A Human- and Male-Specific Protocadherin that Acts through the Wnt Signaling Pathway to Induce Neuroendocrine Transdifferentiation of Prostate Cancer Cells

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Abstract
Protocadherin-PC (PCDH-PC) is a gene on the human Y chromosome that is selectively expressed in apoptosis- and hormone-resistant human prostate cancer cells. The protein encoded by PCDH-PC is cytoplasmically localized and has a small serine-rich domain in its COOH terminus that is homologous to the β-catenin binding site of classical cadherins. Variants of prostate cancer cells that express PCDH-PC have high levels of nuclear β-catenin protein and increased wnt-signaling. In this study, we show that transfection of human prostate cancer cells (LNCaP) with PCDH-PC or culture of these cells in androgen-free medium (a condition that up-regulates PCDH-PC expression) activates wnt signaling as assessed by nuclear accumulation of β-catenin, increased expression of luciferase from a reporter vector promoted by Tcf binding elements and increased expression of wnt target genes. Moreover, LNCaP cells transfected with PCDH-PC or grown in androgen-free medium transdifferentiate to neuroendocrine-like cells marked by elevated expression of neuron-specific enolase and chromogranin-A. Neuroendocrine transdifferentiation was also observed when LNCaP cells were transfected by stabilized β-catenin. Increased wnt signaling and neuroendocrine transdifferentiation of LNCaP cells induced by culture in androgen-free medium was suppressed by short interfering RNAs that target PCDH-PC as well as by dominant-negative Tcf or short interfering RNA against β-catenin, supporting the hypothesis that increased expression of PCDH-PC is driving neuroendocrine transdifferentiation by activating wnt signaling. These findings have significant implications for the process through which prostate cancers progress to hormone resistance in humans. (Cancer Res 2005; 65(12): 5263-71)

Introduction
Prostate cancer develops and progresses under the influence of androgenic steroids. This influence is consistent with the use of androgen depletion therapies to treat patients with advanced disease. Androgen depletion provides palliative relief and clinical trial has proven that it extends life span even though the extension is only a matter of months (1). The transient effectiveness of androgen depletion therapy for patients with prostate cancer is believed to be based upon its ability to suppress proliferation and to induce apoptosis of a fraction of tumor cells (2). Inevitably, however, prostate tumor cells that survive androgen depletion therapy progress to an androgen-refractory state and it is these androgen-insensitive tumor cells that account for the morbidity and mortality of advanced disease.

Efforts to identify the molecular basis for androgen insensitivity of prostate cancer often focus on the androgen receptor gene and gene products (3–5). Hormone-refractory prostate tumors contain cancerous cells with hyperactive androgen signaling. Sometimes, this is associated with the presence of mutations in the androgen receptor gene (that make androgen receptors promiscuous with regards to its ability to bind alternate ligands) or with amplification of the androgen receptor gene (that increases basal expression of androgen receptors). Experimental studies show that androgen receptors of prostate cancer cells can gain ligand-independent activity in the presence of certain coactivators (6, 7) or through mitogen-activated cell-signaling activities (6, 8–10). These latter mechanisms have not yet been sufficiently translated to the human situation to identify the frequency with which perturbations are found in hormone-refractory tumors. Alternatively, given the belief that hormone therapies act by inducing apoptosis of prostate cancer cells, there are reasons to question whether hormone-refractory prostate cancer cells might have defects in their ability to undergo apoptosis in response to androgen-depletion. Bcl-2 expression is frequently up-regulated in hormone-refractory prostate cancers and elevated bcl-2 expression can confer androgen-insensitivity to prostate cancer cells that are normally androgen-sensitive (11–13). Other perturbations of apoptotic pathway regulators found in hormone-insensitive prostate cancer cells include up-regulated nuclear factor κB- and Akt-signaling (14, 15), either of which can contribute to apoptosis resistance.

In an attempt to identify other gene products that might be associated with the acquisition of apoptosis- and hormone-resistance by prostate cancer cells, we had previously developed a model cell system to study this phenomenon by transiently exposing a human androgen–sensitive cell line, LNCaP, to stimuli that induced apoptosis of a majority of these cells (16). By expanding the surviving populations and repeating the exposure/ expansion of survivor paradigm several times, we created two variant cell lines, LNCaP-TR and LNCaP-SSR, that were resistant to apoptosis when compared with parental LNCaP cells. These variants were also found to be androgen-insensitive when tested for their ability to form tumor xenografts in castrated male immunodeficient mice. Use of a comparative genetic screening technique allowed us to identify a gene product that was selectively expressed in the apoptosis-resistant variants but not in parental
LCaP cells (16). The sequence of the major transcript (4.5 kb) of this gene product revealed that it is a unique member of the protocadherin gene family encoded on the human Y-chromosome (at Yp11.2; ref. 17) and because of its association with human prostate cancer, we have named it protocadherin-PC (PCDH-PC). Growth of parental LNCaP cells in medium free of androgens or castration of male mice bearing LNCaP xenograft tumors also induces expression of PCDH-PC (16).

PCDH-PC was evolutionarily derived from a homologue on the human X-chromosome (PCDHX found at Xq21.3) that was duplicated and translocated to the Y-chromosome during the transition from higher primates to humans (17). The coding region of PCDH-PC (also referred to as PCDHY) shares extensive sequence homology with PCDHX. Aside from occasional nucleotide differences throughout the coding region, PCDH-PC has a deletion of a contiguous 13 bp sequence (present in exon 4 of the X-linked gene) as well as complete deletion of three potential exons within PCDHX (7, 8 and 8A, as defined in Blanco-Arias, et al. 18). The 13 bp deletion in PCDH-PC has important consequences for the polypeptide(s) encoded by this gene. This deletion results in a major transcript with an AUG codon embedded within a strong Kozak consensus sequence that preferentially translates to a protocadherin polypeptide lacking a signal sequence (16, 17). This is consistent with the finding that an antibody against PCDH-PC recognizes a protein of appropriate molecular weight that fractionates with the cytoplasm of LNCaP-TR and -SSR cells (16). Thus, the major protein encoded by PCDH-PC is predominantly localized in the cytoplasm rather than membrane bound, as with other members of the cadherin gene family.

Another novel aspect of PCDH-PC is the presence of a small serine-rich domain within the COOH terminus that is homologous to the β-catenin binding domains found in classical cadherins (E-, P-, and N-cadherin; ref. 16). Immunoprecipitation of PCDH-PC from LNCaP-TR and -SSR cell extracts coprecipitated β-catenin, supporting the potential for functional interaction of these two proteins. Moreover, LNCaP variants that express PCDH-PC had anomalies in their intracellular β-catenin distribution (LNCaP-SSR and -TR) that had β-catenin in cytoplasmic and nuclear fractions, whereas LNCaP cells have β-catenin strictly localized in the membrane) and this was consistent with the ability to show enhanced luciferase expression in apoptosis-resistant LNCaP variants using a Tcf-promoted luciferase reporter vector (19). Collectively, these preliminary data support the hypothesis that PCDH-PC encodes a cytoplasmic protein that interacts with β-catenin and induces signaling through the wnt pathway mediated by nuclear accumulation of β-catenin and enhanced transcription from Tcf/LEF-1 binding elements on DNA. This also suggests that the apoptosis-resistant phenotype of LNCaP variants that express PCDH-PC might be related to its ability to stimulate wnt signaling, especially because wnt signaling can induce apoptosis-resistance in other tumor systems (20, 21).

Our previous studies were based on experimentally-derived LNCaP variants that happen to express PCDH-PC, but we also showed previously that transfection of parental LNCaP cells with PCDH-PC increased their apoptosis-resistance and conferred hormone-resistance as evidenced by their ability to form tumors in castrated male immunodeficient mice (16, 22). Finally, studies of clinical specimens of prostate cancer also show that PCDH-PC expression is frequently up-regulated in hormone-resistant prostate tumors (22), supporting the idea that PCDH-PC expression is associated with the development of human hormone-refractory prostate cancer. In the work reported here, we further explore the hypothesis that PCDH-PC expression stimulates wnt signaling in prostate cancer cells by examining for biomarkers of wnt signaling in LNCaP and other human cancer cells that are transiently transfected with PCDH-PC. In the conduct of these studies, we also noted a change in the differentiatation of PCDH-PC transfected prostate cancer cells that has led us to study whether this gene product and its actions on the wnt signaling pathway might also be involved in a transdifferentiation process in which prostate cancer cells acquire phenotypic characteristics of neuroendocrine-like cells. The results of this study have many implications for the progression of human prostate cancer to the advanced or hormone-refractory stage.

Materials and Methods

Cell lines. Human prostate cancer cells, LNCaP, DU145, CWR22rv-1, and PC-3 were obtained from American Type Culture Collection (Manassas, VA) as was the human colon cancer cell line, HCT116. LNCaP and DU145 cells were maintained in RPMI 1640, PC-3 cells in F12K, and HCT116 cells in DMEM medium. All media were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin unless noted. For androgen-free conditions, LNCaP cells were cultured in phenol red–free RPMI containing 10% charcoal-stripped FBS (CS-FBS; ref. 23). Other additives include dibutylyl cyclic AMP (db-cAMP; 1 mmol/L, Sigma Chemical Company, St. Louis, MO), interleukin-6 (IL-6; 50 ng/mL, Upstate Biotechnology, Inc., Lake Placid, NY), or SS-398 (5 mmol/L, Cayman Chemical Co., Ann Arbor, MI) as noted.

Expression vectors, transfection protocols, and luciferase/β-galactosidase assays. PCDH-PC cDNA was inserted into the mammalian expression vectors pcDNA3 (Invitrogen Life Technologies, Inc., Carlsbad, CA) or into pCMV-myc (BD Biosciences, Clontech, Inc., Palo Alto, CA) so that the PCDH-PC product generated from this vector (pPCDH-PC-myc) had a COOH-terminal myc tag. An expression vector containing cDNA encoding a mutated (stabilized) form of human β-catenin (24) was obtained courtesy of Dr. Frank McCormick (Department of Surgery, UCSF, San Francisco, CA). A dominant-negative Tcf expression vector (25) was obtained courtesy of Dr. C.Y. Wang (Department of Biological and Materials Science, University of Michigan, Ann Arbor, MI). The luciferase reporter vectors, pTOP-FLASH and pTOP-FLASH and cytomegalovirus (CMV)-promoted β-galactosidase expression vector were obtained from Upstate Biotechnology. Transfections for RNA or protein extractions were done in 35 cm2 dishes with cells plated 12 hours prior. Aliquots of plasmid (6 μg) were mixed with LipofectAMINE 2000 (Invitrogen Life Technologies) in antibiotic- and serum-free medium and were applied to the cultures. Transfections used for measurement of luciferase and β-galactosidase activities were done in 12-well plates and aliquots of luciferase reporter vectors were mixed with 100 ng of a CMV-promoted β-galactosidase expression vector so that all wells received 1 μg of DNA mixed with LipofectAMINE 2000, as above. Medium was changed after 4 hours to serum-containing medium without antibiotics for the remainder of the 48-hour transfection. Luciferase activity in cell extracts were measured using the luciferase assay system of Promega, Inc. (Madison, WI), β-Galactosidase activity was measured in these same cell extracts using the β-galactosidase enzyme assay system of Promega. All experiments involving luciferase reporter vectors were done in triplicate for each point.

Short interfering RNAs and transfection of cultured human prostate cancer cells. Short interfering RNA (siRNA) targeting human β-catenin and lamin A were purchased from Dharmacon, Inc. (Chicago, IL). Three different siRNAs targeting PCDH-PC were designed using the siRNA Target Finder software program available through Ambion, Inc. (Austin, TX). The anti-PCDH-PC siRNAs targeted sequences at positions 3040 to 3060 (#181), 3217 to 3237 (#190), or 3342 to 3362 (#208) on the PCDH-PC transcript. The anti-PCDH-PC siRNAs targeted sequences at positions 3040 to 3060 (#181), 3217 to 3237 (#190), or 3342 to 3362 (#208) on the PCDH-PC transcript.

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Protein extraction and nuclear isolation procedures. Monolayer cultures were washed and scraped into cold PBS and were pelleted by low-speed centrifugation. Cell pellets were extracted in radioimmunoprecipitation assay buffer (13). Radioimmunoprecipitation assay buffer extracts were centrifuged at 10,000 × g to remove debris. For nuclear isolation, monolayers of 5 × 10^6 cells were washed twice in cold PBS and were scraped into buffer containing 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 1 mmol/L DTT, 10 mmol/L EDTA, and 0.4% polyoxyethylene nonyl phenol (IGEPAL) with 1× protease inhibitor cocktail (Sigma). Cell suspensions were maintained on ice on a rocking platform for 10 minutes and were centrifuged at 15,000 × g for 3 minutes at 4°C. Pellets were suspended in 150 μL of 20 mmol/L HEPES (pH 7.9), 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT with protease inhibitor, and were vortexed. These suspensions were maintained on ice on a rocking platform for 2 hours and insoluble debris was removed by centrifugation at 15,000 × g for 5 minutes. Aliquots of whole cell and nuclear extracts were assayed for protein using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA).

Western blot analysis. Aliquots of cell extracts containing equal amounts of protein were electrophoresed on 10% polyacrylamide gels and proteins in the gel were electrotransferred to PVDF filters. Antibodies used in Western blot analyses were mouse monoclonals obtained from Dako, Inc., Carpinteria, CA (anti-neuron-specific enolase and anti-chromogranin A), Santa Cruz Biotechnology, Inc., Santa Cruz, CA (anti-β-catenin and anti-lamin A/C), or Sigma (anti-β-actin). Antibody dilutions were done according to the manufacturer's recommendations and primary antibody binding was detected using horseradish peroxidase-labeled secondary goat anti-mouse antibody (Santa Cruz Biotechnology). Chemiluminescent detection of secondary antibody was done using Luminol reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibody dilutions were done in 5× blocking buffer (GE Healthcare, Piscataway, NJ). The mRNAs were converted to biotin-16-dUTP-labeled cDNA using the Gene Array Labeling Kit (Superarray Biosciences, Inc., Frederick, MD). The miRNAs were converted to biotin-16-dUTP-labeled cDNA using the GE Array Amplification Kit (Superarray Biosciences). Labeled cDNAs were hybridized to human whole-target gene cDNA microarrays (GE array Q series) from Superarray Biosciences overnight and hybridization was detected using the Gene Array Chemiluminescence Detection Kit (Superarray Biosciences) followed by exposure to Kodak XAR-5 film. All microarrays were processed in batch and exposed on the same film. Films were scanned and analyzed using the Gene Array Analysis Software, Scanalyze, and results from different experimental paradigms were compared with a control array that was hybridized to LNCaP cDNA using the software program Gene Array Analyzer of Superarray Biosciences. Confirmation of increased expression of c-myc, Cox-2, and wnt-7b mRNAs in PCDH-PC-transfected LNCaP cells was done by multicycle RT-PCR using the following primers: (c-myc) forward, 5'-CTCCTGGCAAAAAGGTCAAG-3'; reverse, 5'-AGCTTTTGCTCCTCTGTGG-3'; (Cox-2) forward, 5'-GGAGGATAGCATCTCTGCCT-3'; reverse, 5'-CGATTTGAAAAGATTGGTGTTGGA-3'; (wnt-7b) forward, 5'-TGCTGCTGAGGTCTCAGATT-3'; reverse, 5'-AATCTGGCTCATTGCGCC-3'; reverse, 5'-AAGGATTTGAGCATCTC-3'; (Cox-2) forward, 5'-ATGGATTGGAACAAAGTGATGAG-3'; reverse, 5'-AGAAAACTTGCAGATGTCAG-3' transiently transfected at 94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 4 minutes for 28 cycles). Equal aliquots of PCR reaction products were electrophoresed on agarose gels and were visualized following ethidium bromide staining under UV light.

Results

PCDH-PC expression up-regulates Wnt signaling in prostate and other cancer cell lines. The end point of the canonical Wnt signaling pathway is marked by increased nuclear accumulation of β-catenin protein and increased expression of gene products that are transcriptionally regulated by the Tcf family of transcription factors (25). In order to confirm that Wnt signaling is modulated by expression of PCDH-PC, we first tested whether growth of LNCaP cells in androgen-free medium, a condition that induces expression of PCDH-PC, or direct transfection of LNCaP cells with PCDH-PC increased nuclear β-catenin. Nuclear fractions of parental LNCaP cells or LNCaP cells transfected with an empty plasmid (pcMV-myc) did not have detectable β-catenin (Fig. 1A). However, both PCDH-PC transfected LNCaP cells and LNCaP cells maintained for 10 days in androgen-free medium had high levels of β-catenin in their nuclear fractions (Fig. 1A). Activation of Wnt signaling in androgen-depleted LNCaP cells or in PCDH-PC transfected human prostate (LNCaP, DU145, CWR22r-v1) or colon cancer (HCT116) cells was also ascertained by measurement of normalized luciferase expression from a Tcf-sensitive reporter vector (pTOP-FLASH). Luciferase expression was significantly higher in LNCaP cells in androgen-depleted medium compared with LNCaP cells in normal medium (Fig. 1B). Likewise, luciferase expression was significantly higher in all cancer cell lines cotransfected with PCDH-PC than when transfected with empty vector (pcDNA3; Fig. 1B and C). Luciferase expression from the pTOP-FLASH reporter vector (promoted by mutated, nonfunctional, Tcf binding sites) was not changed by growth of LNCaP cells in androgen-free medium or by cotransfection with the PCDH-PC expression vector (data not shown). Then we used commercially prepared, targeted human Wnt-pathway cDNA microarrays to assess whether Wnt target genes were up-regulated by transfection with PCDH-PC or culture of LNCaP cells in androgen-free medium. The targeted microarray used contains spots for 37 different cDNAs of known canonical Wnt-targets (genes regulated by the Tcf/LEF-1 transcription factor), four gene products referred to as non-canonical Wnt targets (up-regulated in association with a change in cellular Ca^2+ ion metabolism induced by Wnt signaling) as well as 65 other gene products representing proteins potentially involved in the Wnt signaling process. Individual arrays were hybridized to labeled cDNA prepared from control LNCaP cells or from LNCaP cells cultured in androgen-free medium for 10 days as well as to cDNA from LNCaP cells transfected with the PCDH-PC expression vector or a stabilized β-catenin expression vector for 48 hours. Expression patterns on the test arrays were then compared with the control array (hybridized to LNCaP cDNA) to identify differences in gene expression associated with the experimental conditions. From the results of these analyses (Table 1), we found that 18 of 37 known canonical Wnt target genes on the array were up-regulated by at least 2-fold or greater under both test conditions involving increased expression of PCDH-PC (cultured in androgen-free medium or transfected with PCDH-PC). Most of these same genes (with the exception of five, as indicated by the values in boldface in Table 1) were also up-regulated to a similar extent by PCDH-PC and Neuroendocrine Differentiation of Prostate Cancer
transfection with stabilized β-catenin. The remaining 19 canonical Tcf-regulated gene cDNAs on the array were not significantly up-regulated under any of the test conditions. Two of the non-canonical wnt target genes (iNOS and COLIA1) on the array were also up-regulated 2-fold or greater under all three test conditions. Finally, we noted that these three experimental conditions also induced numerous gene products that are mechanistically involved in the wnt signaling process (exemplified by several different wnts and frizzled receptors; Table 1) supporting the concept that wnt signaling might have a feedback loop in prostate cancer cells that further influences wnt signaling. In a further effort to validate the findings of this cDNA microarray analyses, we conducted a semiquantitative RT-PCR analysis on cDNA prepared from parental LNCaP cells from LNCaP cells transiently transfected with PCDH-PC using primers specific for small regions of the human c-myc, Cox-2, and wnt-7b genes (Fig. 1D). This assay was done with three different cycles (24, 28, and 32 cycles) for each primer set and the results were similar for each condition, showing increased levels of PCR product in the cDNA from PCDH-PC-transfected cells.

PCDH-PC expression is also associated with transdifferentiation of prostate cancer cells to a neuroendocrine cell–like phenotype. Culture of LNCaP cells in medium depleted of androgens up-regulates expression of PCDH-PC (16) and this condition is also associated with a transdifferentiation process in which these cells acquire morphologic and other phenotypic characteristics of neuroendocrine-like cells (23). Culture of LNCaP cells in medium supplemented with db-cAMP, IL-6, or NS-398, a selective Cox-2 inhibitor, also induce neuroendocrine transdifferentiation (27–29). The coincidence of these two events led us to examine whether other neuroendocrine transdifferentiation inducer agents might also up-regulate PCDH-PC expression.

Figure 1. PCDH-PC expression increases wnt-mediated signaling in prostate and other cancer cells. A, comparative Western blot analysis of β-catenin protein in nuclear extracts from control (untransfected) and pCMV-myc (empty vector) transfection LNCaP cells or from LNCaP cells maintained for 7 days in androgen-free medium or transfected for 48 hours with a PCDH-PC expression vector (top) or for lamin A/C (bottom) loading control shows that nuclear β-catenin is only detected in cells that express PCDH-PC. B, β-galactosidase-normalized luciferase activity in LNCaP cells subsequent to 48-hour transfection with the Tcf-sensitive pTOP reporter vector. Left, cells maintained for 7 days in normal medium (FBS) or 7 days in androgen-free medium (CS-FBS); right, cells transfected for 48 hours with empty vector (pcDNA3) or PCDH-PC expression vector (pPCDH-PC) as indicated. Columns, mean; bars, SE. C, β-galactosidase normalized luciferase activity in pTOP transfected DU145 (left), CWR22rv-1 (middle), or HCT116 cells (right) cotransfected with empty vector (pcDNA3) or PCDH-PC as indicated. Columns, mean; bars, SE. D, semiquantitative PCR for wnt-7b, Cox-2, c-myc, or actin expression using cDNAs from control (untransfected) LNCaP cells or from PCDH-PC-transfected LNCaP cells.
Western blot survey of proteins from control LNCaP cells or cells cultured in androgen-free medium (10 days) or in normal medium supplemented with 1 mmol/L db-cAMP, 50 ng/mL IL-6, or 5 mmol/L NS-398 (5 days) showed that the expression of neuron-specific enolase and chromogranin-A proteins, two prominent biomarkers of neuroendocrine transdifferentiation were highly up-regulated in each of these conditions (Fig. 2A), as expected based on previous reports. When a second set of cultures treated with these same conditions were extracted for RNA and the RNAs were analyzed by RT-PCR for expression of PCDH-PC, all neuroendocrine transdifferentiated cells had highly up-regulated expression of PCDH-PC mRNA (Fig. 2B). Thus, up-regulated expression of PCDH-PC seems to accompany neuroendocrine transdifferentiation of LNCaP cells induced by a wide variety of stimuli. More significantly, direct transient transfection of LNCaP cells with stabilized β-catenin was sufficient to induce neuroendocrine transdifferentiation. Results of transient transfection of LNCaP cells shown in Fig. 2C confirms that β-catenin transfection is also an efficient inducer of neuron-specific enolase and chromogranin-A expression and supports the idea that increased wnt signaling associated with PCDH-PC expression is involved in neuroendocrine transdifferentiation process of LNCaP cells.

Suppression of PCDH-PC expression blocks increased Wnt signaling and neuroendocrine transdifferentiation of LNCaP cells grown in androgen-free medium. In order to confirm the relationship between PCDH-PC expression and neuroendocrine differentiation of LNCaP cells, we designed and tested three different siRNAs that target unique regions of the PCDH-PC transcript. The design of these siRNAs avoided potential homologies with cadherin box or transmembrane domain sequences. When these siRNAs were cotransfected into LNCaP cells along with the myc-tagged PCDH-PC expression vector, they strongly suppressed expression of PCDH-PC protein, whereas cotransfection of the PCDH-PC expression vector with siRNA targeting the lamin gene product did not suppress expression of PCDH-PC protein (Fig. 3A). Expression of E-cadherin was unaffected by any of the

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<th>Androgen-free medium (fold-increase)</th>
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<td>19.7</td>
</tr>
<tr>
<td>Wnt-10A</td>
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<td>8.9</td>
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<tr>
<td>Wnt-7B</td>
<td>−</td>
<td>5.7</td>
<td>12.3</td>
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PCDH-PC siRNAs (Fig. 3A). Likewise, the siRNAs strongly suppress expression of PCDH-PC mRNA when LNCaP cells are grown in androgen-free medium (Fig. 4A). PCDH-PC siRNAs blocked the ability of PCDH-PC transfection to induce neuron-specific enolase (Fig. 3B), consistent with the idea that suppression of PCDH-PC expression prevents neuroendocrine transdifferentiation. PCDH-PC siRNA also suppresses the ability of exposure to androgen-free medium to induce Wnt signaling in LNCaP cells (Fig. 4B).

Finally, all three PCDH-PC siRNAs strongly suppressed the induction of neuron-specific enolase protein expression in LNCaP cells cultured in androgen-free medium, whereas siRNA against human lamin did not influence neuron-specific enolase expression (Fig. 4C).

Suppression of Wnt-signaling blocks neuroendocrine transdifferentiation of prostate cancer cells induced by PCDH-PC expression. In further tests to prove that activation of the Wnt signaling pathway is critical to the action of PCDH-PC in inducing neuroendocrine transdifferentiation, we evaluated whether suppression of Wnt signaling was sufficient to suppress neuroendocrine transdifferentiation induced by PCDH-PC in transfected or androgen-free LNCaP cells. As shown in Fig. 5A, cotransfection of PCDH-PC and DN-Tcf or β-catenin and DN-Tcf strongly suppressed neuron-specific enolase expression that was induced by PCDH-PC or β-catenin transfection alone. A siRNA that targets human β-catenin and was shown to reduce endogenous β-catenin protein expression in LNCaP cells by 95% (as evaluated by densitometry of the Western blot in Fig. 5B) also significantly reduces neuron-specific enolase expression (Fig. 5C) in PCDH-PC-transfected cells.

Figure 2. PCDH-PC expression is associated with neuroendocrine transdifferentiation of prostate cancer cells. A. LNCaP cells were grown in normal medium (control), androgen-free medium (CS-FBS), or in normal medium supplemented with db-cAMP, IL-6, or NS-398. Western blot of protein extracts were probed with antibody against human neuron-specific enolase (top), human chromogranin-A (middle), or human β-actin (bottom). B, the same cells were extracted for RNAs that were converted to cDNA and subject to PCR for 32 cycles with primers for human β-actin (upper band) or for PCDH-PC (lower band). PCR products from each reaction were mixed together and electrophoresed on an agarose gel that was stained with ethidium bromide and visualized under UV light. C, LNCaP cells grown in normal medium (control LNCaP) or in androgen-free medium (CS-FBS LNCaP) or transfected for 48 hours with empty vector (pCMV-myc), PCDH-PC expression vector or mutant, stabilized β-catenin expression vector were extracted for protein. A Western blot made from these extracts was probed for expression of neuron-specific enolase (top), human chromogranin A (middle), or β-actin (bottom). D, PC-3 cells were transfected for 48 hours with empty vector (pCMV-myc) or with PCDH-PC expression vector and a Western blot made from protein extracts of these cells were probed for expression of neuron-specific enolase (top) or β-actin expression (bottom).

Figure 3. siRNAs against PCDH-PC suppress PCDH-PC expression and neuroendocrine transdifferentiation of LNCaP cells. A. LNCaP cells were transfected with the pPCDH-PC-myc expression vector and were cotransfected with siRNA against human lamin or siRNAs 181, 190, or 208 designed to suppress PCDH-PC expression. A Western blot made against protein extracts from these cells were probed with anti-myc (top) to identify expression of the 110 kDa PCDH myc-tagged protein, anti-β-actin (middle), or anti-human E-cadherin (bottom). B, a repeated experiment that includes control LNCaP cells (no transfection) or pCMV-PCDH-myc transfected LNCaP cells cotransfected with siRNAs against lamin or against PCDH-PC (181, 190, or 208). The Western blot was probed for neuron-specific enolase expression (top) or β-actin expression (bottom).

Figure 5. Coculture of PCDH-PC-expressing cells with LNCaP cells enhances the induction of neuron-specific enolase. A. LNCaP cells were grown in androgen-free medium for 48 hours with or without transfection with pPCDH-PC-myc expression vector, and were either mock-treated or treated with 10 nM db-cAMP, 10 ng/ml IL-6, or 50 μM NS-398. Western blot of protein extracts were probed with antibody against human neuron-specific enolase (top), human chromogranin-A (middle), or human β-actin (bottom).
whereas siRNA against lamin did not affect the ability of PCDH-PC transfection to induce neuron-specific enolase expression. Likewise, transfection of LNCaP cells with siRNA against β-catenin strongly suppressed the ability of culture in androgen-free conditions (CS-FBS) to induce neuron-specific enolase expression (Fig. 5D). Collectively, these data indicate that β-catenin/Tcf-mediated transcription is critical for neuroendocrine transdifferentiation of LNCaP cells induced by PCDH-PC or by culture in androgen-free conditions and implicates wnt signaling as the common mediating factor in neuroendocrine transdifferentiation of prostate cancer cells associated with PCDH-PC expression.

**Figure 4.** siRNAs against PCDH-PC suppress PCDH-PC expression, Tcf-mediated transcription and neuroendocrine transdifferentiation in LNCaP cells grown in androgen-free medium. A. RNAs extracted from LNCaP cells grown in normal medium (Control) or in androgen-free medium (CS-FBS, None) were compared with RNAs from LNCaP cells grown in androgen-free medium transfected with siRNAs against PCDH-PC (181, 190, and 208), or siRNA against lamin by RT-PCR using primers specific for PCDH-PC (top) or β-actin (bottom). PCR reaction products were electrophoresed on agarose gels and were visualized after ethidium bromide staining under UV light. B. LNCaP cells cultured in normal medium (Control) were compared with LNCaP cells grown in androgen-free medium for 7 days without or with transfection with siRNA 181 against PCDH-PC or siRNA against lamin for expression of luciferase from the Tcf-sensitive reporter pTOP for normalized luciferase activity.

**Discussion**

The present work confirms our hypothesis, based on previous observations using naturally selected prostate cancer cell lines (16), that expression of the PCDH-PC protein is associated with up-regulation of wnt signaling in prostate and other human cancer cells. This is shown by our finding that PCDH-PC expression in prostate cancer cell lines (either subsequent to transient transfection with a PCDH-PC expression vector or subsequent to growth of an androgen-sensitive prostate cancer cell line in medium depleted of androgens) leads to nuclear accumulation of β-catenin, increased expression of a luciferase reporter from a Tcf-sensitive promoter and increased expression of wnt-target genes such as c-myc, cyclin D, c-ret, and Cox-2. We do not yet know the mechanism through which PCDH-PC expression induces wnt signaling in prostate cancer cells although we have proposed that PCDH-PC may enable β-catenin, an end molecule of the wnt signaling pathway, to escape the degradative process that regulates its access to the nucleus. Previously, we described a homologous domain within the COOH-terminal region of PCDH-PC and the β-catenin binding sites of classical cadherins and we showed that PCDH-PC co-immunoprecipitates with β-catenin suggesting that there is a functional interaction of these molecules. We also must consider that our wnt gene-specific microarray analysis indicates that PCDH-PC up-regulates upstream wnt signaling molecules (exemplified by Wnt-3, -7B, -10A, and -11) that are not known to be promoted by Tcf transcription factors. Whereas we interpret this finding as supportive of the idea that activation of wnt signaling in prostate cancer cells has a positive-feedback effect, it is also possible that PCDH-PC expression acts primarily to increase the expression of these upstream wnt signaling molecules through some unknown mechanism. Regardless, the finding that PCDH-PC expression in prostate cancer cells is also associated with up-regulated expression of frizzled ligands raises the possibility that prostate cancer cells that express PCDH-PC might gain paracrine wnt signaling activity that can influence surrounding prostate cancer cells. Finally, our studies also indicate that PCDH-PC expression and wnt signaling are also linked to a process in which prostate cancer cells transdifferentiate to a neuroendocrine-like state. This aspect of PCDH-PC also has many implications for the pathway through which prostate cancers might progress to a hormone- and apoptosis-insensitive state.

Like other visceral tissues in the human body, the adult prostate gland contains a small fraction of neuroendocrine cells dispersed throughout the epithelial cell layer (30). These cells have a unique morphology that includes the presence of a large number of dense intracellular secretory granules that store a diverse collection of neurosecretory peptides (exemplified by bombesin, calcitonin, parathyroid-like hormone, serotonin, and proadrenomedullin) that have the potential to influence the growth and survival of other epithelial cells in which they are interspersed. Regardless of their role in normal prostate gland biology, a growing body of literature strongly suggests that these neuroendocrine cells play some role in the biology of prostate cancer progression, especially in the process through which advanced prostate cancer progresses to hormone independence following hormonal therapy. Some prostate cancer patients are diagnosed with homogenous neuroendocrine cell tumors (referred to as small cell carcinoma of the prostate; ref. 31). Although this type of prostate cancer is relatively rare, the prognosis for these patients is poor as the tumors are highly metastatic and poorly responsive to therapies. However, even the
far more common form of prostate cancer, adenocarcinoma of the prostate, shows clinical evidence for the potential influence of neuroendocrine cells. Like the normal epithelium, prostate adenocarcinomas often have neuroendocrine-like cells interspersed among the malignant cells (32). Attempts to quantify the presence of neuroendocrine cells within surgically resected prostate tumors and to correlate neuroendocrine cell populations with clinical variables such as stage, grade, or disease-free survival are controversial; there have been studies that have found such associations (33–35), but just as many as that have not (36–38). However, neuroendocrine cells tend to be regionally clustered within foci of prostate tumors (39, 40). Therefore, the task of correlating prostate tumor characteristics with neuroendocrine cell populations is complicated by the irregular distribution of the cells and tumor sampling limitations may be a reason these studies are so conflicted.

With regards to hormone-refractory prostate cancer, however, there is much more agreement. Studies consistently show that neuroendocrine tumor and serum biomarkers are up-regulated following hormonal therapy of prostate cancer patients (41–43) suggesting that neuroendocrine cells in the tumor are increased by treatment or that tumor cells are increasingly taking on characteristics of neuroendocrine cells. The latter conclusion is consistent with research showing that prostate cancer cells can directly undergo neuroendocrine transdifferentiation (23, 27–29). Whereas the significance of neuroendocrine transdifferentiation for prostate cancer patients is not totally clear, a recent study showing that neuroendocrine differentiated prostate cancer cells xenografted into one flank of a castrated mouse enables tumor growth from androgen-dependent cancer cells xenografted into the opposing flank implies that neuroendocrine-differentiated prostate cancer cells release systemic factors (likely neuropeptide hormones) that support growth of androgen-dependent tumor cells at a distant site (44). This finding greatly raises the value of understanding the neuroendocrine differentiation process of prostate cancer cells.

The results showing that siRNAs against PCDH-PC suppress neuroendocrine transdifferentiation of LNCaP cells in androgen-free medium directly identifies a role for PCDH-PC expression in neuroendocrine transdifferentiation. Other results showing that suppression of wnt signaling (by DN-Tcf or siRNA against β-catenin) blocks neuroendocrine transdifferentiation of LNCaP cells in androgen-free medium or transfected by PCDH-PC supports the idea that neuroendocrine transdifferentiation of prostate cancer cells is driven by increased wnt signaling associated with PCDH-PC expression. It is remarkable then to consider that aberrant wnt signaling is involved in several prominent human cancers (colon and breast cancer as well as melanoma) and that wnt signaling is also important for normal differentiation processes including those of the neural crest derivative cells and bone, muscle, and kidney (25, 45, 46). The fact that wnt targets include gene products involved in promotion of cell proliferation as well as differentiation, a process that is often associated with suppression of proliferation, presents a conundrum with regards to determining the significance of wnt signal activation in any given cell type. However, the results that we have

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**Figure 5.**

A, dominant negative Tcf suppresses the ability of PCDH-PC to induce neuroendocrine transdifferentiation. LNCaP cells were cotransfected with pCMV-myc (empty vector), pPCDH-PC-myc, β-catenin, or pDN-Tcf, as indicated for 48 hours. Protein extracts were analyzed by comparative Western blotting for expression of neuron-specific enolase (top) or expression of β-actin (bottom). B, siRNA against β-catenin suppresses β-catenin (top), but not β-actin (bottom) expression in LNCaP cells. C, siRNA against β-catenin suppresses the ability of PCDH-PC expression to induce neuroendocrine transdifferentiation of LNCaP cells. Comparative Western blot analysis of untransfected (Control) LNCaP cells or cells transfected with pPCDH-PC-myc and no siRNA or with siRNA against β-catenin or lamin for expression of neuron-specific enolase (top) or expression of β-actin (bottom). D, comparative Western blot analysis of LNCaP cells grown in normal medium (Control LNCaP) or in androgen-free medium (CS-FBS) for 7 days without (no siRNA) or with transfection with siRNA against β-catenin or lamin for expression of neuron-specific enolase (top) or β-actin (bottom).
obtained here suggests that activation of the wnt signaling pathway via increased PCDH-C expression in hormonally-derived prostate cancer cells has the potential to significantly alter the biological properties of these cells in a manner that increases their potential for aggressiveness in treated prostate cancer patients. Analyses of human prostate tumors have already identified the presence of (wnt) activating mutations in β-catenin in a relatively small proportion of the tumors analyzed (47, 48). Clinical studies citing evidence of more widespread nuclear β-catenin and wnt signaling in hormone-refractory prostate cancers (19, 49, 50) cannot account for the small proportion of prostate tumors with mutated β-catenin. PCDH-C expression associated with hormone therapies may be another mechanism through which wnt is activated in prostate cancer. This consideration is consistent with the concept that novel therapeutic agents that suppress PCDH-C expression or wnt signaling activation might have value in treatment of advanced prostate cancer.

Acknowledgments

Received 1/18/2005; revised 3/17/2005; accepted 4/1/2005.

Grant support: Supported by grants from the DOD Prostate Cancer Research Program (DAMD17-98-68011), the TJ. Martell Foundation, New York, NY (R. Buttayan), and from the Association de Recherche contre le Cancer, Association pour la Recherche sur les Tumeurs de la Prostate, France (D.K. Chopin). X. Yang is an Irwin White Fellow for Prostate Cancer Research. D.L. Bemis is the Charles Royce Fellow.

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References

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