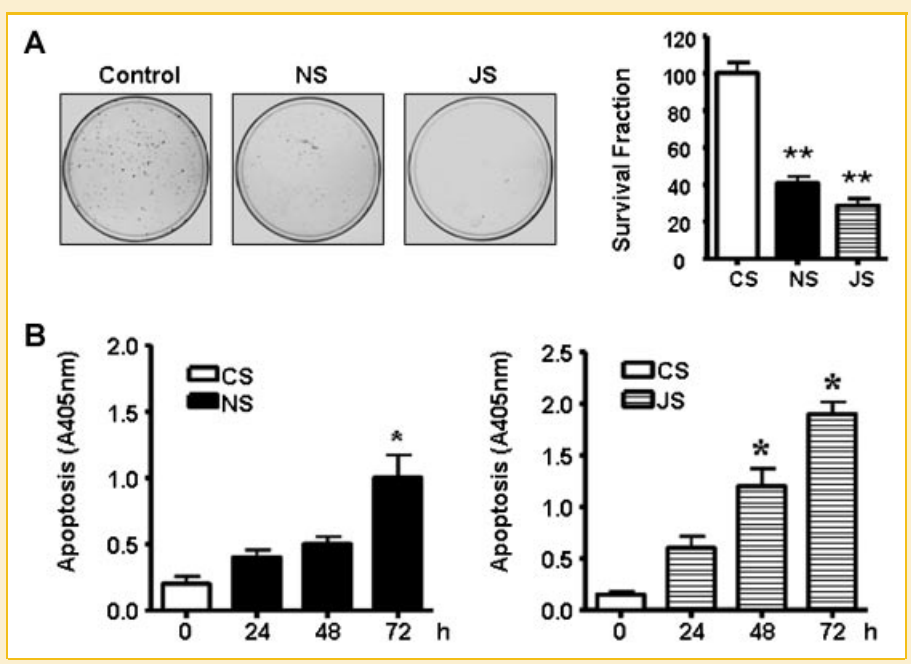

Fig. 2. Effect of Notch-1 siRNA or Jagged-1 siRNA on PC-3 prostate cancer cell death. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. A: Left, cell survival of human PC-3 prostate cancer cell line. Notch-1 siRNA or Jagged-1 siRNA transfected PC-3 cells were evaluated by the clonogenic assay. Right, photomicrographic difference in colony formation in siRNA transfected cells compared to control. *P* values represent comparisons between cells transfected with siRNA and control using the paired *t*-test. B: Apoptosis was measured by Histone DNA ELISA in PC-3 cells transfected with Notch-1 siRNA or Jagged-1 siRNA for different periods of time as indicated in the figure. Values are reported as mean ± SD. $^{*}P < 0.05$, compared to the control. We found that down-regulation of Notch-1 and Jagged-1 induced the apoptosis.

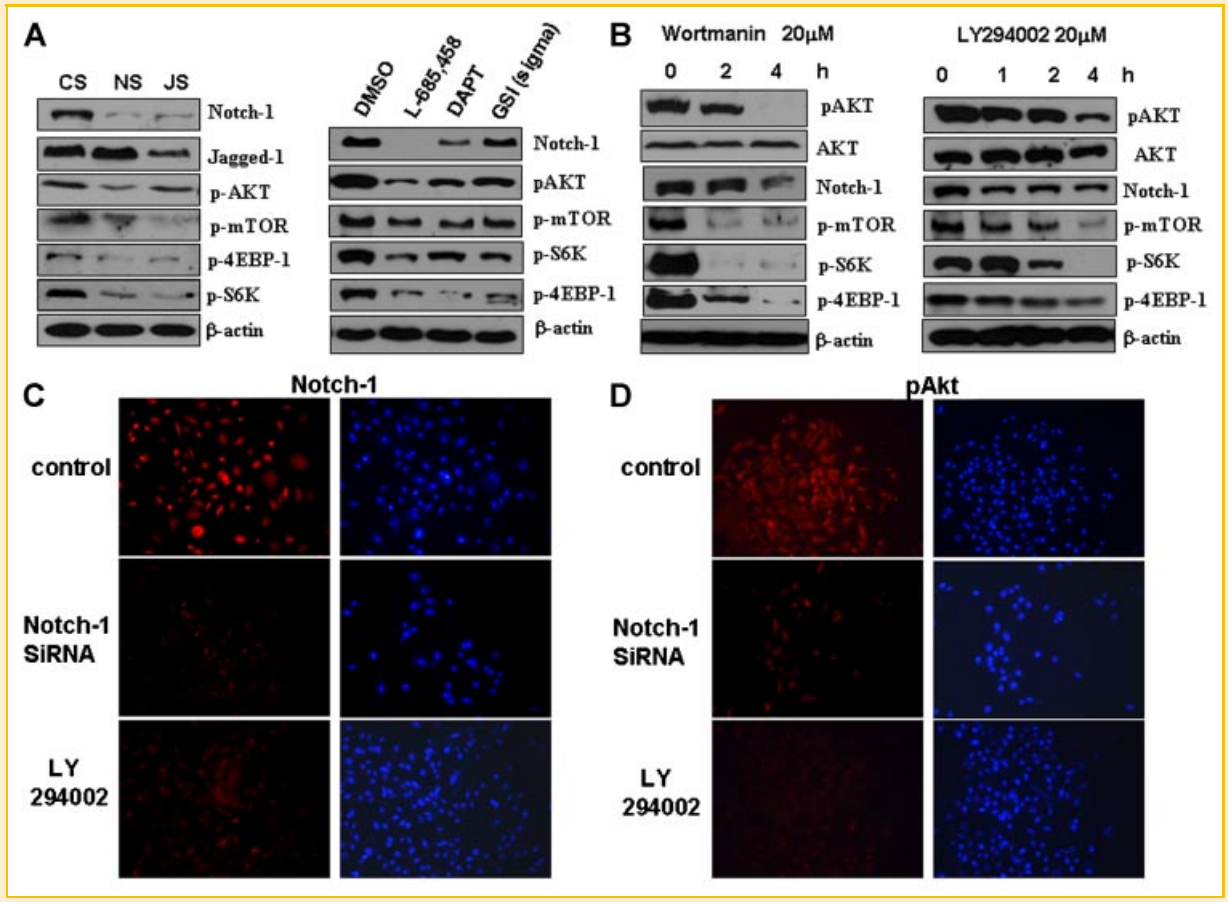

Fig. 3. Down-regulation of Notch-1 inhibited Akt and mTOR pathway in PC-3 cells. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. A: Down-regulation of Notch-1 by siRNA or GSI inhibited the activity of Akt and mTOR pathways as assessed by Western blot analysis. B: Inactivation of Akt by PI3K inhibitors (20 μM LY294002, 20 μM Wortmanin) inhibited the expression of Notch-1 and phospho-mTOR as assessed by Western blot analysis. C,D: Immunofluorescent staining showing lower levels of Notch-1 protein in the nucleus and pAkt in the cytoplasm and nucleus in the Notch-1 siRNA-transfected PC-3 cells. LY294002 eliminated Notch-1 expression and mTOR phosphorylation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

immunofluorescent staining. Indeed, we observed lower level of Notch-1 protein in the nucleus and pAkt in the cytoplasm and nucleus in the Notch-1 siRNA-transfected PC-3 cells (Fig. 3C,D). In contrast, up-regulation of Notch-1 by NICD transfection increased Akt phosphorylation and mTOR pathway (Supplementary Fig. 2B). It has been shown that Akt can control Notch-1 expression in melanoma (Bedogni et al., 2008), and thus we sought to determine whether Notch-1 expression could be controlled by Akt in PC-3 cells. We found that LY294002 and Wortmanin, the PI3K inhibitors, eliminated the expression of Notch-1, the phosphorylation of mTOR, S6K and 4EBP-1 (Fig. 3B), suggesting the existence of an interesting reciprocal regulation of Notch-1 and Akt pathways in prostate cancer cells. Moreover, we found that the expression of pAkt and its down-stream gene mTOR pathway was increased in Akt transfected PC-3 cells. Furthermore, inhibition of pAkt and mTOR pathway by Notch-1 siRNA was abrogated by Akt transfection (Supplementary Fig. 2C). Notch-1 could induce Akt signaling, but Notch-1 is also downstream of Akt and our results are consistent with previous findings in T-ALL cell lines and melanomas (Bedogni et al., 2008; Calzavara et al., 2008).

NOTCH-1 WAS DECREASED IN AKT KNOCK-OUT MEF CELL LINES

To further confirm our results showing that Notch-1 is a downstream effector of Akt, we examined the expression of Notch-1 in Akt WT, Akt-1KO, and Akt DKO MEF cell lines. We found that Notch-1 and Jagged-1 were decreased significantly in Akt DKO cell lines, which showed decreased pAkt and mTOR pathway (Fig. 4A,B). Moreover, Notch-1 siRNA and GSI decreased pAkt and p-mTOR in Akt WT cell lines. Further experiments were done where Akt WT and DKO MEFs were deprived of serum for 24 h and then stimulated by addition of 20% FBS, which showed that the Notch activity was impaired in Akt DKO cells (Fig. 4C), suggesting that many growth stimulating factors could increase Notch-1 activity through Akt signaling pathway.

DOWN-REGULATION OF NOTCH-1 OR JAGGED-1 BY 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 in prostate cancer. Nuclear proteins from transfected cells were subjected to analysis for NF-κB p65 DNA-binding activity as measured by EMSA. The results showed that down-regulation of

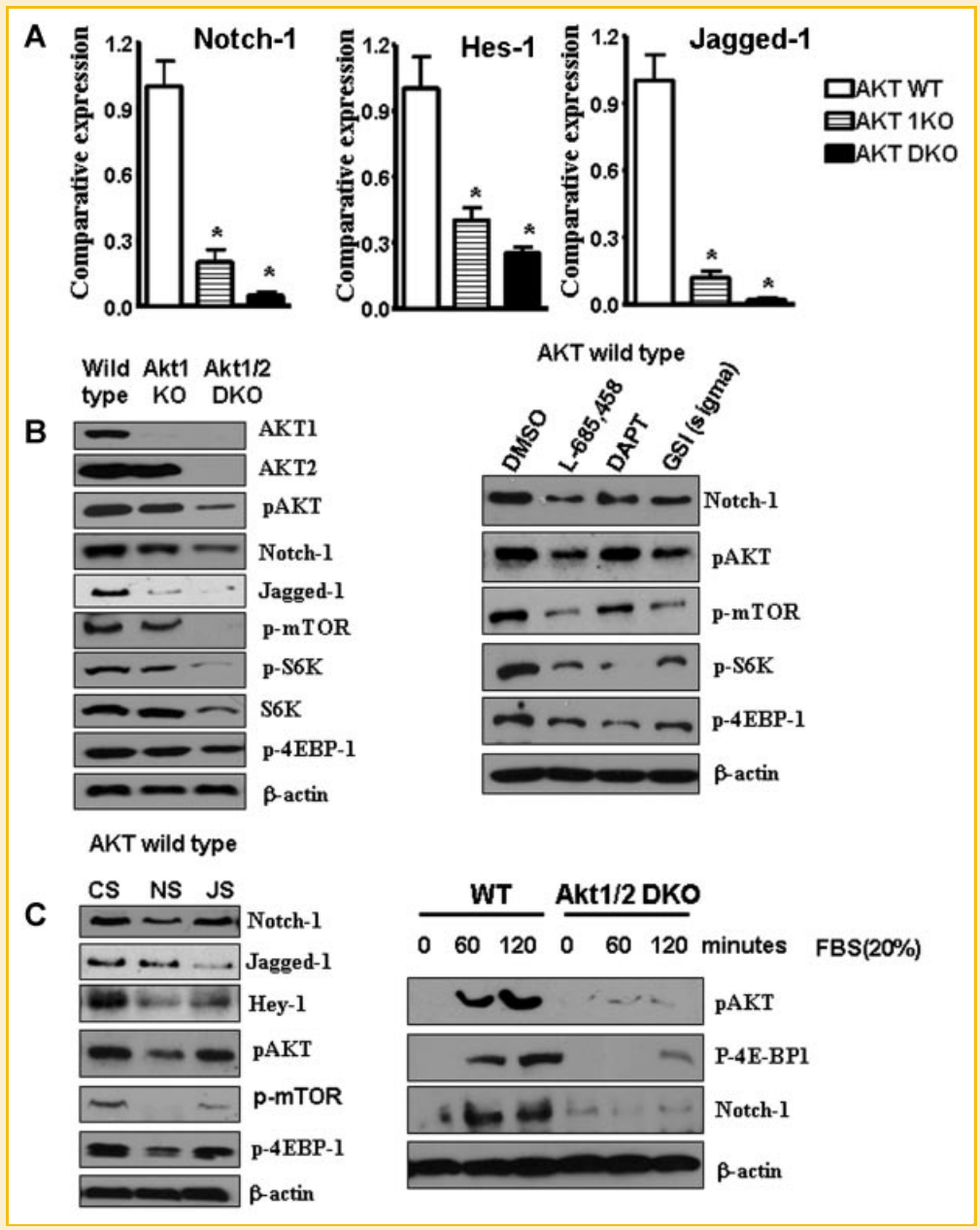

Fig. 4. Notch-1 was decreased in Akt knock-out MEF cell lines. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. A: Notch-1, Hes-1 and Jagged-1 mRNA were decreased significantly in Akt DKO cell lines using Real-time RT-PCR. B: Notch-1 and Jagged-1 proteins were decreased in Akt DKO cell lines using Western blot analysis. Notch-1 siRNA and GSI (L-685,458, DAPT) decreased pAkt and p-mTOR in Akt WT cell lines. C: Notch-1 siRNA and Jagged-1 siRNA decreased pAkt and p-mTOR in Akt WT cell lines using Western blot analysis. Akt WT and DKO MEFs were deprived of serum for 24 h and then stimulated by addition of 20% FBS for different time periods. We found that Notch activity was impaired in Akt DKO cells.

Notch-1 or Jagged-1 significantly inhibited NF-κB p65 DNA-binding activity compared to control (Fig. 5C). The effect of Notch-1 siRNA on p65 DNA-binding activity was not due to modulation of the p65 total protein level (Fig. 5B). Further, we found that Notch-1 siRNA or Jagged-1 siRNA reduced the basal levels of IKKα, IκBα and pIκBα (Fig. 5A). In contrast, up-regulation of Notch-1 activity by NICD transfection increased NF-κB p65 DNA-binding activity (data not shown). These results provided direct evidence in support of a mechanistic cross-talk between Notch-1 and NF-κB in prostate cancer. Furthermore, we also found that down-regulation of Notch-1 or Jagged-1 inhibited NF-κB downstream gene expression, such as MMP-9, VEGF, and uPA (Fig. 5D).

DOWN-REGULATION OF NOTCH-1 OR JAGGED-1 DECREASED MMP-9, VEGF, AND uPA GENE TRANSCRIPTION AND THEIR ACTIVITIES

To explore whether Notch-1 or Jagged-1 siRNA transfection could decrease the transcription of MMP-9, VEGF, and uPA, real-time RT-PCR was employed. VEGF exists in at least six isoforms with variable amino acid residues produced through alternative splicing:

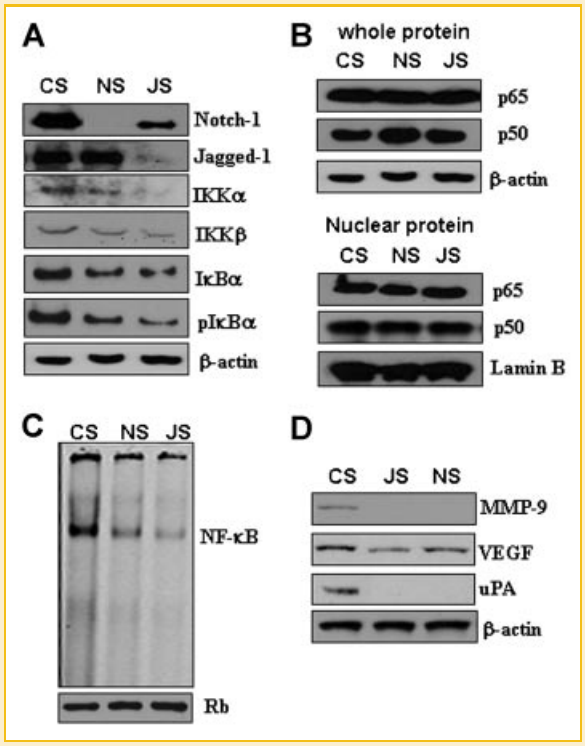

Fig. 5. Notch-1 siRNA and Jagged-1 siRNA inhibited the NF-κB DNA binding activity. A: Western blot analysis showed that Notch-1 siRNA and Jagged-1 siRNA inhibited the expression of IKKα, IκBα and pIκBα. B: Notch-1 siRNA and Jagged-1 siRNA did not change the protein expression of p65 and p50 using Western blot analysis. C: Notch-1 siRNA and Jagged-1 siRNA inhibited the NF-κB DNA binding activity in PC-3 cells as assessed by EMSA. D: Western blot analysis showed that Notch-1 siRNA and Jagged-1 siRNA inhibited the expression of MMP-9, VEGF, and uPA genes.

VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206. VEGF121, VEGF165, and VEGF189 are the major forms secreted by most cell types. Therefore, we detected the transcription of three major forms (VEGF121, 165, 189). We found that MMP-9, uPA, VEGF total mRNA, VEGF121, VEGF165, and VEGF189 were dramatically decreased in the siRNA transfected cells (Fig. 6A), which we believe is due to inactivation of NF-κB activity. Next, we examined whether the down-regulation of Notch-1 or Jagged-1 could also lead to a decrease in their activities in PC-3 prostate cancer cells. We found a marked decrease in the activity of MMP-9, uPA, and VEGF in Notch-1 and Jagged-1 siRNA transfected cells (Fig. 6B), which is also consistent with transcriptional inactivation of these genes.

DOWN-REGULATION OF NOTCH-1 OR JAGGED-1 DECREASED PROSTATE CANCER CELL MIGRATION AND INVASION

MMP-9, VEGF, and uPA are believed to be critically involved in the processes of tumor cell migration, invasion and metastasis. Since Notch-1 siRNA and Jagged-1 siRNA inhibited the expression and activity of MMP-9, VEGF, and uPA, we tested the effects of Notch-1 and Jagged-1 down-regulation on cancer cell migration and invasion. We found that down-regulation of Notch-1 or Jagged-1 decreased prostate cancer cell migration. Moreover, as illustrated in Figure 6C, Notch-1 siRNA or Jagged-1 siRNA transfected cells showed a lower level of penetration through the matrigel-coated membrane compared with the control cells. The value of fluorescence from the invaded PC-3 prostate cancer cells was decreased about three- to fourfold compared with that of control cells (Fig. 6C), suggesting a direct role of Notch signaling in prostate cancer cell migration and invasion.

DISCUSSION

Aberrant expression of Notch pathway has been found in a variety of human cancers including breast, brain, cervical, lung, colon, head and neck, renal cell carcinoma, acute myeloid, Hodgkin and Large-cell lymphomas and pancreatic cancer (Miele, 2006; Miele et al., 2006). It has been reported that Notch-1 was over-expressed in prostate cancer cell lines and human prostate cancer tissues (Zayzafoon et al., 2004; Bin et al., 2009). Moreover, Notch-1 expression in human prostate cancer tissues increased with increasing tumor grade (Bin et al., 2009). However, the role of Notch pathway in prostate carcinogenesis remains poorly understood. Therefore, in the present study, we investigated the role of Notch-1 and Jagged-1 in cell proliferation in prostate cancer cells. In our study, down-regulation of Notch-1 and Jagged-1 elicited a dramatic effect on cell growth inhibition of PC-3 prostate cancer cells, as demonstrated by MTT assay and clonogenic assay. In addition, Notch-1 siRNA and Jagged-1 siRNA caused induction of apoptotic cell death, suggesting the growth inhibitory activity of Notch-1 or Jagged-1 down-regulation is in part attributed to an increase in cell death.

Hyperactivation of Akt pathway has previously been observed in human prostate cancer (de Souza et al., 2009). Recently, Notch has been shown to regulate the Akt pathway. Liu et al. (2006) have reported that Notch-1 activation enhanced melanoma cell survival and such effects of Notch signaling were mediated via activation of the Akt pathway. Palomero et al. (2008) found that Notch-1 induced up-regulation of the PI3K-Akt pathway via Hes-1, which negatively controls the expression of phosphatase and tensin homolog on chromosome 10 (PTEN) in T-ALL. Since Notch-1 has been reported to cross-talk with Akt pathway in human cancer cell lines (Gutierrez and Look, 2007; Bedogni et al., 2008; Meurette et al., 2009), we postulated whether cell growth inhibition was indeed due to the regulation of Akt pathway. We found that down-regulation of Notch-1 by siRNA or GSI decreased Akt phosphorylation. Interestingly, we also observed that inactivation of Akt by LY294002 and Wortmanin eliminated Notch-1 expression and mTOR phosphorylation. These results suggest the existence of an interesting reciprocal regulation of Notch-1 and Akt pathways. In other words Notch-1 could induce Akt signaling, but Notch-1 is also downstream of Akt pathway. Our results are consistent with previous findings in T-ALL cell lines, melanomas and breast cancer cells (Bedogni et al., 2008; Calzavara et al., 2008; Meurette et al., 2009).

The regulation mTOR activity by growth factors is mediated by the PI3K/Akt signaling pathway. The mTOR protein kinase has emerged as a critical player for controlling many cellular processes,

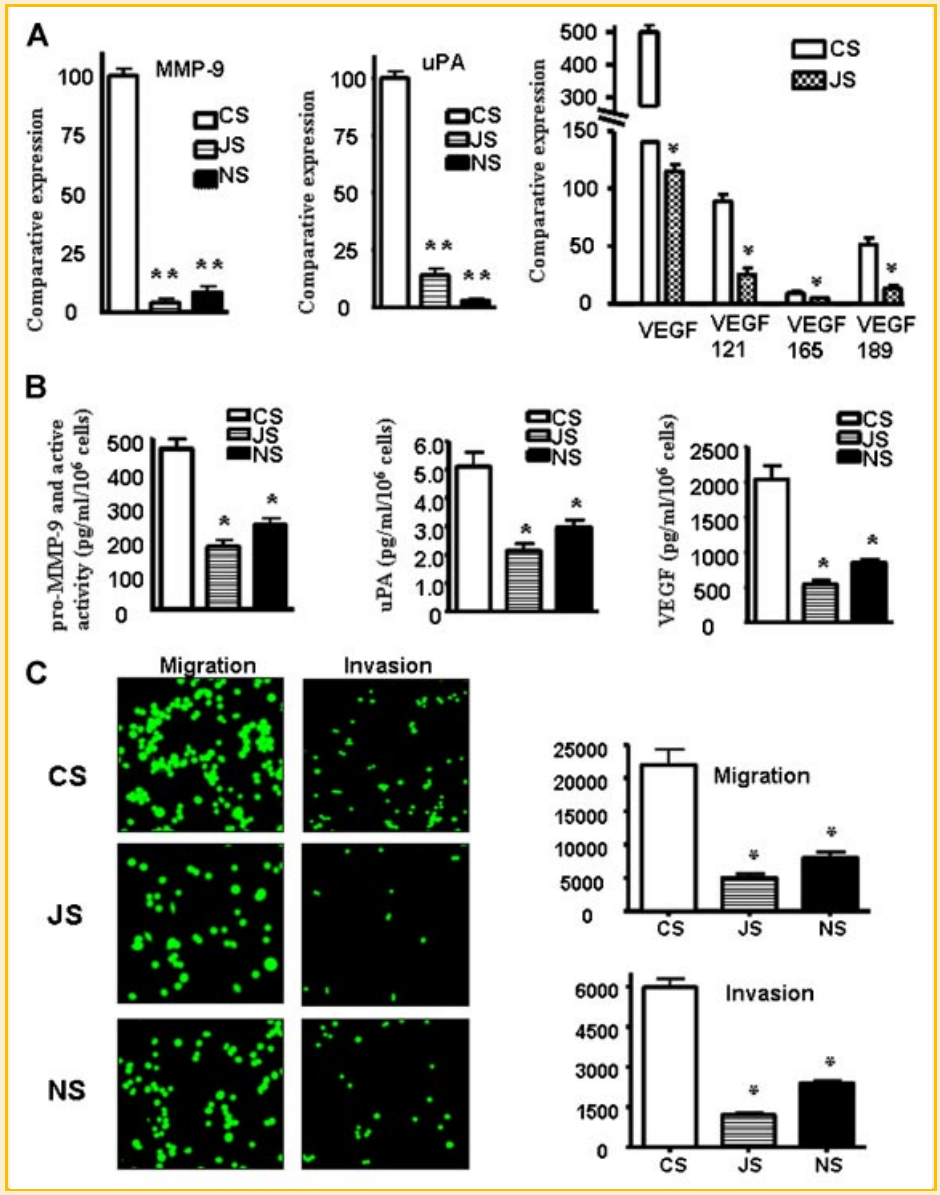

Fig. 6. Down-regulation of Notch-1 or Jagged-1 decreased prostate cancer cell migration and invasion. CS: control siRNA; NS: Notch-1 siRNA; JS: Jagged-1 siRNA. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to control. A: Real-time RT-PCR showed that Notch-1 or Jagged-1 siRNA inhibited the expression of MMP-9, uPA, and VEGF genes at mRNA levels in PC-3 cells. B: Notch-1 siRNA or Jagged-1 siRNA inhibited the activity of MMP-9, uPA, and VEGF in PC-3 cells as assessed by ELISA. C: Left; Notch-1 siRNA or Jagged-1 siRNA decreased PC-3 prostate cancer cell migration and invasion. Right; values of fluorescence from the migrated cells or invaded cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

such as cell growth and cell division by receiving stimulatory signals from Notch and Akt (Mungamuri et al., 2006; Chan et al., 2007). It has been reported that inhibition of tumor suppressor protein p53 by ICN mainly occurs through mTOR using the PI3K/Akt pathway, as rapamycin treatment abrogated ICN inhibition of tumor suppressor protein p53 and reversed the chemo-resistance of breast cancer and T-ALL (Mungamuri et al., 2006). It is well known that mTOR regulates translation rates and cell proliferation in part by phosphorylating two major targets, 4E-BP1 and ribosomal protein S6 kinases (S6K1 and S6K2). In deed, we found that down-regulation of Notch-1 by siRNA or GSI decreased mTOR phosphorylation and its target gene 4EBP-1 and S6K phosphorylation. We also found that inactivation of Akt by LY294002 and Wortmanin eliminated mTOR and its target 4EBP-1, s6K phosphorylation.

Akt regulates a number of downstream effectors including NF-κB. NF-κB has been reported to cross-talk with Notch pathway. The cross-talk between NF-κB and Notch is extraordinarily complex. Constitutive levels of Notch activity are essential to maintain 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 (Osipo et al., 2008). Recently, Song et al. (2008) reported that Notch-1 stimulated NF-κB activity in cervical cancer cells by associating with the IKK signalosome through IKKα. NF-κB was also previously shown to increase Notch-1 activity indirectly by

increasing the expression of Jagged-1 in lymphoma and myeloma cells (Bash et al., 1999). These reports suggest that, at least, in some contexts stimuli that activate NF-κB could lead to Notch activation, and conversely inactivation of Notch could inhibit NF-κB. In our study, we found that down-regulation of Notch inhibited the NF-κB DNA binding activity. Moreover, we found that Notch-1 siRNA or Jagged-1 siRNA reduced the basal levels of IKKα, IκBα, and pIκBα, which are consistent with previous findings in cervical cancer cells (Song et al., 2008). However, further in-depth molecular mechanistic studies are required in order to determine how Notch-1 cross-talk with NF-κB.

It is well accepted that many important molecules, such as MMP-9, VEGF, uPA, are involved in tumor cell invasion and metastasis. Since we found that down-regulation of Notch-1 and Jagged-1 inhibited the expression and activities of NF-κB downstream genes such as, MMP-9, VEGF, and uPA, we tested the effects of down-regulation of Notch-1 and Jagged-1 on the migration and invasion of prostate cancer cells. We found that down-regulation of Notch-1 or Jagged-1 inhibited migration and invasion of prostate cancer cells through matrigel, and these results are consistent with previous findings of Bin et al. (2009). Our results also suggest that down-regulation of Notch-1 or Jagged-1 could inhibit cancer cell migration and invasion, which was in part due to down-regulation of NF-κB and its downstream target genes such as MMP-9, uPA, and VEGF.

In summary, we presented experimental evidence which strongly suggest the role of Notch-1 down-regulation as a potential anti-tumor and anti-metastatic approach in prostate cancer. Down-regulation of Notch-1 or Jagged-1 inhibited cell growth with reduced Akt phosphorylation, its downstream gene mTOR phosphorylation and inactivated NF-κB signaling. From these results, we conclude that down-regulation of Notch-1 or Jagged-1 could potentially be an effective therapeutic approach for the inactivation MMP-9, uPA, and VEGF, which is likely to result in the inhibition of cell growth, migration, invasion and metastasis of prostate cancer. On the basis of our results, we propose a hypothetical pathway by which Jagged-1 and Notch-1 inactivation may induce apoptotic cell death and inhibit invasion of PC-3 cells, which is in part mediated via dysregulation of Akt, mTOR, and NF-κB signaling pathway (Supplemental Fig. 2). Taken together, these data provide a rationale for targeting the Notch signaling pathways for the treatment of prostate cancer in the future.

ACKNOWLEDGMENTS

This work was funded by grants from Department of Defense Postdoctoral Training Award W81XWH-08-1-0196 (Zhiwei Wang) and the National Cancer Institute, NIH (5R01CA083695, 1R01CA101870) to F.H.S.

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