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Notch-1 Signaling Is Lost in Prostate Adenocarcinoma and Promotes PTEN Gene Expression

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ABSTRACT

Prostate tumorigenesis is associated with loss of PTEN gene expression. We and others have recently reported that PTEN is regulated by Notch-1 signaling. Herein, we tested the hypothesis that alterations of the Notch-1 signaling pathway are present in human prostate adenocarcinoma and that Notch-1 signaling regulates PTEN gene expression in prostate cells. Prostate adenocarcinoma cases were examined by immunohistochemistry for ligand cleaved (activated) Notch-1 protein. Tumor foci exhibited little cleaved Notch-1 protein, but expression was observed in benign tissue. Both tumor and benign tissue expressed total (uncleaved) Notch-1. Reduced Hey-1 expression was seen in tumor foci but not in benign tissue, confirming loss of Notch-1 signaling in prostate adenocarcinoma. Retroviral expression of constitutively active Notch-1 in human prostate tumor cell lines resulted in increased PTEN gene expression. Incubation of prostate cell lines with the Notch-1 ligand, Delta, resulted in increased PTEN expression indicating that endogenous Notch-1 regulates PTEN gene expression. Chromatin immunoprecipitation demonstrated that CBF-1 was bound to the PTEN promoter. These data collectively indicate that defects in Notch-1 signaling may play a role in human prostate tumor formation in part via a mechanism that involves regulation of the PTEN tumor suppressor gene. J. Cell. Biochem. 107: 992–1001, 2009.

KEY WORDS: NOTCH-1; PTEN; PROSTATE

Notch-1 is part of an evolutionarily conserved signaling pathway that regulates cell differentiation, proliferation, and growth [Mumm and Kopan, 2000]. Humans have four Notch receptors (Notch-1, -2, -3, and -4) and at least five Notch ligands (Jagged-1, Jagged-2, Delta-1, Delta-3, and Delta-4). Notch signaling has been reported to be a requirement for normal murine prostate re-growth and branching [Wang et al., 2004, 2006]. In addition, Notch-1 signaling may act to repress androgen receptor signaling [Belandia et al., 2005]. Notch-1 signaling has been linked with regulation of prostate tumor cell motility [Scorey et al., 2006]. Notch-1 and Notch-ligands have been reported to be overexpressed in prostate tumor cell lines as compared with prostate cell lines derived from normal tissue [Shou et al., 2001]. In a comprehensive study of 154 men, the Notch-ligand, Jagged-1, was overexpressed in advanced malignant and recurrent prostate tumors [Santagata et al., 2004].

The 300 kDa Notch protein is cleaved in the trans-golgi to generate a 180 kDa extracellular fragment and a 120 kDa fragment that contains a transmembrane domain and an intracytoplamsic region [Blaumueller et al., 1997; Logeat et al., 1998]. Upon interaction with ligand, Notch-1 undergoes two distinct cleavages that involve (1) a TACE metalloprotease and (2) a γ-secretase

Abbreviations used: ICN, intracytoplasmic Notch; ChIP, chromatin immunoprecipitation; IHC, immunohistochemistry; PTEN, phosphatase and tensin homolog commonly deleted on chromosome 10.

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complex that includes presenilin-1 and -2, Aph-1, and nicastrin [Mumm et al., 2000; Brou et al., 2000; Fraering et al., 2004]. This results in the release of the intracytoplasmic Notch fragment (ICN) which then translocates to the nucleus where it interacts with the CBF-1 (also termed RBP-Jk) transcription factor [Jarriault et al., 1995; Struhl and Adachi, 1998]. The ICN fragment is constitutively active.

CBF-1 is the main transcriptional effector of Notch-1 receptor activation [Furukawa et al., 1992; Jarriault et al., 1995; Lai, 2002]. CBF-1 recognizes the consensus DNA sequence C/G TGGGAA A/C. In the absence of Notch-1 activation, CBF-1 acts as a transcriptional repressor by forming a repression complex that includes Skip, SMRT/N-CoR, and HDAC1 [Kao et al., 1998; Hsieh et al., 1999]. Intracytoplasmic Notch-1 binds to CBF-1 and/or Skip, displacing SMRT/N-CoR and HDAC1, which results in the recruitment of MAML, CBP/p300, and histone acetyl transferases. This converts the CBF-1 complex into a transcriptional activation complex [Lai, 2002; Wu and Griffin, 2004]. Thus, CBF-1 functions as a Notch-responsive transcriptional switch.

PTEN (phosphatase and tensin homolog commonly deleted on chromosome 10) is a 54 kDa protein originally identified through a common deletion on chromosome 10 in a variety of tumor types [Li et al., 1997; Myers et al., 1997]. Three distinct functions have been assigned to the PTEN gene product: (1) lipid phosphatase activity [Maehama and Dixon, 1998], (2) protein phosphatase activity [Li et al., 1997; Myers et al., 1997], and (3) a phosphorylation substrate [Torres and Pulido, 2001; Torres et al., 2003]. PTEN is best characterized as a lipid phosphatase that acts as an antagonist to PI3K/Akt signaling. Because PI3K/Akt signaling is the basis for many survival and proliferative signals in most cell types, regulation of this pathway is one major way in which PTEN exerts its role as a tumor suppressor [Salmena et al., 2008]. PTEN has also been reported to participate in the regulation of other signaling pathways such as Ras/Raf/Mek/ERK, focal adhesion kinase, and GSKbeta [Salmena et al., 2008].

Many prostate cancers exhibit loss of PTEN heterozygosity [Ozen and Pathak, 2000]. Mouse models have indicated that the level of PTEN gene expression is directly linked with the incidence and severity of prostate tumors [Di Cristofano et al., 2001; Trotman et al., 2003; Ma et al., 2005]. The lower the dose of PTEN expression, the more aggressive and widespread the prostate tumors. Loss of PTEN expression is also closely linked with other epithelial carcinomas such as breast cancer and colon cancer [Salmena et al., 2008].

Despite the central role of PTEN as a tumor suppressor and as a central upstream control point of several major signaling pathways, regulation of PTEN gene expression is not well characterized. The PTEN promoter has been reported to contain a p53 responsive element [Stambolic et al., 2001]. We have recently reported that signaling through the Notch-1 pathway results in increased expression of PTEN in 293 fibroblasts [Chappell et al., 2005]. This was mediated by the binding of the Notch-activated transcription factor CBF-1 to the PTEN minimal promoter [Whelan et al., 2006]. Another group has also reported Notch-1 regulation of PTEN via c-myc in acute T-cell leukemia [Palomero et al., 2007].

We have examined Notch-1 signaling in prostate adenocarcinoma. Prostate tumor foci exhibited a loss of Notch-1 signaling as compared with surrounding benign tissue. We also demonstrate in prostate cells, that Notch-1 promotes PTEN gene expression via the CBF-1 transcription factor. Our data provide novel mechanistic insight how loss of Notch-1 signaling promotes prostate tumor development through its role as a regulator of the PTEN tumor suppressor gene.

MATERIALS AND METHODS

CELL CULTURE

DU145 [Stone et al., 1978] and 22Rv1 [Sramkoski et al., 1999] cells were purchased from the American Tissue Type Culture Collection (ATTC). Cells were maintained in T-75 flasks with RPMI supplemented with 10% HFCS, 10 U/ml penicillin/streptomycin and 2 mM L-glutamine.

RETROVIRAL VECTORS

The Notch-1 IRES-GFP vector bearing human intracytoplasmic Notch-1 and the MigR1 IRES-GFP vector were the kind gift of Warren Pear, University of Pennsylvania [Pui et al., 1999]. Retroviral constructs expressing wild-type CBF-1 (CBF-1wt) or a mutated, R218H, CBF-1, (CBF-1(R218H)), have been previously described [Whelan et al., 2006]. The R218H mutation has been described previously to generate a DNA binding deficient CBF-1 [Chung et al., 1994]. Both CBF-1(R218H) and CBF-1wt bear the V5 epitope tag.

RETROVIRAL TRANSDUCTION

Retroviral transduction was carried out as described previously [Chappell et al., 2005; Whelan et al., 2006]. Transduced cells were selected in the presence of 2 mg/ml G418 (for the CBF-1 constructs) or FACS purified based on GFP expression (for the Notch-1 and MigR1 constructs).

WESTERN BLOT ANALYSIS

Western blotting was performed as described [Chappell et al., 2005; Whelan et al., 2006]. Membranes were probed sequentially with the indicated antibodies.

POLYMERASE CHAIN REACTION

RT-PCR was performed as described previously [Bertrand et al., 2000]. For PTEN the primer pair, (5′-CAG AAA GAC TTG AAG CGT AT-3′ and 5′-AAC GGC TGA GGG AAC CTC-3′), was used with an annealing temperature of 50°C for 30 cycles. For Hes-1, the primer pair (5′-TGG AAA TGA CAG TGA AGC ACC T-3′ and 5′-GTT CAT GCA CTC GCT GAA GC-3′) [Varnum-Finney et al., 2000] was used with an annealing temperature of 58°C for 30 cycles. For GAPDH, the primer pair, (5′-ATG GCC TTC CGT GTC CCC ACT G-3′ and 5′-TGA GTG TGG CAG GGA CTC CCC A-3′), was used with an annealing temperature of 60°C for 25 cycles.

ELECTROPHORETIC MOBILITY SHIFT ASSAYS

Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) as directed. Electrophoretic mobility shift assays (EMSA) were performed using the DIG Gel Shift Kit, 2nd Generation (Roche Diagnostics) as per

manufacturer's protocol, using a 70 bp probe spanning the PTEN minimal promoter from −1,174 to −1,104 [Stambolic et al., 2001]. In some experiments, excess unlabeled probe was added as a competitor. For supershift experiments, anti-V5 (Invitrogen) was added to the binding reaction and incubated at room temperature for 5 min prior to the addition of labeled probe.

LUCIFERASE ASSAYS

Luciferase assays were performed as described [Chappell et al., 2005]. HES AB and HES ΔAB were the kind gift of Jon Aster, Harvard University [Jarriault et al., 1995]. Generation of the 70 bp minimal PTEN promoter luciferase reporter been described previously [Whelan et al., 2006]. Luciferase was read using a Turner Systems luminometer.

CHROMATIN IMMUNOPRECIPITATION

2×10^6 DU145/CBF-1wt or DU145/CBF-1(R218H) cells were plated in a 60 mm culture dish, cultured for 24 h, and then used to perform ChIP, as described previously [Shewchuk et al., 2006]. Immunoprecipitation was performed with an anti-V5 antibody (Invitrogen) overnight at 4°C with rotation. Negative control immunoprecipitations contained no antibody. PCR was performed using primers to the PTEN promoter region (5′-CTC GAG TCA GTG ACA CTG CT-3′ and 5′-TCT ACT GAG CAT GCC CAG TGT A-3′) and to GAPDH (5′-ATG GCC TTC CGT GTC CCC ACT G-3′ and 5′-TGG TGT GGC AGG GAC TCC CA-3′).

WOUNDING ASSAYS

Wounding assays were performed on cultured cells as described [Denker and Barber, 2002]. Wounds were photographed at 0, 8, 16, and 24 h under phase contrast with a 10× objective. For analysis, photographs of each wound were printed in an 8.5 in. × 11 in. format. The width of the wound was measured every inch for 8 in. These values were used to determine the average width of the wound and the standard deviation.

PROLIFERATION ASSAYS

Cell proliferation was evaluated via the MTT assay using CellTiter 96® reagent (Promega, Madison, WI) as per manufacture's instruction. Absorbance was measured at 492 nm and is expressed as relative proliferation. All experiments were performed in triplicate.

CO-CULTURE WITH DELTA LIGAND

Purified Delta ligand extracellular domain fused to the Fc region of human IgG has been reported previously [Varnum-Finney et al., 2000] and was the kind gift of Dr. Irwin Bernstein, Fred Hutchinson Cancer Research Center, Seattle WA. Non-tissue culture coated 96-well plates were coated with increasing concentrations of the Delta-Fc protein and incubated at 4°C overnight. The plates were washed extensively with PBS + 2% bovine serum albumin. Fifty thousand DU145 cells per well, were seeded into the plates and were incubated for 24 h at 37°C in standard tissue culture conditions. Cells were then harvested for RT-PCR.

IMMUNOHISTOCHEMICAL STAINING

Archived paraffin-embedded prostate adenocarcinoma cases were selected for IHC in accordance with the Brody School of Medicine Institutional Review Board (IRB). Serial sections cut from the paraffin blocks were dewaxed in xylene and rehydrated through a series of graded alcohols to water. The slides were subjected to antigen retrieval using 10 mM citrate buffer, pH 6, by steamer method. Endogeneous peroxidase activity was blocked by exposure to hydrogen peroxide. Slides were then blocked (Sniper Block, BioCare Medical) and incubated with primary antibody or control antibodies. Antibody detection was performed with a biotin-free polymer based detection system (Mach 3 Rabbit-Probe HRP Polymer Kit, BioCare Medical). Impact DAB (Vector Laboratories) served as the final chromagen and slides were counterstained with hematoxylin prior to mounting. Species and isotype matched Igs served as negative controls. Antibodies used were: Notch-1: C-20 rabbit polyclonal affinity purified antibody, Santa Cruz Biotechnology; Cleaved Notch-1: Val-1744, rabbit polyclonal, affinity purified, Cell Signaling; ImmunoPure Rabbit IgG, Whole Molecule: control, Thermo Scientific/Pierce Biotechnology. Sections were scored independently for Notch staining and Gleason Grade (A.K. and K.H-L.) with differences resolved at the microscope. The following scoring criteria were used: (1) negative; (2) 5–20% cells positive; (3) 20–70% cells positive; and (4) >70% cells positive. These scores were then averaged and used to calculated statistical significance via the unpaired *t*-test.

RESULTS

LOSS OF CLEAVED NOTCH-1 IN PROSTATE ADENOCARCINOMA

Reduced PTEN gene expression is common in prostate cancer and because Notch-1 signaling regulates PTEN gene expression in fibroblasts and T-ALL, we sought to determine the status of Notch-1 signaling in prostate adenocarcinoma. Ligand induced Notch-1 signaling results in cleavage of Notch-1 at VAL1744. The VAL1744 epitope is only revealed upon ligand induced Notch-1 signaling [Brou et al., 2000; Mumm et al., 2000]. Paraffin embedded prostate sections from 16 clinical cases of prostate adenocarcinoma were subjected to IHC using and total Notch-1 (C-20, Upstate Biotechnology; Fig. 1A) and antibodies against cleaved Notch-1 (Cell Signaling; Fig. 1B). Immunoreactivity with the anti-Notch-1 VAL1744 antibody indicates the presence of activated Notch-1 signaling, whereas the total Notch-1 antibody is a measure of Notch expression, regardless of signaling status. Sections were also graded according to the Gleason scale. Basal epithelial cells exhibited strong Notch-1 expression (Fig. 1A, left) and Notch-1 signaling (Fig. 1B, left). Benign secretory cells exhibited weaker total Notch-1 expression (Fig. 1A, left) and Notch-1 signaling (Fig. 1B, left). However, adenocarcinoma foci were devoid of immunoreactive cleaved Notch-1 (Fig. 1B, right).

Expression of the basic-loop-helix transcription factor Hey-1 is increased by Notch-1 signal transduction. To further verify that prostate adenocarcinoma foci lack Notch-1 signaling transduction, Hey-1 expression was measured in prostate adenocarcinoma by IHC. These were the same 16 cases as described above. Consistent with our findings that prostate adenocarcinoma lacks measurable cleaved

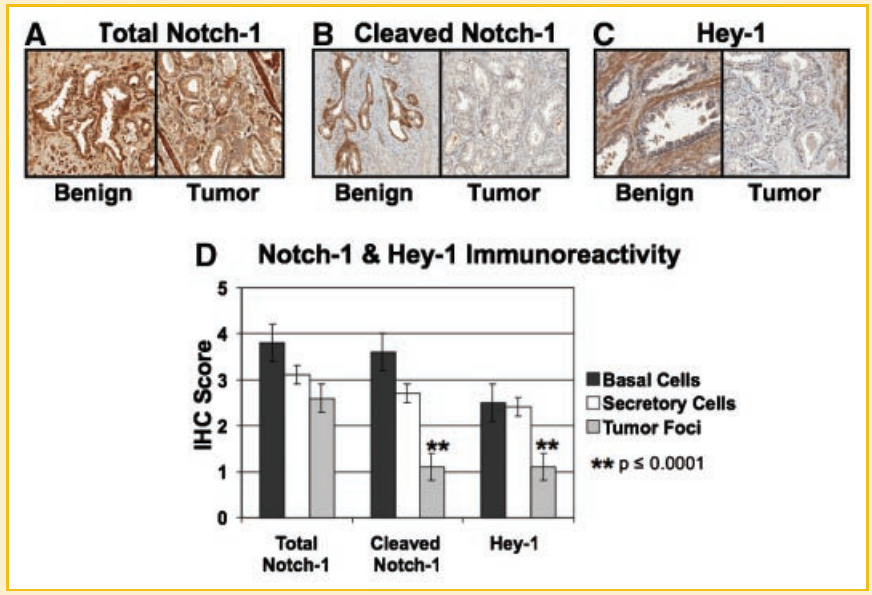

Fig. 1. Loss of cleaved Notch-1 and Hey-1 protein in prostate tumor foci. A: Total Notch-1. Representative photomicrograph of prostate sections stained with anti-Notch-1 antibody (C-20, Santa Cruz Biotechnology). B: Cleaved Notch-1. Representative prostate section stained for cleaved (activated) Notch-1 using the anti-VAL 1744 antibody (Cell Signaling). C: Hey-1 IHC. Representative IHC using anti-Hey-1 (Millipore). For each example (A–C) Benign (left) and tumor (right) are from the same section. Antibody staining is brown (DAB), nuclear stain is hematoxylin (light blue). D: Notch-1 and Hey-1 immunoreactivity. IHC slides from 16 prostate adenocarcinoma cases were scored as described in Materials and Methods Section. Plotted is the average score for the 16 cases and the standard deviation. Immunostaining is grouped into benign basal cells (black column), benign secretory cells (white column), and adenocarcinoma tumor foci (gray column). Loss of cleaved activated Notch-1 and Hey-1 immunoreactivity in tumor foci reached statistical significance with a *P*-value $\leq$0.0001 as compared with benign tissue.

Notch-1, indicating loss of Notch-1 signaling, adenocarcinoma foci had reduced levels of Hey-1 expression (Fig. 1C, right) as compared with surrounding benign tissue (Fig. 1C, left). These results confirm our observations above that Notch-1 signaling, at the level of loss of Notch-1 cleavage, is impaired in human prostate adenocarinoma.

Figure 1D summarizes the IHC immunoreactivity for the 16 clinical cases of prostate adenocarcinoma. While Notch-1 expression was slightly reduced in tumor foci as compared with benign basal and secretory cells, loss of immunoreactivity of cleaved Notch-1 and the Notch-1 target gene Hey-1 reach statistical significance ($P \leq 0.0001$). These data indicate that prostate adenocarcinoma and its surrounding microenvironment have a defect in Noch-1 signaling, resulting in a failure to generate constitutively active cleaved Notch-1 that is essential for Notch signal transduction.

DU145 PROSTATE TUMOR CELLS EXPRESSING CONSTITUTIVELY ACTIVE NOTCH-1 AND CBF-1

To examine Notch-pathway mediated regulation of PTEN in prostate tumors lacking functional p53, DU145 cells were retrovirally transduced with constitutively active Notch-1 (DU145/Notch-1), the empty vector MigR1 (DU145/MigR1), wild-type CBF-1 (DU145/CBF-1wt), or a mutated CBF-1 (DU145/CBF-1(R218H)) that bears a point mutation (R218H) in the DNA binding region. Notch expression in the DU145/Notch-1 cells was verified by Western blotting (Fig. 2A), as was V5-tagged CBF-1 expression in the DU145/CBF-1wt and DU145/CBF-1(R218H) cells (Fig. 2B). Notch-1 signal transduction in DU145/Notch-1 cells was confirmed using a Hes-1 promoter reporter (HES AB), which contains two CBF-1 binding sites fused upstream of the luciferase gene [Jarriault et al., 1995]. A reporter construct, HES ΔAB, in which the two CBF-1 binding sites are mutated was used as a negative control [Jarriault et al., 1995]. Expression of constitutively active Notch-1 in DU145 cells resulted in robust activation of the Hes-1 reporter (Fig. 2C), indicating that the activated Notch-1 retrovirus was capable of activating the Notch-1 pathway in DU145 prostate cells.

REDUCED MIGRATION IN THE PRESENCE OF CONSTITUTIVELY ACTIVE NOTCH-1

The growth characteristics of DU145/Notch-1, DU145/MigR1, and parental DU145 cells were studied by performing wounding assays. These experiments measure the migratory and proliferative abilities of cells to repopulate a cleared area in a confluent monolayer. DU145/Notch-1 cells exhibited a reduced ability to repopulate the wounded monolayers, as compared with DU145/MigR1 cells and parental DU145 cells (Fig. 3). Twenty-four hours post-wounding, the DU145/Notch-1 cells had only repopulated about 71% of the wound, whereas, the parental DU145 and DU145/MigR1 cells had repopulated 93% and 88%, respectively.

Proliferation assays were performed to determine if DU145/Notch-1 cells had lower proliferative rates, which would account for the decreased ability to repopulate the wounded area in the preceding experiments. As seen in Figure 3C, DU145/Notch-1 cells exhibited a subtle increase in proliferation as compared with parental DU145 cells or DU145/MigR1 cells that was not statistically significant. These data suggest that the inability of DU145/Notch-1

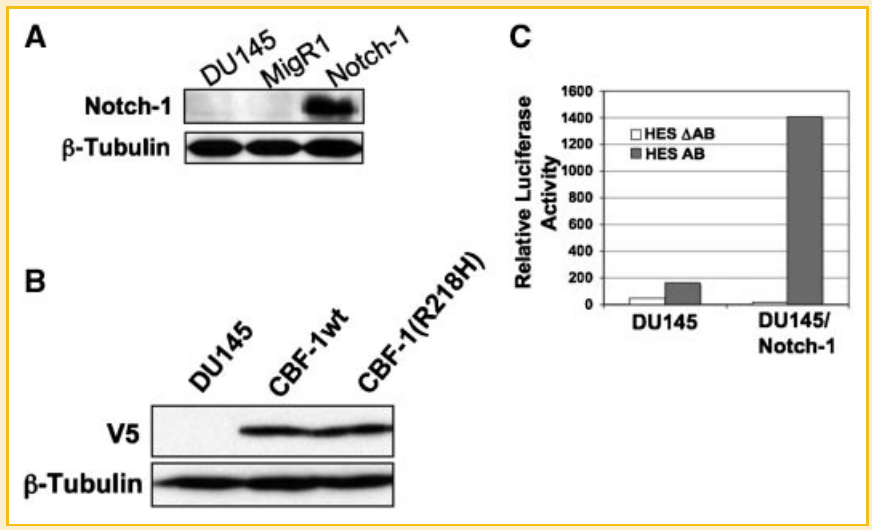

Fig. 2. DU145 cells expressing activated Notch-1. A: DU145 prostate tumor cells were transduced with a constitutively active Notch-1 retrovirus as described in Materials and Methods Section. Notch-1 expression was verified by Western blotting. DU145, parental DU145 cells; MigR1, DU145 cells transduced with an empty vector; Notch-1, DU145 transduced with the constitutively active intracytoplasmic Notch-1 retrovirus. B: DU145 cells were retrovirally transduced with the indicated CBF-1 construct and selected in the presence of neomycin. Whole cell lysates were analyzed by Western blotting to detect the V5 epitope tag to confirm CBF-1 construct expression. CBF-1wt, wild-type CBF-1 construct. CBF-1(R218H), mutated CBF-1 construct that has altered DNA binding capability. C: The Notch-1 signaling pathway is activated in DU145/Notch-1 cells. Parental DU145 or DU145/Notch-1 cells were transiently transfected with a CBF-1 reporter HES AB (shaded bars) or HES ΔAB (white bars), in which the CBF-1 binding site has been mutated. Cells were co-transfected with a vector expressing β-galactosidase as a normalization control. Data are shown as relative luciferase activity normalized to β-galactosidase expression.

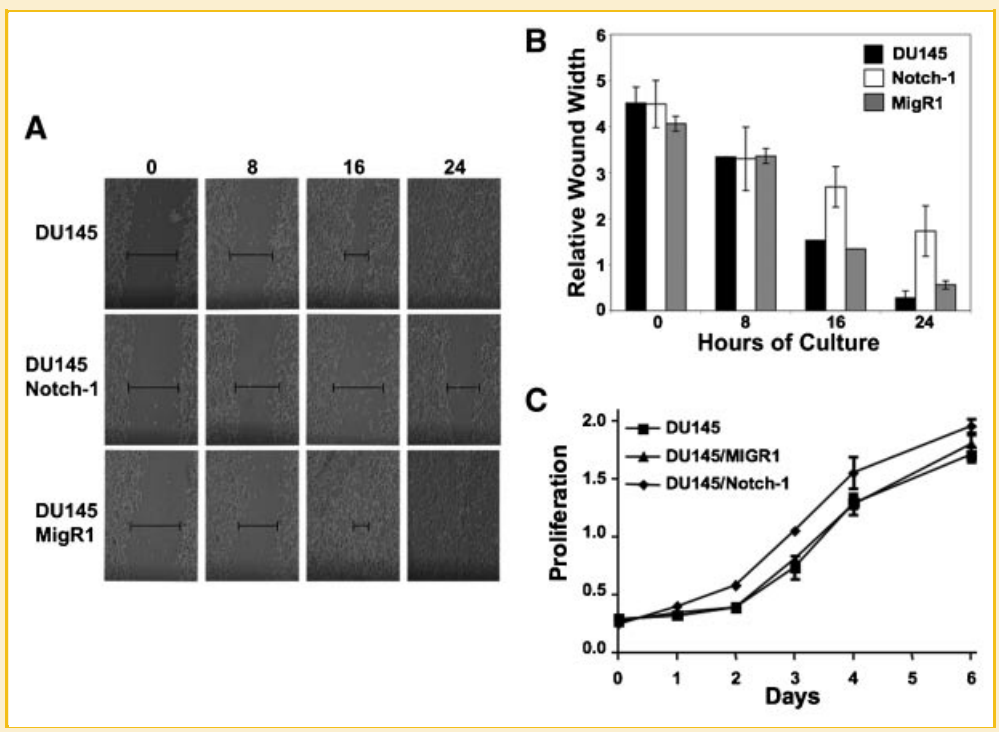

Fig. 3. Constitutively active Notch-1 inhibits wound repopulation. A: Representative photomicrographs of wounds in DU145, DU145/Notch-1, and DU145/MigR1 cells were taken at the indicated time points under phase contrast with a 10× objective. Parental DU145 cells (top row) and DU145/MigR1 cells (bottom row) repopulated the wound between 16 and 24 h. DU145/Notch-1 cells (middle row) still exhibited a visible wound following 24 h of culture. B: Wound width is plotted for each time point. DU145/Notch-1 cells (white bars); parental DU145 cells (black bars); DU145/MigR1 cells (gray bars) (N = 8). C: Parental DU145 cells (squares), DU145/MigR1 cells (triangles), and DU145/Notch-1 cells (diamonds) were cultured over a 6-day period and assayed daily via the MTT assay for cell proliferation.

cells to repopulate cleared areas (Fig. 3A,B) is likely a result of reduced migration and not reduced proliferation.

NOTCH-1 SIGNALING RESULTS IN INCREASED PTEN GENE EXPRESSION

RT-PCR was used to examine the effects of Notch-1 signaling on PTEN gene expression in parental DU145/MigR1 cells (empty vector), DU145/Notch-1 cells, DU145/CBF-1wt cells, and DU145/CBF-1(R218H) cells. As seen in Figure 4A, DU145/Notch-1 cells exhibited increased PTEN expression as compared with cells expressing an empty vector alone (DU145/MigR1). Prostate tumor cells overexpressing wild-type CBF-1 had PTEN gene expression levels less than that of DU145/Notch-1 cells, consistent with the role of CBF-1 as a repressor in the absence of Notch-1 signaling. In contrast, cells overexpressing CBF-1(R218H) exhibited PTEN gene expression levels similar to that seen in DU145/Notch-1 cells. This is consistent with the fact that in the DU145/CBF-1(R218H) cells, the mutated CBF-1 does not bind to DNA and thus cannot function as a repressor in the absence of Notch-1 signaling, and is likely sequestering co-factors necessary for PTEN repression away from the endogenous wild-type CBF-1.

To further verify that Notch-1 mediated increases in PTEN gene expression are a general feature of prostate tumor cells, 22Rv1 cells were transduced with the constitutively active Notch-1 retrovirus. 22Rv1 is a human prostate adenocarcinoma cell line that was established from a xenograft serially passaged in mice [Sramkoski et al., 1999]. 22Rv1 has wild-type PTEN and functional p53, although one p53 allele bears a point mutation. RT-PCR analysis revealed that PTEN gene expression was increased in 22Rv1 cells bearing constitutively active Notch-1 (Fig. 4B). These experiments corroborate our findings in the DU145 cell line.

NOTCH-1 SIGNALING RESULTS IN PTEN PROMOTER TRANSACTIVATION

Luciferase assays were conducted using a minimal PTEN promoter reporter to determine if Notch-1 signaling would transactivate the PTEN promoter. As seen in Figure 4C, DU145/Notch-1 cells exhibited increased transactivation of the 70 bp PTEN promoter, as compared with parental DU145 cells. These data indicate that Notch-1 regulates the PTEN gene directly through regulation of the PTEN promoter in prostate tumor cells.

CBF-1 BINDS TO THE MINIMAL PTEN PROMOTER

EMSA were performed to determine if CBF-1 binds to the minimal PTEN 70 bp promoter. A specific mobility shift (Fig. 5A, lane 1) that was competed with excess unlabeled probe (Fig. 5A, lane 3) was observed in DU145 cells stably transfected with V5-tagged wild-type CBF-1 (DU145/CBF-1wt). The addition of anti-V5

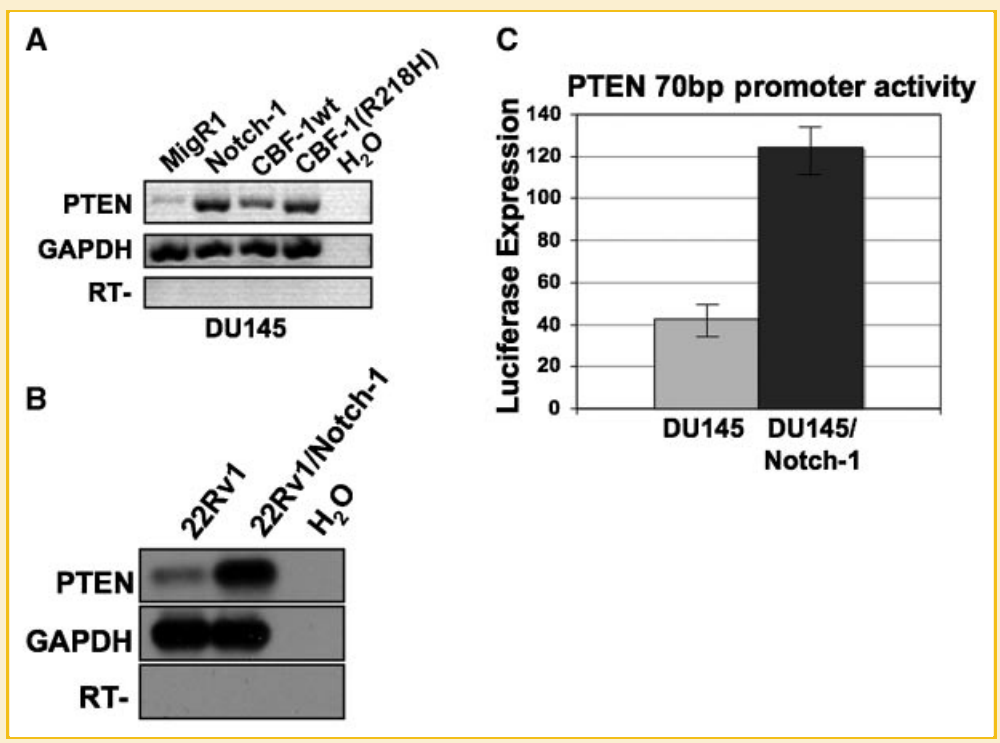

Fig. 4. PTEN gene expression is regulated by Notch-1 signaling. A: Expression of constitutively active Notch-1 results in increased PTEN gene expression. DU145 prostate tumor cells retrovirally transduced with the indicated retroviral vectors were examined by RT-PCR for PTEN expression. GAPDH was used as a normalization control. RT– means no reverse transcriptase. H_2O means a PCR reaction without template. Shown is the negative image of an ethidium bromide stained gel. B: Activated Notch-1 increases PTEN gene expression in 22Rv1 cells. 22Rv1 cells were stably transduced with a constitutively active Notch-1 retrovirus as described above. RT-PCR was performed to detect PTEN gene expression. PCR products were separated on a 1.5% agarose gel, followed by Southern blotting. GAPDH is a loading control. H_2O means no template and RT– means no reverse transcriptase. C: Notch-1 activation of the PTEN promoter. Parental DU145 and DU145/Notch-1 cells were transiently transfected with pGL-Basic or a minimal PTEN promoter reporter. Cells were cotransfected with a plasmid expressing β-galactosidase as a normalization control. Data are shown as normalized fold of 70 bp PTEN luciferase activity over normalized pGL2-Basic (a promoter-less control) luciferase activity.

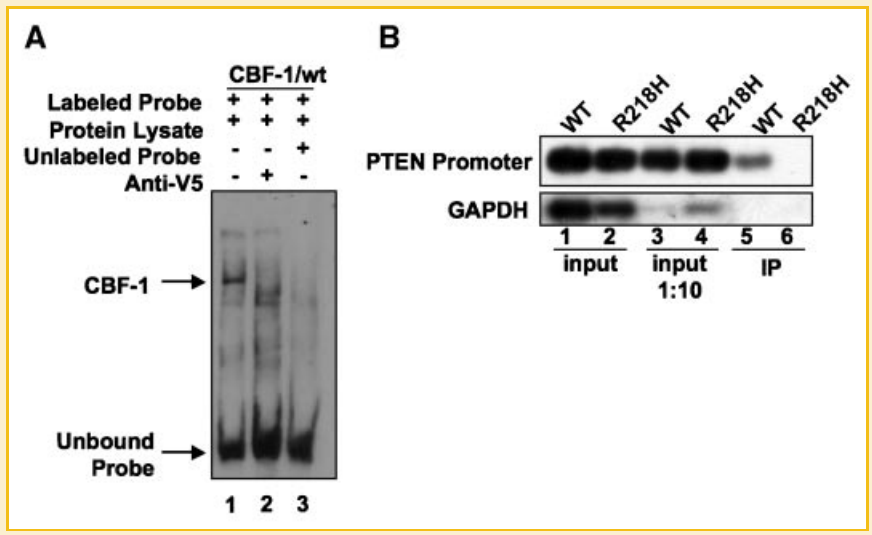

Fig. 5. CBF-1 binds to the PTEN promoter. A: EMSA analysis of CBF-1 binding to the PTEN promoter. EMSA analysis was performed using the DIG Gel Shift Kit, 2nd Generation as per instruction (Roche Diagnostics). The 70 bp minimal PTEN promoter sequence was labeled with digoxigenin and incubated with nuclear extract from DU145/CBF-1wt cells in the absence (lanes 1 and 2) or presence (lane 3) of excess unlabeled probe. Lane 2 also contained anti-V5 antibody that blocked CBF-1 binding to the shifted complex and provided confirmation that CBF-1 is in the mobility shift observed in lane 1. B: CBF-1 binds to the PTEN promoter in DU145 prostate tumor cells. ChIP assay for CBF-1 binding to the PTEN promoter. Lanes 5 and 6 were immunoprecipitated with anti-V5. Only DU145 cells expressing CBF-1wt exhibited immunoprecipitation of the PTEN promoter DNA (lane 5). DU145/CBF-1(R218H) cells that express CBF-1 with a mutated DNA binding site did not exhibit PTEN promoter DNA immunoprecipitation (lane 6). Lanes 1 and 2 are input DNA prior to immunoprecipitation and serve as positive control for PCR amplification. Lanes 3 and 4 are a 10-fold dilution of the input DNA. WT, DU145/CBF-1wt. R218H, DU145/CBF-1(R218H). Input means DNA prior to immunoprecipitation. IP means immunoprecipitated DNA. Amplification of GAPDH was performed as a negative control to confirm the specificity of the immunoprecipitation in that CBF-1 does not bind to the GAPDH gene, therefore no GAPDH should detected in the immunoprecipitated (IP) samples (lanes 5 and 6).

antibody to the binding reaction resulted in a less intense and a faster migrating mobility shift (Fig. 5A, lane 2). This indicated that CBF-1 was part of the protein complex binding to the PTEN promoter.

CBF-1 BINDS TO THE PTEN PROMOTER IN VIVO

Chromatin immunoprecipitation (CHIP) was performed to determine if CBF-1 binds to the PTEN promoter region in DU145 cells in vivo. For these experiments we made use of DU145/CBF-1wt and DU145/CBF-1(R218H) because these constructs have a V5 epitope tag fused to the expressed CBF-1 protein that permits immunoprecipitation with an anti-V5 antibody. As seen in Figure 5B, anti-V5 immunoprecipitation with lysates from DU145/CBF-1wt cells resulted in amplifiable material from the PTEN promoter (Fig. 5B, lane 5). In contrast, no PTEN promoter DNA was detected in anti-V5 immunoprecipitated DNA/protein complexes from the DU145/CBF-1(R218H) cells (Fig. 5B, lane 6). These cells bear CBF-1 that has been mutated in the DNA binding region resulting in a reduced capacity to bind the CBF-1 DNA sequence and thus served as a control to verify the specificity of the CHIP in lane 5. DU145/CBF-1wt and DU145/CBF-1(R218H) express similar levels of CBF-1wt or CBF-1(R218H) as seen in the Western blot in Figure 2B.

ENDOGENOUS NOTCH-1 SIGNALING RESULTS IN INCREASED PTEN GENE EXPRESSION

To test if activation of the endogenous Notch pathway in DU145 cells would result in increased PTEN gene expression, DU145 cells were incubated for 24 h in 96-well plates that had been coated with soluble Delta-1 ligand (Delta-1^{Ext-Ig}). Delta-1^{Ext-Ig} consists of the extracellular portion of Delta-1 fused to the Fc region of human IgG [Varnum-Finney et al., 2000] (kind gift of I. Bernstein, Fred Hutchinson Cancer Research Center). As seen in Figure 6, culture with increasing concentrations of Delta-1^{Ext-Ig} resulted in increased expression of the Notch pathway target gene Hes-1. In addition, PTEN gene expression also increased with increasing amounts of Delta-1^{Ext-Ig}. These experiments demonstrate that endogenous

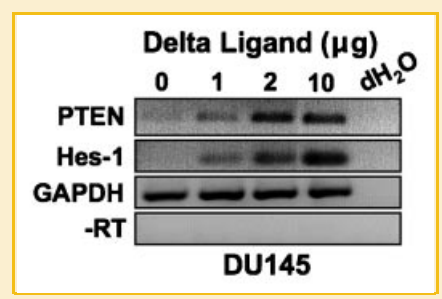

Fig. 6. Endogenous Notch signaling increases PTEN gene expression. Parental DU145 cells were cultured for 24 h on plates coated with increasing amounts of Delta-1^{Ext-Ig} ligand. PTEN, Hes-1, and GAPDH gene expression was analyzed by RT-PCR. PTEN gene expression increased with increasing amounts of ligand up to 2 µg. Hes-1 gene expression also showed a dose-dependent increase in response to ligand. The loading control GAPDH did not. RT− means no reverse transcriptase. dH_2O means no cDNA template. Shown is the negative image of ethidium bromide stained gels.

Notch signaling regulates PTEN gene expression similar to over-expression of constitutively active Notch-1.

DISCUSSION

Our findings indicate that Notch-1 regulates PTEN gene expression in prostate cancer cells and that Notch-1 signaling is aberrant in prostate adenocarcinoma. We report herein that regulation of PTEN gene transcription in the prostate is mediated by binding of the CBF-1 transcription factor to the PTEN minimal promoter. p53 has also been reported to regulate the PTEN gene [Stambolic et al., 2001]. DU145 cells lack functional p53 due to mutations in both p53 alleles that abrogate DNA binding [Isaacs et al., 1991]. Because several tumor types, including prostate tumors, bear p53 mutations, our observation that PTEN is regulated by Notch-1 in the absence of p53 is important when considering clinical applications for these findings. Notch-1 signaling has been reported to result in increased p53 function in a murine model of T-cell lymphoma [Beverly et al., 2005]. Our observations in the p53-deficient DU145 cell line permit us to rule out an important alternative hypothesis; that Notch-1 may be directly regulating p53, which in turn regulates PTEN.

We observed loss of PTEN expression in several prostate tumor samples that also exhibited loss of Notch-1 signaling (data not shown). However, it was not clear if loss of PTEN in those samples was due to loss of Notch-1 signaling or allelic loss of the PTEN gene. We also observed PTEN gene expression in some tumor foci that exhibited loss of Notch-1 signaling, illustrating that other factors such as p53 and c-myc also participate in regulation of PTEN gene expression.

Several studies point to a role for the Notch-1 pathway in normal prostatic development and in prostate cancer [Leong and Gao, 2008]. In a recent study of tumor samples from 154 men, the Notch ligand, Jagged-1, was overexpressed in metastatic prostate cancer as compared with localized and benign disease [Santagata et al., 2004]. That study, however, only examined expression of Jagged-1, and did not address the status of Notch-1 signaling in prostate tumors.

The Notch-targeted gene, Hey-1, has been shown to be excluded from the nucleus in prostate adenocarcinoma [Belandia et al., 2005]. In comparison, benign tissue exhibited nuclear localization of Hey-1. A separate study reported that the Hey-1 gene was down-regulated in prostatic adenocarcinoma [Wang et al., 2006]. Hey-1 is a direct target of the Notch-1 signaling, and our findings are consistent with these previous studies that involved Hey-1.

The mechanistic basis for loss of cleaved Notch-1 in prostate tumor foci is unknown at present. Preliminary IHC to detect the Notch-regulator, Numb [LeBorgne et al., 2005], indicated that Numb is expressed in both benign and malignant prostate [Fred E. Bertrand, unpublished work], as is the Numb regulator Musashi [Okano et al., 2002]. Alterations in Notch-ligand expression combined with altered glycosylation of Notch receptors could account for loss of Notch-1 cleavage. Fringe modified Notch-1 is more responsive to signaling through interactions with Delta [Haines and Irvine, 2003]. In those studies, it was proposed that both Delta and Jagged bind to fringe modified Notch-1, but that Jagged-1 does not bind with sufficient strength to effect signal transduction, and may in fact be inhibitory by competing with other ligands for the Notch-binding site [reviewed in D'Souza et al., 2008]. We and others have observed Delta expression in prostate tissue and cell lines [Wang et al., 2004; Fred E. Bertrand, data not shown]. Santagata et al. [2004], have reported over-expression of the Jagged-1 ligand in recurrent metastatic prostate cancer. In our model, Notch-1 in the prostate may have been modified by Fringe to be Delta responsive. High levels of Jagged-1 in prostate foci, block Delta from binding, and prevent further Notch-1 signaling. This, in turn, lowers PTEN expression. It has recently been shown that PTEN is required for genomic stability [Yin and Shen, 2008]. Thus, loss of Notch-1 signaling may contribute to the accumulation of genetic alterations that result in prostate cancer through reduced PTEN gene expression. We are presently testing this model to determine if the apparent Notch-pathway defect in prostate adenocarcinoma is at the level of ligand or Notch-1 glycosylation.

A recent article from Hafeez et al. [2009], describes increased expression of extracellular Notch-1 in prostate cancer samples. Using a model of late-stage metastatic disease, PC-3 cells, the authors go on to report that siRNA knockdown of Notch-1 inhibits the metalloprotease, MMP9, and invasion of PC-3 in in vitro assays. While the biological significance of increased surface Notch-1 receptor expression in the absence of data regarding signal transduction is not clear, the data presented by Hafeez et al. [2009] and our data herein illustrate the emerging complexity of Notch in the prostate.

The data presented herein focuses on a mechanistic pathway in which Notch-1 signaling in the prostate exerts it effects through transcriptional regulation of the PTEN tumor suppressor gene. It has been reported that constitutively active Notch-1 inhibited proliferation in the PTEN-null prostate tumor cell line, LNCAP [Leong and Gao, 2008 and reference therein]. This illustrates that Notch-1 likely has PTEN dependent and PTEN independent effects in prostate cells, although in prostate cancer homozygous deletion of PTEN is rare [Ozen and Pathak, 2000; Salmena et al., 2008].

Further complexity between Notch-1 and PTEN was revealed by studies from Palomero et al. [2007], in which Notch-1 signaling was reported to decrease PTEN expression in T-ALL. This was mediated by Hes-1 binding to the PTEN promoter and luciferase assays demonstrated that expression of Hes-1 would decrease PTEN promoter activation. The presence of c-myc appeared to reduce the inhibitory effect of Hes-1 on the PTEN promoter. In contrast, our data herein indicate that PTEN expression is increased through binding of CBF-1 to the PTEN promoter. These differences illustrate the complexity and likely the tissue/cell type specificity of PTEN gene regulation and Notch-1 signal transduction.

Our study herein provides, to our knowledge, the first direct evidence that diminished Notch-1 signal transduction is associated with adenocarcinoma in the human prostate. We have examined the functional status of Notch-1 signaling in prostate adenocarcinoma through specific IHC detection of Notch-1 cleaved at VAL1744. Our data point to a novel tumor suppressor pathway in the human prostate.

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