Altered Expression of Neurotensin Receptors Is Associated with the Differentiation State of Prostate Cancer

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Abstract

In prostate cancer, traditional treatments such as androgen response manipulation often provide only temporary resolution of disease, with emergence of a more aggressive, androgen-independent tumor following initial therapy. To treat recurrent disease, cell surface proteins that are specifically overexpressed on malignant cells may be useful for generating targeted therapeutics. Recent evidence suggests that neurotensin receptors (NTR) are recruited in advanced prostate cancer as an alternative growth pathway in the absence of androgens. In this study, we assessed the potential use of these receptors as targets by analyzing NTR expression patterns in human prostate cell lines and primary prostate tumor cell cultures derived from patient samples. In primary tumor cell cultures, NTR1 was upregulated in cells with a basal phenotype (cytokeratin 1/5/10/14+), whereas NTR2 and NTR3 were upregulated in cells with luminal phenotype (cytokeratin 18+). Similar patterns of NTR expression occurred in benign prostate tissue sections, implicating differentiation state as a basis for the differences observed in tumor cell lines. Our findings support the use of NTRs as tools for therapeutic targeting in prostate cancers composed of both poorly differentiated and/or well-differentiated cells. Cancer Res; 70(1); 347–56. ©2010 AACR.

Introduction

In human prostate cancer, differences in tumor heterogeneity, androgen dependence, differentiation state, and Gleason score argue strongly for an individually tailored treatment approach because eradication of the entire prostate tumor depends on the ability to target multiple cellular compartments, including epithelia (basal, neuroendocrine, and luminal cells) as well as stromal cells (1). Peptide receptors represent attractive candidates for therapeutic tumor targeting, particularly those that play a significant role in determining cell fate, and can be exploited in direct cell surface binding approaches via existing ligand interactions (2, 3).

Neurotensin receptors (NTR) are recruited in advanced prostate cancer as an alternative growth pathway in the absence of androgens. NTR1 and NTR2 are structurally similar cell surface G-protein–coupled receptors containing seven transmembrane domains (14). NTR1 is a high-affinity receptor (KD = 0.56 ± 0.1 nmol/L), whereas NTR2 binds with a lower affinity (KD = 3.7 ± 0.2 nmol/L; refs. 15, 16). NTR3 is a structurally distinct type 1 sorting receptor with a single transmembrane domain, predominantly present within intracellular compartments such as the trans-Golgi network (17). A minority (5–10%) of NTR3 is, however, expressed at the cell surface; this proportion varies relative to the rate of NTR1 surface turnover or external stimuli (18, 19).

To assess the potential application of NTRs as therapeutic targets for human prostate cancer, we have analyzed expression patterns of NTR1, NTR2, and NTR3 in a panel of prostate cell lines and in primary, patient tissue–derived epithelial cell cultures maintained at different stages of differentiation. NTR1 and NTR2 were overexpressed in malignant samples relative to benign controls, and a specific relationship between prostate epithelial differentiation and the expression pattern of NTR isoforms was also identified.

Materials and Methods

Cell culture. LNCaP, DU145, PNT1a, PNT2.C2, and BPH-1 cells were cultured in RPMI (Invitrogen) supplemented with 10% FCS (PAA) and 2 mmol/L L-glutamine (Invitrogen). PC-3 cells were maintained in Ham’s F-12 (Lonza) supplemented with 7% FCS and 2 mmol/L L-glutamine. P4E6 cells were...
cultured in keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with 2% FCS, 2 mmol/L L-glutamine, 5 ng/mL epidermal growth factor (EGF; Invitrogen), and 5 μg/mL bovine pituitary extract (Invitrogen). PC346C cells were maintained in a 1:1 mix of DMEM/Ham’s F-12 supplemented with 100 μg/mL streptomycin, 100 units/mL penicillin G, 2% FCS, 0.01% (w/v) bovine serum albumin, 10 ng/mL EGF, 1% (v/v) ITS-G, 0.1 nmol/L R1881, 1.4 μmol/L hydrocortisone, 1 nmol/L triiodothyronine, 0.1 mmol/L phosphatebuffered saline (PBS), 50 ng/mL cholera toxin, 0.1 μg/mL fibronectin, and 20 μg/mL futhan. Androgen response experiments were carried out by 24- or 48-h pre-culture in charcoal-stripped serum, followed by culture in medium supplemented with 10 nmol/L DHT or vehicle alone for 24 h. LNCap, PC-3, and DU145 cells were purchased from American Type Culture Collection (ATCC), whereas PNT1a, PNT2.C2, and P4E6 were generated and characterized in our own laboratory (20, 21). PC346C and BPH-1 cells were a gift from Guido Jenster (Erasmus University, Rotterdam MC, the Netherlands) and Simon Hayward (Vanderbilt University, Nashville, TN), respectively. Cells were handled under good laboratory practice conditions in defined passage windows, were monthly certified free of Mycoplasma, and were genotyped (using the ATCC-approved Powerplex 1.2 system [Promega]) to ensure authenticity.

Patient prostate tissue was taken with informed patient consent and ethical approval from the York Research Ethics Committee from patients undergoing trans-urethral resection of the prostate, cystectomy, or radical prostatectomy (Supplementary Table S1). Epithelial cells were isolated as follows: NTR1 FW, 5′-AGTGAAGGTTGGTACATACA-3′; NTR1 RV, 5′-CCAGAGTTAAAAGCAGCCCTG-3′; NTR2 FW, 5′-GATGATGAACCTTGTTG-3′; NTR2 RV, 5′-CAGCCGCTCTATCAATGT-3′; NTR3 FW, 5′-AGTGAATAAGATTGAGGTTCCTC-3′; PSA FW, 5′-TGTGTCTTCAAGGTTACGTCTAC-3′; PSA RV, 5′-CTGATCCCCCAACCATGGCCC-3′; GAPDH FW, 5′-AAGTGAAGGTTGGTACATACA-3′; GAPDH RV, 5′-CCAGAGTTAAAAGCAGCCCTG-3′; HPRT FW, 5′-GATGATGAACCTTGTTG-3′; HPRT RV, 5′-CAGCCGCTCTATCAATGT-3′. The concentration of input cDNA per primer pair was 10 ng for GAPDH/HPRT (and supplementary experiments) and 50 ng for NTR1/NTR2. Experiments were run in triplicate using an Applied Biosystems 7000 real-time PCR thermal cycler, and expression levels were standardized to GAPDH or HPRT.

**Quantitative reverse transcriptase-PCR analysis.** RNA was isolated using the GenElute Total Mammalian RNA extraction kit (Sigma) from cells cultured in KSFM or DH10. Total RNA (7.5 μg) was used as the template for cDNA reverse transcription. SYBR Green quantitative reverse transcriptase-PCR (RT-PCR) standard curves and primer efficiencies were initially optimized on two prostate cell lines, LNCap and PC-3 (data not shown). Primer sequences were as follows: NTR1 FW, 5′-ACGCGTCAGCTGTCACTACA-3′; NTR1 RV, 5′-ATGTTGTTCCAGGAGGAGTA-3′; NTR2 FW, 5′-AAGAAGACAATTCATCCAGGG-3′; NTR2 RV, 5′-GGACGTCTTATAGCTTACCC-3′; NTR3 FW, 5′-CAGCCGCTCTATCAATGT-3′; NTR3 RV, 5′-AGTGAATAAGATTGAGGTTCCTC-3′; PSA FW, 5′-TGTGTCTTCAAGGTTACGTCTAC-3′; PSA RV, 5′-CTGATCCCCCAACCATGGCCC-3′; GAPDH FW, 5′-AAGTGAAGGTTGGTACATACA-3′; GAPDH RV, 5′-CCAGAGTTAAAAGCAGCCCTG-3′; HPRT FW, 5′-GATGATGAACCTTGTTG-3′; HPRT RV, 5′-CAGCCGCTCTATCAATGT-3′. The concentration of input cDNA per primer pair was 10 ng for GAPDH/HPRT and supplementary experiments and 50 ng for NTR1/NTR2. Experiments were run in triplicate using an Applied Biosystems 7000 real-time PCR thermal cycler, and expression levels were standardized to GAPDH or HPRT.

**SDS-PAGE and Western blot analysis.** Whole-cell lysates from standardized numbers of cells were prepared by direct lysis in SDS sample buffer [10% glycerol, 62.5 mmol/L Tris-HCl (pH 6.8), 35 mmol/L SDS, 65 mmol/L DTT] containing Complete Protease Inhibitor (Roche), Biotin-labeled (Cell Signalling Tech) and Kaleidoscope (Bio-Rad) ladders were included for sizing. Samples were resolved on a 10% Tris-SDS gel and transferred onto an Immobilon-P membrane (Millipore) at 100 V for 2 h. Membranes were blocked for 1 h at room temperature in 5% milk (Marvel), and primary antibodies [NTR1 (Santa Cruz), NTR2 (Abcam), and NTR3 (Alomone)] were incubated overnight at 4°C. Further TBS-T washes were followed by a short blocking step in 2.5% Marvel Horseradish peroxidase (HRP)–conjugated secondary antibodies (Dako/Sigma) were added for 1 h at room temperature. TBS-T washes were done, followed by the addition of HRP substrate (Roche). Membranes were exposed to preflashed Hyperfilm ECL (GE Healthcare) and manually processed using developer and fixer solutions (GBX, Kodak). When reprobing with GAPDH antibody (Abcam) as an internal control, membranes were stripped in buffer [20 mmol/L Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 20 mmol/L DTT] for 30 min at 55°C. Semiquantitation of band densities relative to GAPDH was done using ImageJ software.

**Indirect immunohistochemistry.** Paraffin-embedded sections of benign prostate tissue were dewaxed in xylene and antigen retrieval was done in 0.01 mol/L sodium citrate (pH 6.0). Slides were rehydrated in TBS, followed by blocking with 5% serum. Primary antibodies were added for 1 h [NTR1 (Santa Cruz), NTR2 (Abcam), NTR3 (Alomone), negative isotype control (Sigma), and pan-cytokeratin (Sigma)] followed by TBS washes. Secondary biotin-conjugated antibodies (Dako) were added for 30 min followed by TBS washes. Streptavidin-HRP conjugates (Dako) were used as the secondary antibody.
incubated for 30 min, followed by TBS washes and the subsequent addition of 3,3′-diaminobenzidine substrate (Sigma). Slides were counterstained with Hematoxylin QS (Vector Labs), dehydrated, and mounted in DPX mounting medium (Sigma). Images were captured using a Nikon FXA microscope.

**Immunohistochemistry for confocal microscopy.** Cells were maintained in eight-well chamber slides in KSFM or DH10 for 4 d, then fixed in 4% paraformaldehyde (pH 7.4) for 10 min, permeabilized in 0.5% Triton X-100 for 10 min, and blocked for 1 h in 10% serum. Primary antibody staining [cytokeratin 18 (CK18; Sigma), NTR2 (Abcam), NTR3 (Alomone), and pan-cytokeratin (Sigma)] was done for 30 min in 10% serum. A secondary Alexa 488 antibody (Invitrogen) was added in 10% serum for 30 min in the dark. This was followed by the addition of 4′,6-diamidino-2-phenylindole (DAPI) for 1 min to enable nuclear visualization. Cells were overlaid with PBS and visualized using a Zeiss LSM 510 meta confocal microscope using equivalent exposures.

**Dual staining for flow cytometry.** Cells cultured in KSFM or DH10 were detached using 0.02% EDTA, fixed in 4% paraformaldehyde (pH 7.4) for 10 min, and permeabilized in 0.5% Triton X-100 for 10 min. Cells were blocked for 1 h in 10% serum. Primary antibody staining [CK18 (Sigma) plus NTR3 (Alomone) or GAPDH (Abcam)] was done for 30 min in 10% serum. Secondary antibodies [Alexa Fluor 488 or Alexa Fluor 680 (Invitrogen)] were added in 10% serum for 30 min in the dark. Cells were suspended in 0.02% EDTA (in PBS). Flow cytometric analysis was done in duplicate using a CyAn ADP, with 10,000 singlet events collected per sample.

**Results**

**Expression levels of NTR1 and NTR2 are increased in malignant compared with nonmalignant samples.** Analysis of NTR mRNA expression was done on a panel of malignant and nonmalignant prostate cell lines at the transcriptional and translational levels. These cell lines were chosen because they displayed a range of differentiation stages based on AR, prostate-specific antigen (PSA), and basal/luminal cytokeratin expression (25–27). In malignant cell lines, RT-PCR analysis showed high NTR1 expression in less differentiated (AR−) cell lines, PC-3, DU145, and P4E6, whereas NTR1 was detected at lower levels in nonmalignant AR+ cell lines, PNT1a, P4E6, PNT2.C2, and BPH-1 (Fig. 1A). NTR2 expression was detectable only in malignant well-differentiated (AR+) cell lines, LNCaP and PC346C (Fig. 1A), as two forms in the PCR fragments analyzed (the expected size of 429 bp and as a smaller fragment of 339 bp). The two amplified NTR2 gene products were further characterized through cloning and sequencing analysis; the smaller form of NTR2 lacked exon 3 and was a splice variant of the NTR2 gene (Supplementary Fig. S1). NTR3 was expressed in all malignant and nonmalignant cell lines at consistent levels relative to GAPDH.

From primary patient material, nine malignant and three benign epithelial cell samples were cultured in serum-free medium to maintain a basal, proliferating phenotype (22). RT-PCR analysis detected NTR1 expression in the majority (8 of 9) of malignant samples, whereas NTR2 was detectable in only a small number (4 of 9) of malignant samples under these conditions (Fig. 1B). In contrast, all benign samples expressed low or undetectable levels of NTR1 and NTR2 (Fig. 1B), whereas NTR3 was consistently expressed in both benign and malignant samples relative to GAPDH controls.

NTR protein levels were also analyzed by SDS-PAGE and Western blot analysis in two malignant prostate cell lines (LNCaP and PC-3) and two nonmalignant prostate cell lines (PNT1a and PNT2.C2). All tested cell lines expressed detectable levels of NTR1, NTR2, and NTR3 (Fig. 1C), with no demonstrable change in total protein expression between malignant and nonmalignant cell lines. NTR2 was detected in both monomeric and dimeric forms in all cell lines; these dimers were maintained in the presence of urea, suggesting that quaternary structure was maintained by strong covalent bonds (data not shown). Interestingly, in PNT1a, monomeric NTR2 was almost absent, whereas the dimer was expressed at levels consistent with other cell lines. Levels of NTR3 were relatively consistent among all cell lines except PNT2.C2, where expression was elevated (Fig. 1C). There were some inconsistencies in cell line data between RNA and protein expression despite several repetitions.

**Patterns of NTR expression in basal and luminal primary cell phenotypes.** To functionally show a relationship between cellular differentiation and NTR expression, monolayer cultures of primary epithelial cells with either basal or luminal phenotypes were generated through the use of different culture conditions (23). In serum-free medium (KSFM), cells were maintained in a basal, proliferating state and were positive for high molecular weight basal cytokeratins CK1, CK5, CK10, and CK14 (data not shown). In medium high in calcium, serum, and androgens (DH10), a luminal phenotype was induced, and cells expressed higher levels of CK18 (data not shown).

Both basic RT-PCR and SYBR Green quantitative RT-PCR were done to analyze changes in NTR expression in basal (KSFM) or luminal (DH10) cells originating from two malignant patient samples (patients 13 and 14). In both patient samples, RT-PCR analysis showed a clearly higher level of NTR1 expression in epithelial cells cultured in KSFM compared with cells cultured in DH10 (Fig. 2A). However, changes in NTR2 and NTR3 expression were ambiguous. Thus, quantitative RT-PCR was done for these genes, relative to a GAPDH control, for further quantitative analysis. Quantitative RT-PCR results showed a statistically significant overexpression of NTR2 (P < 0.05) and NTR3 (P < 0.001) in DH10-cultured epithelial cells in both patient samples (Fig. 2B). Whereas there was some patient variability in the fold upregulation for each gene, NTR3 was upregulated to the highest degree (8- to 12-fold) and NTR2 was upregulated at 3- to 10-fold.

Western blot analysis of NTR expression done on matched samples of total protein cell lysates derived from patient 13...
confirmed that the changes in NTR expression seen at the mRNA level in basal- or luminal-like cells were conserved at the protein level (Fig. 2C). These changes were quantified relative to GAPDH controls, showing a 1.71-fold upregulation of dimerized NTR1 protein (90 kDa) in KSFM-cultured cells (data not shown). NTR2 was detected in both monomeric and dimeric forms. The dimeric form was detectable as two bands of 95 and 100 kDa; in DH10-cultured cells, an additional smaller (80-kDa) form was also evident (Fig. 2C). Dimeric NTR2 was more highly expressed (2-fold) in DH10-cultured cells, whereas NTR3 was 1.83-fold overexpressed in DH10-cultured cells (data not shown). The fold change at the protein level was not as large as that seen at the mRNA level, which may be due to translational inefficiency or protein instability.

**Luminal cells expressing high levels of CK18 also express the highest levels of NTR3.** Immunocytochemistry was done in primary cells cultured in KSFM or DH10. Cells derived from patient 13 were analyzed by confocal microscopy for CK18 or NTR3 expression. Cells cultured in KSFM expressed a low background level of CK18, whereas cells cultured in DH10 exhibited a small population (∼30% within the field of view) that were highly CK18 positive, indicative of luminal differentiation (Fig. 3A). In KSFM-cultured cells, NTR3 was expressed at the cell surface and throughout the cytoplasm (Fig. 3A). However, in DH10-cultured cells, not only was NTR3 more highly expressed but also its location of expression became concentrated within perinuclear compartments (Fig. 3A).

Dual immunocytochemistry for both CK18 together with NTR3, or GAPDH as a control, was then done in cells derived from patient 15 and analyzed by flow cytometry, confirming that KSFM-cultured cells were homogeneous with regards to

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**Figure 1.** NTR expression analysis in prostate cell lines and primary epithelial cell cultures. **A** and **B,** RT-PCR analysis of NTR expression was carried out in five malignant (lanes 1–5; LNCaP, PC-3, DU145, PC346C, and P4E6) and three nonmalignant (lanes 6–8; PNT1a, PNT2.C2, and BPH-1) prostate cell lines (A) and in three benign (patients and lanes 1–3) and nine malignant (patients and lanes 4–12) primary patient epithelial cell samples (B). Patient details can be found in Supplementary Table S1. For primary samples, positive controls were PC-3 (NTR1) or LNCaP (NTR2 and NTR3) cell lines. Images have been color inverted to enable clearer visualization. **C,** Western blot analysis of NTR1, NTR2, and NTR3 protein expression was done in two malignant (LNCaP and PC-3) and two nonmalignant (PNT1a and PNT2.C2) prostate cell lines. Total protein lysates derived from equivalent numbers of cells were loaded per cell line. In all experiments, expression of GAPDH was analyzed as a control.
CK18, NTR3, and GAPDH expression, as evidenced by a tightly concentrated, clustered population (Fig. 3B). Cells cultured in DH10 represented a heterogeneous population, with a greater range of CK18 and NTR3 expression levels representative of basal, early-intermediate, and intermediate-late phenotypes (Fig. 3B). A comparison of NTR3 expression levels in the major cell population in KSFM versus DH10 cultures confirmed a >4-fold higher expression in DH10-cultured compared with KSFM-cultured cells (mean values of 234.23 ± 45.73 compared with 52.46 ± 0.06, respectively; Supplementary Table S2). Conversely, GAPDH was expressed at comparable levels in DH10-cultured (192.83 ± 5.91) and KSFM-cultured (181.97 ± 8.93) cells (Supplementary Table S2).

Furthermore, within the heterogeneous DH10 epithelial cell culture, a distinct cell population (14.67%) was identified that expressed the highest level of mean CK18 fluorescence (749.78 ± 57.03; Fig. 3B; Supplementary Table S2). This population represented the most differentiated phenotype and was found to express more than 2-fold higher levels of NTR3 protein compared with the major population (486.5 ± 167.96 compared with 234.23 ± 45.73). Therefore, the cells expressing the highest levels of CK18 within DH10 cultures also expressed the highest levels of NTR3. GAPDH controls showed an increased level of expression within the minor population (11.5%) of most differentiated cells, but this change was not of the same magnitude as that seen for NTR3 (Fig. 3B; Supplementary Table S2).

**Differentiation-related changes in NTR expression in bilayer primary models of differentiation.** Bilayer models of primary epithelial cell differentiation were generated with cells derived from patient 16 or 17, and levels of NTR expression analyzed via immunocytochemistry. Results showed that in the basal cell layer, NTR2 was expressed at a moderate intensity and was diffusely distributed throughout the cytoplasm (Fig. 4A). However, in the
luminal cell layer, NTR2 was expressed in an intense, punctate pattern. NTR3 was expressed at a low level within the basal cell layer and was diffusely distributed throughout the cytoplasm (Fig. 4B). However, in the luminal cell layer, NTR3 was more highly expressed and concentrated in a perinuclear location, indicating potential trans-Golgi network localization. Conversely, pan-cytokeratin staining was similar in both basal and luminal cell layers (Fig. 4C). Negative and secondary antibody-only controls showed minimal nonspecific fluorescence, which was mainly localized to keratinized cellular protrusions (Fig. 4D).

**NTR expression in benign patient tissue sections.** Tissue sections of benign prostate glands, which retain distinctive architecture, were next analyzed to identify whether the basal-luminal segregation of NTR expression was a unique feature of prostate tumorigenesis or existed as a functional difference in the normal prostate gland. Paraffin-embedded benign tissue sections (from patient 18) stained for NTRs compared with pan-cytokeratin positive controls.

NTR1 was expressed in an evenly distributed pattern within both the basal and luminal epithelial compartments, similar to the positive control, pan-cytokeratin, which also showed uniform distribution throughout basal and luminal epithelia (Fig. 5A and B). NTR1 was also detected in the prostate stroma. NTR2 was expressed in both basal and luminal epithelial compartments but was highly concentrated around the apical cell surface of luminal cells (Fig. 5C). Similarly, NTR3 was expressed in both the basal and luminal compartments but was concentrated within the luminal cell layer, particularly at the extremity of the apical cell surface (Fig. 5D). Negative isotype controls did not show staining...
apart from a nonspecific signal associated with blood vessels (Supplementary Fig. S2).

Discussion

Changes in NTR expression associated with prostate tumorigenesis and androgen independence have previously been reported in a small number of well-characterized cell lines (5, 6, 9, 24). In this study, initial experiments analyzed the expression of NTRs in both poorly differentiated (AR−) and well-differentiated (AR+) malignant and benign prostate cell lines. In malignant cell lines, NTR1 expression was confined to poorly differentiated cell lines, in agreement with previous findings from other groups (5, 24), and was expressed at the highest level in malignant cell lines. NTR2 was detected only in well-differentiated malignant cell lines, in two splice variant forms. NTR2 splice variants have previously been described in the mouse and rat (28, 29), but this was the first known observation in human cells. NTR3 was detected in all cell lines, implying that NTR3 has an essential role in cell survival and function and may be intimately involved with growth responses. The lack of correlation between mRNA and protein levels in cell lines is likely to be a function of the complex interactions involved, precluding an obvious linear relationship between mRNA levels and translation rate (30, 31). In patient-derived, nonimmortalized epithelial cell cultures, NTR1 and NTR2 were more highly expressed at the mRNA level in malignant samples compared with benign equivalents. These results, as well as the strong body of evidence in the literature, firmly implicate the neurotensin/NTR pathway in prostate cancer growth and progression (5, 6, 8, 9, 32, 33).

A potential relationship between NTR expression and cellular differentiation has not been previously documented in

Figure 4. Expression of NTR2 and NTR3 in bilayer epithelial cultures. Malignant epithelial cells derived from patient 16 or 17 were used to generate bilayer cultures. A and B, cells from patient 16 were stained using antibodies against NTR2 and NTR3 bound to Alexa Fluor 488; C, cells from patient 17 were stained for pan-cytokeratin (bound to Alexa Fluor 488) as a positive control. D, negative and secondary antibody-only controls (patient 16) are also shown. Z-stack confocal microscopy was used to image through basal layers (at a z-distance of 6.11 μm from the bottom position) and luminal layers (at a z-distance of 18.33 μm from the bottom position). All samples except the negative control were counterstained with DAPI to enable nuclear visualization. Images were taken at ×200 magnification. Bar, 50 μm. Arrows, areas of strong protein localization.
the prostate gland. To further test this relationship, primary epithelial cells were cultured in different media to generate basal-like or luminal-like cell phenotypes, with the hypothesis that changes in the expression level of specific NTR subtypes could be driven by inducing cellular differentiation. At both the mRNA and protein levels, NTR1 was more highly expressed in basal-like cells, whereas NTR2 and NTR3 were more highly expressed in luminal-like cells. Bilayer primary epithelial cell cultures provided further evidence of an overexpression of NTR2 and NTR3 in luminal compared with basal cells. In particular, NTR3 became concentrated in a perinuclear location, indicating potential trans-Golgi network localization in luminal cells. Basal cells do not express AR (34) and thus may require an alternative growth stimulation signal to proliferate. In this context, overexpression of the high-affinity NTR1 may supply this mitogenic stimulus. Of particular interest was the identification of differentiation-specific changes in NTR1 and NTR2 expression only at the dimeric level. For NTR2, three similarly sized bands at ~90 kDa were detected, which may reflect differences in levels of posttranslational modification or correspond to the presence of two species of dimer, NTR2 homodimers and NTR2/NTR1 heterodimers, which have previously been described in the rat (35). This implied that cells were resistant to changes in the levels of cell surface–bound NTR2 dimer, and that the majority of NTR2 was maintained in the long term as a dimer, in agreement with Perron and colleagues (36).

Analysis of benign tissue sections confirmed that a differentiation-specific pattern of NTR expression also existed in the normal prostate gland, implying that during prostate carcinogenesis, this relationship may be enhanced and corrupted and that the previous observations were not culture induced.

The differentiation-related changes in NTR expression we observed could be indicative of direct androgen regulation of NTR expression. To test this hypothesis, we carried out different in silico searches for androgen response elements in all three NTR genes (detailed in Supplementary Table S3), but these failed to find evidence of either full palindromic or curtailed consensus sequences (37, 38). The direct androgen responsiveness of NTR transcription was also tested under androgen stimulation. No significant changes in NTR expression were observed, whereas PSA was upregulated (16-fold) as expected (Supplementary Tables S3 and S4). Control genes HPRT and GAPDH were unchanged by androgen treatment. Thus, we concluded that differences in NTR expression in different prostate epithelial compartments are due to the overall differentiated state (basal versus luminal), which may be induced by androgens, but that NTR expression was not directly androgen influenced. This is not unexpected, given that NTR expression is seen in other non-AR-expressing tissues.

The presence of high levels of NTR1 in basal-like cells and the transition to high levels of NTR2 and NTR3 in luminal-like cells suggest that NTRs represent excellent targets for the eradication of multiple cellular compartments within the heterogeneous prostate tumor. These differentiation-specific changes in NTR expression were consistent in independent patient epithelial cell cultures and were not AR dependent. Future experiments will focus on further characterization of NTR expression patterns in the prostate stroma because carcinogenic stromal factors play a role in prostate tumor growth and progression, and secretion of neurotensin ligand from nerve terminals located in the prostate stroma has been reported (39). Finally, given the cancer-specific upregulation of NTRs, the generation of prostate-specific

![Figure 5](http://example.com/figure5.png)
immunotherapeutics and gene therapy vectors directed toward the NTRs should be feasible.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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