Targeted Knockdown of *Notch1* Inhibits Invasion of Human Prostate Cancer Cells Concomitant with Inhibition of Matrix Metalloproteinase-9 and Urokinase Plasminogen Activator

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Abstract

Purpose: *Notch*, a type 1 transmembrane protein, plays a key role in the development of many tissues and organ types. Aberrant *Notch* signaling, found in a wide variety of human cancers, contributes to tumor development. Because *Notch1* was found to be overexpressed in prostate cancer (PCa) cells and human PCa tissue, we therefore tested our hypothesis that overexpression of *Notch1* in PCa promotes tumor invasion.

Experimental Design: *Notch1* expression was evaluated in human PCa cells and human PCa tissues. PCa cells were transiently transfected with *Notch1*-specific small interfering RNAs in concentrations ranging from 30 to 120 nmol/L and subsequently evaluated for effects on invasion and expression analysis for molecules involved in invasion.

Results: Small interfering RNA–mediated knockdown of *Notch1* in PC3 and 22Rν1 PCa cells dramatically decreased their invasion. Focused cDNA array revealed that *Notch1* knockdown resulted in significant reduction in the expression of urokinase plasminogen activator (uPA) and matrix metalloproteinase-9 (*MMP9*) gene transcripts. These data were further verified by reverse transcription-PCR, real-time reverse transcription-PCR, and immunoblot analysis. Knockdown of *Notch1* was also observed to significantly reduce the mRNA expression and protein levels of uPA and its receptor uPAR. A significant reduction in *MMP9* expression in *Notch1* knockdown cells suggested a role for *Notch1* in augmenting *MMP9* transcription.

Conclusions: Our data show the involvement of *Notch1* in human PCa invasion and that silencing of *Notch1* inhibits invasion of human PCa cells by inhibiting the expression of MMP9 and uPA. Thus, targeting of *Notch1* could be an effective therapeutic approach against PCa.

Notch signaling has been known for decades to developmental biologists as a key player in cell fate determination (1, 2) and tissue homeostasis by maintaining the self-renewal potential of some tissues and inducing differentiation of others (3), including formation of the prostate gland (4). In humans, notch family of transmembrane proteins consists of four receptors, *Notch1* through *Notch4*, and five ligands, *Jagged1* and *Jagged2* and Delta-like ligand 1 (*Dll1*), *Dll3*, and *Dll4* (5). Broadly, the modular structure of notch consists of an extracellular ligand binding domain, a hydrophobic transmembrane domain, and a notch intracellular domain. Notch receptors undergo a series of programmed proteolytic events, first by α-secretase at the extracellular surface, which leads to liberation of the extracellular fragment, and then by intramembraneous cleavage mediated by γ-secretase. Notch intracellular domain is then released from the inner surface of cell membrane and is translocated into nucleus where it activates transcription of the target genes (6). It has been shown that *Notch1* receptor ligand *Jagged1* is overexpressed in metastatic human prostatic tissue compared with localized prostate cancer (PCa) or benign prostatic tissue (7), implicating *Notch1* in PCa progression. A recent study has shown that down-regulation of *Jagged1* inhibits growth of PCa cells (8). Elevated expression of *Notch1* was observed in highly metastatic PCa cell lines as compared with normal prostate epithelial cells (9). *Notch1* level was reported to be elevated in malignant prostatic epithelial cells of primary and metastatic tumors of transgenic mouse model of PCa (10, 11). Recent studies show the involvement of notch signaling in cancer angiogenesis and metastasis (12–14); however, mechanisms for these effects remain unknown. In the present study, we provide evidence that *Notch1* plays an important role in invasiveness of human PCa concomitant with decrease in the expression of matrix metalloproteinase 9 (MMP9) and urokinase plasminogen activator (uPA).

Materials and Methods

Cell culture. Human PCa cell lines PC3, DU-145, LNCaP, and 22Rν1 were obtained from American Type Culture Collection and

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

In this study we have analyzed human prostate cancer (PCa) cells and tissues for the expression of *Notch1*. Based on the experimental studies done using specific depletion of *Notch1* protein in PCa cells, it becomes evident that *Notch1* is in fact overexpressed in PCa cells where it promotes cell invasion by a mechanism involving increased expression of matrix metalloproteinase-9 and urokinase plasminogen activator (uPA) and its receptor uPAR. Extensive histochemical analyses involving both normal and prostate tumor tissue were undertaken to screen for *Notch1* protein expression. In light of these findings, our data strongly suggest that *Notch1* protein is significantly overexpressed in a large cohort of PCa tissues as compared with normal human prostate tissue. Moreover, the expression was significantly concentrated in tumor areas situated near vasculature, which further indicates that *Notch1* could augment prostate tumor cell invasion and metastasis. In summary, these findings suggest that *Notch1* not only could potentially serve as a strong surrogate marker for PCa diagnoses but could also be developed to help screen for patients with high propensity for PCa metastasis. In addition, developing novel strategies to inhibit *Notch1* signaling could pave the way to effectively target PCa.

cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin solution. Prostate epithelial cells and their growth media were procured from Cambrex BioScience. Cells were grown in a humidified incubator containing 5% CO_2 at 37°C.

Immunohistochemistry. Human prostate tissues were obtained from the Department of Pathology, University of Wisconsin-Madison under an institutional review board approval. Immunohistochemical staining was done using an automated benchmark immunostainer (Ventana Medical Systems). Tissue sections were subjected to antigen retrieval, incubation with specific primary antibody of full-length *Notch1* (Santa Cruz Biotechnology, Santa Cruz, CA), which is a rabbit polyclonal antibody raised against amino acids 20 to 150 mapping within an extracellular domain of *Notch1* of human origin at a dilution of 1:50, followed by incubation with appropriate horseradish peroxidase–conjugated secondary antibody. Immunoreactive complexes were detected using 3,3′-diaminobenzidene and visualized under a Zeiss-Axiophot DM HT microscope and captured with an attached camera.

Histopathologic grading of PCa specimens. The Gleason system and the WHO grading system were used for evaluation of *Notch1* expression in the prostate tissues. Prostate adenocarcinoma was first graded in Gleason patterns 2, 3, and 4. The intensity of immunoperoxidase staining for *Notch1* was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) based on the Gleason patterns. The primary Gleason pattern and the secondary Gleason pattern were added to arrive at a Gleason score, ranging from 6 to 8. A total of 318 core tissue samples were from 41 patients. Normal glandular tissues, high-grade prostatic intraepithelial neoplasia (HGPIN), and blood vessels were from the areas adjacent to the cancerous tissue.

Notch1 small interfering RNA transfection. Validated *Notch1*-specific and scrambled small interfering RNAs (siRNA) were purchased from Santa Cruz Biotechnology. The *Notch1* siRNA contains the pool of three independent sequences: (*a*) sense 5′-CACCAGUUUGAAUGGUCAAtt-3′ and antisense 5′-UUGACCAUUCAAACUGGUGtt-3′; (*b*) sense 5′-CCCAUGGUACCAAUCAUGAtt-3′ and antisense 5′-UCAUGAUUGGUACCAUGGGtt-3′; and (*c*) sense 5′-CCAUGGUACCAAUCAUGAAtt-3′ and antisense 5′-UUCAUGAUUGGUACCAUGGtt-3′. Using electroporation (Amaxa, Inc.), cells were transiently transfected with *Notch1* siRNA in concentrations ranging from 30 to 120 nmol/L and scrambled

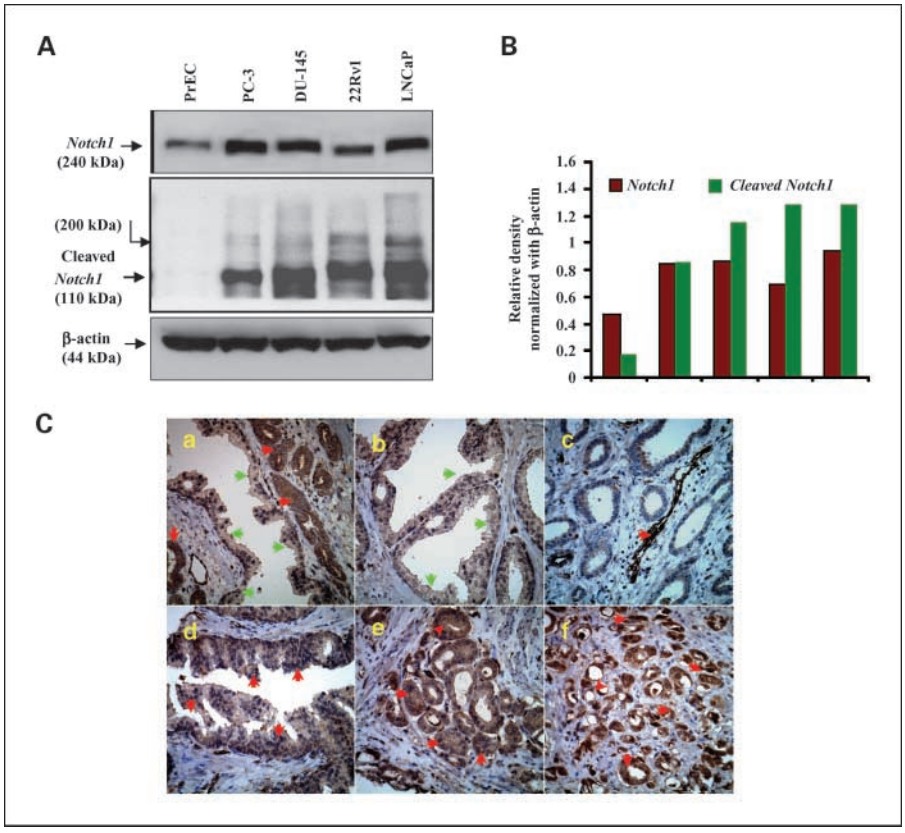

Fig. 1. Expression of *Notch1* in normal and PCa cells and in prostate tumor tissue. *A,* whole cell lysates were prepared and Western blot analysis was done to evaluate the protein levels of *Notch1* and cleaved *Notch1* in normal prostate epithelial cells and PCa cell lines PC3, DU-145, LNCaP, and 22Rv1. Blots were reprobed with β-actin antibody to analyze the equal loading of proteins. *B,* histogram represents density of bands in *A* normalized with β-actin. *C,* representative photomicrographs of prostate tumor biopsy specimen showing immunostaining for *Notch1* in cancerous as well as normal adjacent tissue (*a*); normal prostate tissue (*b*); blood vessels in cancerous tissue (*c*); HGPIN (*d*); and Gleason pattern 3 (*e*) and Gleason pattern 4 (*f*). Green arrows, normal tissue with none to low staining; red arrows, cancerous tissue with moderate to high *Notch1* expression.

Table 1. Expression of Notch1 in human normal prostate, HGPIN, and adenocarcinoma specimens

Samples	*n*	None (%)	Weak (%)	Moderate (%)	Strong (%)	*P*
Normal glands	91	1 (1)	33 (37)	53 (58)	4 (4)	—
High grade PIN	69	0 (0)	7 (10)	41 (60)	21 (30)	0.001*
Gleason pattern 3	130	0 (0)	5 (4)	57 (44)	68 (52)	0.001*
Gleason pattern 4	28	0 (0)	0 (0)	10 (36)	18 (64)	0.001*

NOTE: The expression of Notch-1 was evaluated as staining of the tissue as none (0), weak (1), moderate (2), and strong (3). Fisher's exact test was used to examine the association between staining intensity and tissue type or staining intensity and tumor grade (for tumor specimen only).
*$P < 0.001$.

siRNA (120 nmol/L). Cells were allowed to grow further in a CO_2 incubator for 24 h and later harvested for further analysis.

Western blot analysis. Forty micrograms of protein resolved over 4% to 20% Tris-glycine gels (Invitrogen) were transferred onto nitrocellulose membranes and probed with appropriate primary antibody against *Notch1* (Santa Cruz Biotech) and cleaved *Notch1* (Val1744 Cell Signaling Technology). The cleaved Notch1 antibody detects endogenous levels of the cytosolic domain of *Notch1* only when cleaved between Gly1743 and Val1744. This antibody does not recognize full-length *Notch1* or cleaved *Notch1* at other positions. Pro-MMP9 and uPA antibodies were purchased from Chemicon International, Inc., and uPAR antibody was obtained from R&D Systems. Expression levels of proteins were analyzed as described (15). Densitometric measurements of the bands in Western blot analysis were done using digitalized Scientific Software program, UN-SCAN-IT, purchased from Silk Scientific Corporation.

Gene expression analysis. Gene expression analysis was done using pathway focused Human Extracellular Matrix and Adhesion molecules oligo gene array (Superarray) containing 96 genes encoding proteins important for the attachment of cells to their surroundings. The array consisted of 96 cDNA in a 8 × 14 grid of tetraspots printed on a nylon membrane. The array was hybridized with total cellular cDNA that was reverse transcribed from total cellular RNA obtained either from PC3 cells transfected with scrambled siRNA or from *Notch1* siRNA. After hybridization, membrane was developed as per manufacturer's instructions. Data were analyzed using GEArray Expression Analysis Suite software (Superarray).

In vitro *chemoinvasion assay.* *Notch1* siRNA– or scrambled siRNA–transfected cells were resuspended in fresh culture medium and incubated in chemoinvasion chamber containing polycarbonate filter coated with Matrigel (Chemicon International) for 24 h. In the upper chamber, 30,000 cells were seeded in fetal bovine serum–free culture media and the lower chamber contained culture media containing 10% fetal bovine serum as a chemoattractant. The cells were allowed to migrate for 24 h, following which the chamber was washed with PBS and cells were visualized as per manufacturer's instruction. To quantitate the migratory cells, the invasion chamber was dipped in 10% acetic acid, and the resultant solution was spectrophotometrically read at 540 nm according to the vendor's protocol (16).

Gelatin zymography. Equal number of PC3 cells was transiently transfected with *Notch1*-siRNA and control siRNA for 24 h. The conditioned media were collected, concentrated using Amicon filter (Millipore) as per manufacturer's protocol, and electrophoresed (40 μg protein) under nonreducing conditions. The gelatinolytic activity of MMP9 was determined with a zymography kit (Invitrogen) as per vendor's protocol.

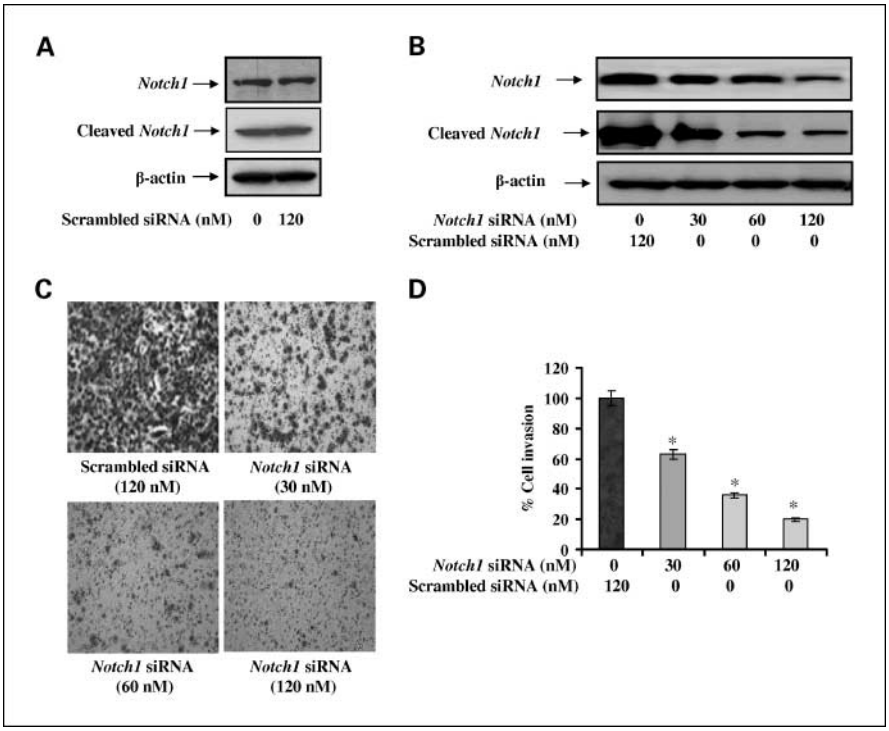

Fig. 2. Effect of *Notch1* knockdown on invasive behavior of PC3 cells. *A, Notch1* expression in control and scrambled siRNA – transfected cells. *B,* effect of *Notch1* siRNA on the expression of *Notch1* and cleaved *Notch1*. *C, Notch1* expression was knocked down in PC3 cells using *Notch1* siRNA and subjected to invasion assay using a two-chambered invasion apparatus as described in Materials and Methods. The photomicrograph shows the number of migratory cells transfected with varying concentrations of *Notch1* siRNA. *D,* histogram showing percent inhibition of PC3 cell invasion. The experiment was done in triplicate and the value obtained from scrambled siRNA transfected cells was set as 100%.

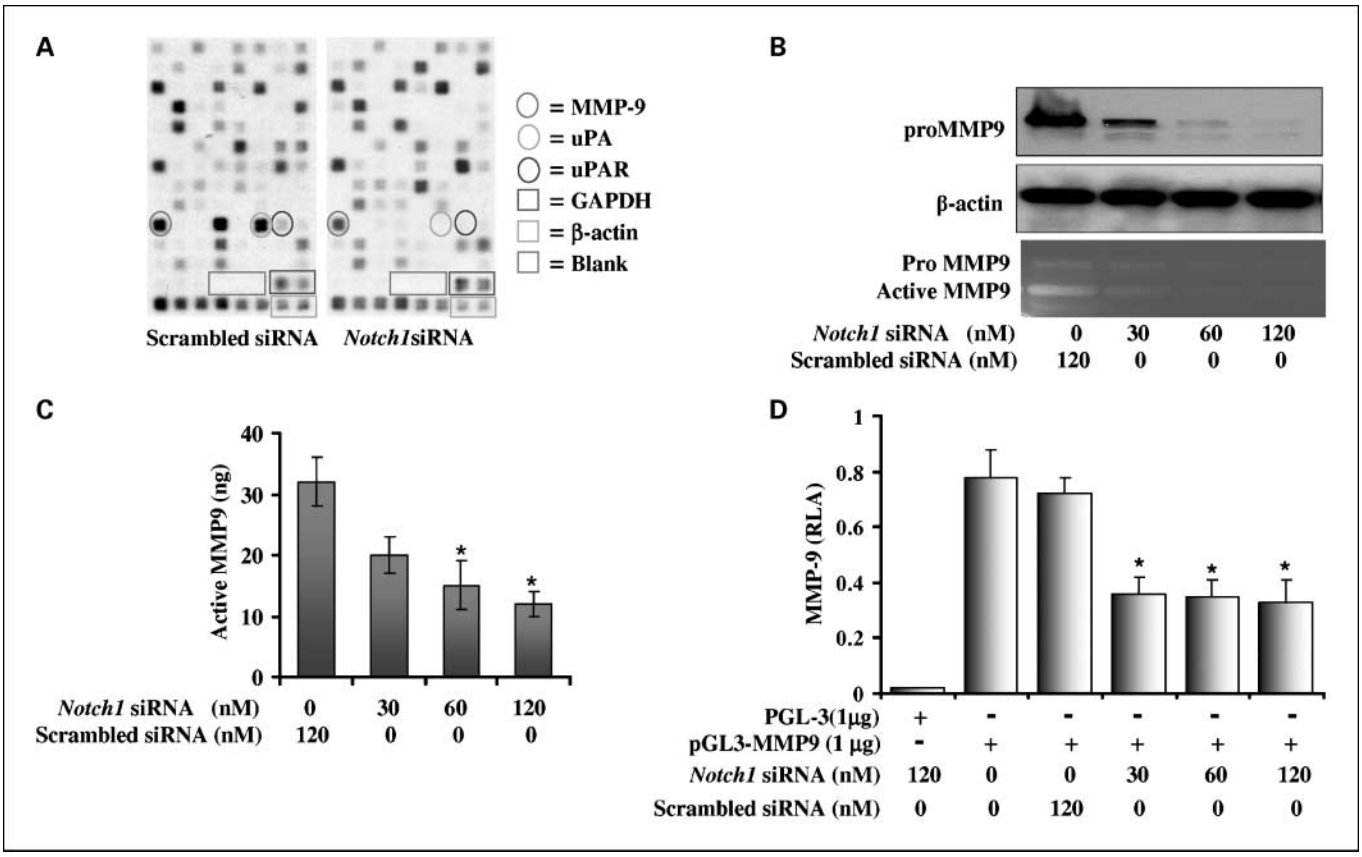

Fig. 3. Effect of *Notch1* knockdown in PC3 cells on the expression of genes involved in extracellular matrix degradation and cell adhesion. *A,* autoradiographic image of a cDNA array from scrambled siRNA – transfected control (*left*) and *Notch1* knockdown cells (*right*). Encircled tetraspots indicate the position of genes: red, MMP9; green, uPA; brown, uPAR. *B,* Western blot analysis to evaluate pro-MMP9 protein expression in *Notch1* knockdown cells. Blot was reprobed with β-actin antibody to analyze the equal loading of proteins. *C,* gelatin zymogram showing activity of pro-MMP9 and active MMP9 in scrambled siRNA – and *Notch1* siRNA – transfected cells. *D,* quantification of MMP9 secretion using MMP9-specific ELISA done in the culture media of PC3 cells transfected with either scrambled or *Notch1* siRNA. *E,* effect of *Notch1* knockdown on the promoter activity of *MMP9* gene expression in PC3 cells. PC3 cells (2×10^6) cotransfected with either scrambled or *Notch1* siRNA along with 1 μg of pGL3 or 1 μg of MMP9 luciferase reporter plasmid and 50 ng of renilla luciferase reporter plasmid as an internal control as described in Materials and Methods. Columns, mean of quadruple experiments; bars, SD. *, $P < 0.01$.

Luciferase assay. Cells (2×10^6) were nucleofected with human MMP9 luciferase reporter plasmid (pGL3-MMP9, 1 μg), a gift from Dr. Dougles D. Boyden (M. D. Anderson Cancer Center, Houston, TX), along with 50 ng of renilla luciferase reporter plasmid pRL-TK (Promega), which was used as an internal control to normalize transfection efficiency and varying concentrations of *Notch1* siRNA (30, 60, and 120 nmol/L). In parallel, cells were also transfected with empty pGL3 reporter vector and a scrambled siRNA (120 nmol/L) with no validated target sequence in the human genome. After nucleofection, 30,000 cells were distributed per well of a 24-well plate and allowed to grow for another 24 h. Dual luciferase assay reagent kit was procured from Promega and luciferase activity was measured according to the manufacturer's protocol.

MMP9 ELISA assay. Twenty-four hours posttransfection, the cell culture media were collected and used for quantifying MMP9 levels by using an MMP9-specific ELISA kit from Amersham Bioscience and following the vendor's protocol.

Semiquantitative PCR. PCR reactions were carried out using forward and reverse primer combinations for uPA (forward 5′-gtgaagaagggcgtccaaag-3′; reverse 5′-tcggcagtcaatgaggaaagt-3′), uPAR (forward 5′tctatccggagcagctgaaaa-3′; reverse 5′-cgtgtagacgcctggcttgt-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward 5′-aatccatcaccatcttccaggag-3; reverse 5′-gcattgctgatgatcttgaggctg-3). PCR reaction standardization kits were obtained from Epicentre. The cDNA was amplified with an initial denaturation at 94°C for 2 min followed by sequential cycles of denaturation at 94°C for 45 s, annealing at 59°C for 45 s, and extension at 72°C for 1 min for 30 cycles, with a final extension at 72°C for 7 min.

Quantitative reverse transcription-PCR. Real-time amplification of MMP9, uPA, and uPAR was done from 2 μL cDNA prepared from 2 μg of total RNA. The following primers were used: MMP9, forward 5′-atttctgccaggaccgcttctact-3′ and reverse 5′-cagtttgtatccggcaaactggct-3′; uPA, forward 5′-tcacaccaaggaagagaatggcct-3′ and reverse 5′-aatgacaaccagcaagaaagcggg-3′; uPAR, forward 5′tgtggctcatcagacatgagctgt-3′ and reverse 5′-ttgttgtggaaaccattggagccc-3′; and β-actin, forward 5′-atctggcaccacaccttctacaatgagctgcg-3 and reverse 5′-cgtcatactcctgcttgctgatccacatctgc-3. PCR reaction standardization kits were obtained from Epicentre. The cDNA was amplified with an initial denaturation at 95°C for 10 s followed by sequential cycles of denaturation at 94°C for 45 s, annealing at 55°C for 10 s, and extension at 72°C for 1 min for 30 cycles, with a final extension at 72°C for 7 min.

Statistical analysis. All measures were summarized as mean ± SE. Associations of categorical variables were evaluated using the Fisher exact test. All tests were two-sided and conducted at the $\alpha = 0.05$ significance level. All statistical analyses were done with the S-plus, Professional version 6.2 (Insightful Corp.) software.

Results

Notch1 is overexpressed in human PCa cells. *Notch1* has been implicated in many malignancies but its role in prostate

Table 2. List of genes modulated by knockdown of Notch1 in PC3 cells

UniGene	Ref Seq #	Symbol	Description	Fold change
Genes down-regulated by specific knockdown of Notch1				
Hs.77274	NM_002658	*PLAU*	Plasminogen activator, urokinase	34.66
Hs.514412	NM_000442	*PECAM1*	Platelet/endothelial cell adhesion molecule (CD31 antigen)	18.16
Hs.643357	NM_006988	*ADAMTS1*	ADAM metallopeptidase with thrombospondin type 1 motif, 1	11.00
Hs.592171	NM_002214	*ITGB8*	Integrin, β_8	8.68
Hs.632226	NM_000213	*ITGB4*	Integrin, β_4	8.12
Hs.74034	NM_001753	*CAV1*	Caveolin 1, caveolae protein, 22 kDa	6.81
Hs.482077	NM_002203	*ITGA2*	Integrin, α_2 (CD49B, α_2 subunit of VLA-2 receptor)	6.46
Hs.55279	NM_002639	*SERPINB5*	Serpin peptidase inhibitor, clade B (ovalbumin), member 5	5.02
Hs.58488	NM_003798	*CTNNAL1*	Catenin (cadherin-associated protein), α-like 1	4.58
Hs.643447	NM_000201	*ICAM1*	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	4.00
Hs.133397	NM_000210	*ITGA6*	Integrin, α_6	3.82
Hs.466871	NM_002659	*PLAUR*	Plasminogen activator, urokinase receptor	3.40
Hs.609663	NM_002293	*LAMC1*	Laminin, γ1 (formerly LAMB2)	2.93
Hs.371147	NM_003247	*THBS2*	Thrombospondin 2	2.43
Hs.297413	NM_004994	*MMP9*	Matrix metallopeptidase 9 gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase	4.20
Hs.159581	NM_016155	*MMP17*	Matrix metallopeptidase 17 (membrane-inserted)	2.20
Hs.411312	NM_000419	*ITGA2B*	Integrin α_{2b} (platelet glycoprotein IIb of IIb/IIa complex, antigen CD41)	2.08
Genes up-regulated by specific knockdown of Notch1				
Hs.313	NM_000582	*SPP1*	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activator)	9.07
Hs.73800	NM_003005	*SELP*	Selectin P (granule membrane protein 140 kDa, antigen CD62)	6.66
Hs.21422	NM_005010	*NRCA<*	Neuronal cell adhesion molecule	5.07
Hs.537417	NM_006690	*MMP24*	Matrix metallopeptidase 24 (membrane-inserted)	5.01
Hs.300774	NM_005141	*FGB*	Fibrinogen β-chain	4.38
Hs.375129	NM_002422	*MMP3*	Matrix metallopeptidase 3 (stromelysin 1, progelatinase)	3.29
Hs.652397	NM_00362	*TIMP3*	TIMP metallopeptidase inhibitor 3 (Sorsby fundus dystrophy, pseudoinflammatory)	3.21
Hs.2936	NM_002427	*MMP13*	Matrix metallopeptidase 13 (collagenase 3)	3.10
Hs.161985	NM_019894	*TMPRSS4*	Transmembrane protease, serine 4	2.67
Hs.143434	NM_001843	*CNTN1*	Contactin 1	2.64
Hs.204732	NM_021801	*MMP26*	Matrix metallopeptidase 26	2.60
Hs.461086	NM_004360	*CDH1*	Cadherin 1, type 1, E-cadherin (epithelial)	2.50
Hs.445981	NM_001903	*CTNNA1*	Catenin (cadherin-associated protein), α_1, 102 kDa	2.16

carcinogenesis is not well defined. Therefore, we examined the expression of *Notch1* in different PCa cell lines and also in normal prostate epithelial cells. Western blot analysis showed increased expression of *Notch1* and cleaved *Notch1* in human PCa cell lines. A significantly higher level of *Notch1* expression was observed in androgen-independent PC3, DU-145, and 22R*v*1 cells and androgen-dependent LNCaP cells compared with prostate epithelial cells (Fig. 1A). *Notch1* protein in 22R*v*1 cells migrated faster than in other PCa cells, which could be a splice variant of the native *Notch1* protein.

Notch1 expression increases in human prostate tumor specimens with increasing tumor grade. To further establish the contextual role of *Notch1* in PCa, immunohistochemistry was done in human prostate tumor specimens of normal, HGPIN, and PCa representing different tumor grades. A total of 318 specimens were examined. We observed enhanced immunoreactivity of *Notch1* in tumor cells compared with normal epithelial cells in adjacent areas of the same tissue (Fig. 1C, *a*). In addition, enhanced expression of *Notch1* was also prominent in the cells surrounding blood capillaries in cancerous tissue (Fig. 1C, *c*). Based on the scoring patterns, a significant difference was observed in *Notch1* expression between cancer and normal tissues (Table 1). The staining for *Notch1* protein was moderate to strong in PCa specimens as compared with normal prostate specimens, which exhibited either none or weak to moderate staining (Table 1). In a total of 91 specimens of normal tissue obtained from adjacent regions of tumor tissues, the staining for *Notch1* was weak in 33 (36%), moderate in 53 (58%), and negative in the remaining 1 (1%) specimen (Table 1). HGPIN specimens (n = 69) showed strong staining for *Notch1* in 21 (30%), moderate staining in 41 (60%), and weak staining in the remaining 7 (10%) specimens (Table 1). The percentage of HGPIN specimens exhibiting strong *Notch1* staining was 7-fold higher than that in normal gland. Because HGPIN has been identified as the most significant risk stage for PCa development and the expression of *Notch1* protein was found to be significantly increased in both HGPIN and PCa specimens, a strong link could be suggested between the expression of *Notch1* protein and development of human PCa. Staining for *Notch1* was observed in epithelial cells; however, in stromal cells the staining was either occasional or negative in normal as well as in cancer specimens. The immunoreactivity of *Notch1* was observed in a coarsely granular pattern in the cytoplasm of epithelial cells of normal, HGPIN, and Gleason pattern 3 (Gleason score 5-6) to Gleason pattern 4 (Gleason score 7-10) prostatic adenocarcinomas (Fig. 1B, *c-f*). Accumulative analysis of all PCa specimens (n = 158) suggested higher levels of *Notch1*, with moderate staining in 67 (42%) to strong staining in 86 (54%) specimens, weak staining in 5 (3%) specimens, and no staining in 0 (0%) specimens (Table 1). These results indicate that *Notch1* is overexpressed in human PCa.

Notch1 knockdown decreases cell invasion. To address the role of *Notch1* in PCa invasion, knockdown of *Notch1* was achieved by transfecting PC3 cells with three independent pools of *Notch1* siRNA compared with scrambled siRNA where no effect on *Notch1* and cleaved *Notch1* expression was observed (Fig. 2A). Because we had hypothesized that *Notch1* is involved in PCa invasion, and the fact that PC3 cells represent advanced metastatic cancer, we selected this particular cell line for our studies. *Notch1* siRNA dose-dependently decreased both *Notch1* and cleaved *Notch1* expression with maximum effect observed at a concentration of 120 nmol/L at 24 hours posttransfection (Fig. 2B). To further show the effect of *Notch1* knockdown, PC3 cells were subjected to invasion assay. *Notch1* knockdown cells showed only a marginal invasion through the extracellular matrix compared with cells transfected with nonspecific siRNA (Fig. 2C). *Notch1* knockdown caused 40% to 80% decrease in cell invasion (Fig. 2D), suggesting an essential role of *Notch1* in conferring invasive properties to PCa cells.

cDNA array identifies decreased transcripts of genes implicated in tumor invasion. To define the role of *Notch1* in PC3 cell invasion, a specifically designed cDNA gene array was used. This array contained 96 cDNA fragments specifying genes with role in cell invasion and metastasis. A significant difference in gene expression of CD31, MMP9, uPA, and uPAR (Fig. 3A) was observed in *Notch1* knockdown cells. To ascertain fold changes between scrambled and *Notch1* siRNA knockdown cells, we performed a detailed analysis of gene expression profile by clustergram, and scatter plot indicated a 35-fold down-regulation in the expression of uPA, 18-fold in CD31, and >3-fold in the expression of MMP9 and uPAR (Table 2). These results indicate that *Notch1* regulates the expression of these downstream target genes that are involved in extracellular matrix degradation, suggesting that *Notch1* might play a determining role in cell invasion.

Notch1 knockdown decreases MMP9 expression. To further validate the results of microarray analysis, *Notch1* knocked down cells were subjected to Western blot analysis. We observed a decrease in pro-MMP9 protein levels (Fig. 3B), suggesting that *Notch1* may be involved in MMP9 activation either by enhancing its expression or by stabilizing the protein. Gelatin zymography was done to assess MMP9 activity in cultured medium from *Notch1* knockdown cells and we observed a significant decrease in MMP9 activity (Fig. 3B). To further strengthen our findings, MMP9-specific ELISA was done to quantify secreted MMP9 protein levels. Results indicated a consistent decrease in MMP9 secretion compared with scrambled control (Fig. 3C), further emphasizing the role of *Notch1* in regulation of MMP9. To further elucidate the involvement of *Notch1* in MMP9 expression, luciferase assay was done to evaluate the effect of *Notch1* on MMP9 promoter activity. *Notch1* knockdown cells showed a marked 2-fold decrease in reporter activity ($P < 0.01$), indicating the involvement of *Notch1* in MMP9 expression (Fig. 3D). Surprisingly, the decrease observed in MMP9 promoter activity was not dose dependent. This suggests that although *Notch1* is involved in MMP9 transcription, it may not be an exclusive mechanism that regulates MMP9 expression and indicates the existence of a posttranslational stabilization mechanism. However, real-time reverse transcription-PCR (RT-PCR) data showed a dose-dependent decrease in mRNA expression of MMP9 in *Notch1*

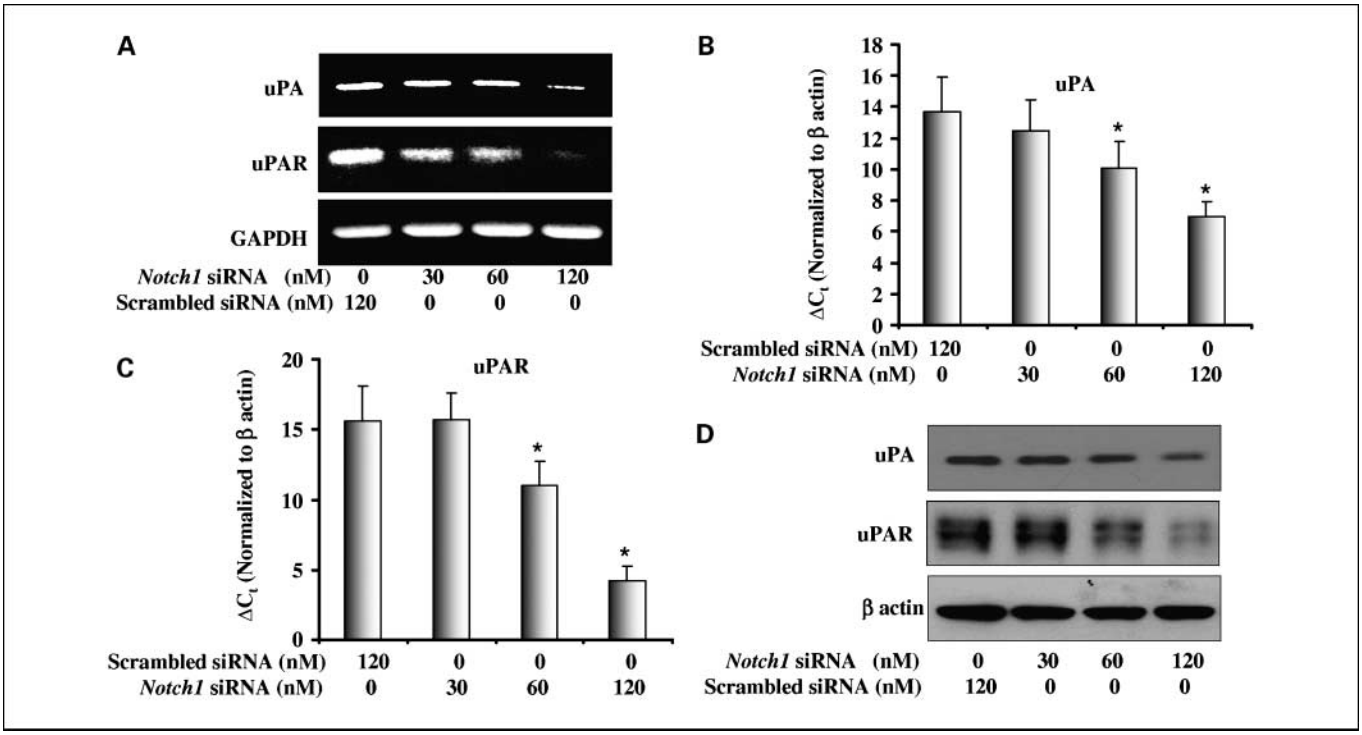

Fig. 4. Effect of *Notch1* knockdown on mRNA and protein expressions of uPA and uPAR. *A,* semiquantitative RT-PCR analysis shows mRNA expression of uPA and uPAR in cells transfected with increasing concentration of *Notch1* siRNA. Scrambled siRNA was used as a control in parallel. GAPDH was used as an internal control. *B,* Western immunoblot analysis of uPAR and uPA expression in PC3 cells transfected with increasing concentration of *Notch1* siRNA. Blots were reprobed with β-actin antibody to analyze the equal loading of proteins. *C* and *D,* real-time RT-PCR analysis showing mRNA expression of uPA and uPAR.

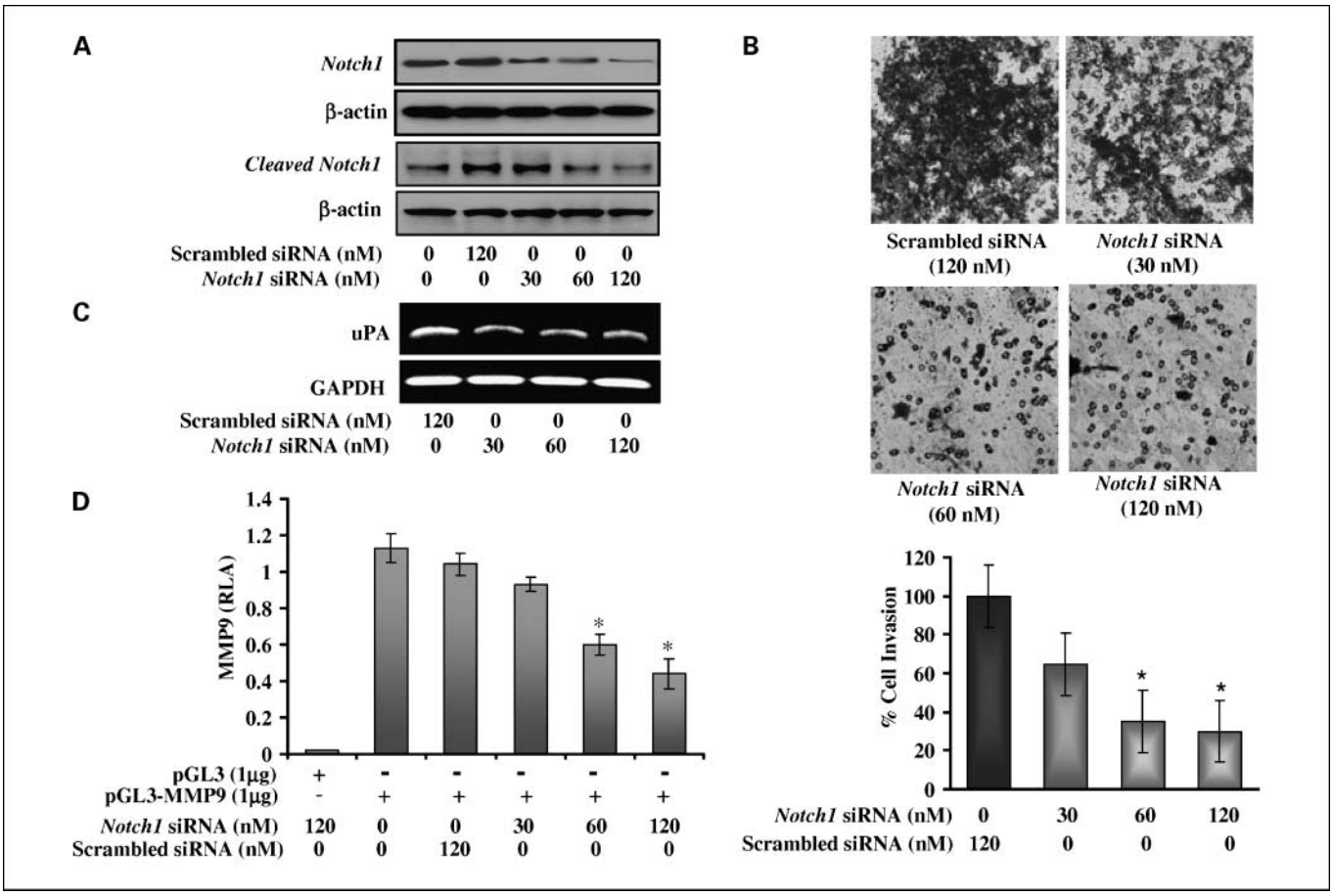

Fig. 5. Effect of *Notch1* knockdown on invasion, mRNA expression of uPA, and MMP9 activity in 22Rv1 cells. *A,* protein levels of *Notch1* and cleaved *Notch1* in control, scrambled siRNA –, and Notch1 siRNA – transfected 22Rv1 cells as determined by Western blot analysis. *B,* the photomicrograph shows the number of migratory cells transfected with varying concentrations of *Notch1* siRNA and scrambled siRNA. *Bottom,* quantification of migratory cells in scrambled siRNA – and *Notch1* siRNA – transfected cells. The experiment was done in triplicate and the value obtained from scrambled siRNA transfected cells was set as 100% migration. *C,* semiquantitative RT-PCR analysis shows mRNA expression of uPA in cells transfected with increasing concentration of *Notch1* siRNA. Scrambled siRNA was used as a control in parallel. GAPDH was used as an internal control. *D,* effect of *Notch1* knockdown on the promoter activity of *MMP9* gene expression in PC3 cells. 22Rv1 cells (2×10^6) cotransfected with either scrambled or *Notch1* siRNA along with 1 μg of pGL3 or 1 μg of MMP9 luciferase reporter plasmid and 50 ng of renilla luciferase reporter plasmid as an internal control as described in Materials and Methods. Columns, mean of quadruple experiments; bars, SD. *, $P < 0.01$.

knockdown cells, which further confirms our microarray data (Supplementary Fig. S1).

Notch1 knockdown decreases the expression of uPA and its receptor uPAR. uPA is another marker for invasion and metastasis and has been shown to be up-regulated in many malignancies including PCa. uPA regulates the conversion of pro-MMPs to their active forms, which are involved in extracellular matrix degradation. RT-PCR and real-time RT-PCR were done to confirm the microarray data, which showed down-regulation of uPA and uPAR transcripts in *Notch1* knockdown cells (Fig. 4A-C). We also observed that knockdown of *Notch1* in PC3 cells inhibited protein levels of the ligand uPA and its receptor uPAR (Fig. 4D). These data were in direct agreement with the microarray data and suggest the involvement of *Notch1* in regulation of uPA and uPAR.

Notch1 knockdown decreases invasion and inhibits the expression of MMP9, uPA, and uPAR in another human PCa 22Rv1 cell line. To ascertain whether *Notch1* knockdown produces similar effects in other PCa cells, we selected androgen-sensitive 22Rv1 cells and transiently transfected with scrambled siRNA and specific *Notch1* siRNA. Knockdown of *Notch1* in 22Rv1 cells significantly decreased protein expression of *Notch1* and cleaved *Notch1* (Fig. 5A), invasion of cells through the extracellular matrix (Fig. 5B), uPA expression (Fig. 5C), and MMP9 promoter activity (Fig. 5D) compared with cells transfected with nonspecific siRNA. RT-PCR and real-time RT-PCR were done and showed down-regulation of MMP9, uPA, and uPAR transcripts in *Notch1* knockdown cells (Supplementary Fig. S5A-C). We also observed that knockdown of *Notch1* in 22Rv1 cells inhibited protein levels of the ligand uPA and its receptor uPAR (Supplementary Fig. S5D). These data suggest that the effect of *Notch1* knockdown in PCa cell invasion could be comprehensive because we observed similar mechanism in both the PCa androgen-sensitive 22Rv1 and androgen-insensitive PC3 cells.

Discussion

Aberrant expression of *Notch1* has been detected in various types of human cancers including T-cell acute lymphoblast leukemia (17), breast carcinoma (18), and brain tumor (19). Whereas some studies have shown the involvement of *Notch1*

in cancer progression, others have suggested antiproliferative effect of *Notch1* in some cancer types (3). *Notch1* has also been reported to be overexpressed in malignant phenotype including moderately differentiated adenocarcinoma of TRAMP mice (11). In spite of these studies, the role of *Notch1* in prostate carcinogenesis remains poorly understood. In this study, we report overexpression of *Notch1* in PCa cell lines, consistent with previous findings of Zayzafoon et al. (20). We also observed significantly elevated expression of *Notch1* in human PCa tissues. *Notch1* expression increased with increasing tumor grade, with specimens of Gleason pattern 3 and 4 exhibiting significantly higher percentage of strong expression. An interesting observation was a significant induction of *Notch1* in vascular endothelial cells of these tissues, consistent with earlier reports (21), suggesting that *Notch1* may facilitate angiogenesis of PCa cells to neighboring and distant organs. We observed that targeted disruption of *Notch1* in PC3 cells resulted in significant decrease in cell invasion across artificial matrix, which mimics *in vivo* extracellular matrix. In cDNA array, we observed significant modulation of genes that are involved in cell invasion and angiogenesis. We observed significant decreases in the expression of CD31, uPA, uPAR, and MMP9. MMPs have been shown to be involved in extracellular matrix degradation and are overexpressed in advanced stage PCa. We examined the functional activity of MMP9 in *Notch1* knockdown cells and observed concentration-dependent decrease in MMP9 expression and activity, indicating that *Notch1* might directly regulate the expression of MMP9 by enhancing its promoter activity. Intracellular domain of *Notch 1* vests a transactivation function, and it is possible that it might help recruit transcriptional machinery to MMP9 regulatory element to enhance its expression. Although a progressive decrease in *Notch1* was observed with increasing concentration of *Notch1* siRNA, the decrease in MMP9 promoter activity was not concentration dependent, in contrast to MMP9 protein expression, which rules in the possibility for the existence of a posttranslational stabilization mechanism. We suggest that *Notch1* controls MMP9 expression directly by enhancing its transcriptional activity where it functions as a transcription factor. Our data correspond to a previous report by Wang et al. (22), which showed the regulation of MMP9 via *Notch1* in pancreatic cancer cells. An additional mechanism that is commonly involved in promoting tumor cell invasion is the uPA-uPAR system, which is one of the most frequent alterations observed in invasive type of PCa (23). We also observed decrease in uPA and uPAR expression in *Notch1* knockdown cells, suggesting that *Notch1* can also regulate the expression of uPA and its receptor via a distinct gene expression mechanism. These findings suggest that *Notch1* is involved in invasion and metastasis of PCa by regulating the expression of MMPs in addition to the uPA-uPAR system, which might work in synergy to enhance tumor cell invasion and metastasis. To illustrate the broader relevance of our hypothesis, we performed additional studies on androgen-sensitive human PCa 22Rv1 cells, which also showed significantly higher expression of *Notch1*. Our results indicate that the disruption of *Notch1* in these cells also leads to a decrease in their invasiveness accompanied by a decrease in MMP9 and uPA expression and suggest the versatile role of *Notch1* in PCa cell invasion. We suggest that *Notch1* could be a target for intervention of human PCa.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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