Analysis of Wnt Gene Expression in Prostate Cancer: Mutual Inhibition by WNT11 and the Androgen Receptor

Hanneng Zhu, Michal Mazor, Yoshiaki Kawano, et al.

Cancer Res 2004;64:7918-7926.

[CANCER RESEARCH 64, 7918–7926, November 1, 2004]

Analysis of Wnt Gene Expression in Prostate Cancer: Mutual Inhibition by WNT11 and the Androgen Receptor

Hanneng Zhu,[1] Michal Mazor,[1] Yoshiaki Kawano,[1] Marjorie M. Walker,[2] Hing Y. Leung,[3] Kelly Armstrong,[3] Jonathan Waxman,[1] and Robert M. Kypta[1]

[1]Prostate Cancer Research Group, Department of Cancer Cell Biology, Division of Medicine, Imperial College London; [2]Department of Histopathology, Division of Investigative Science, Imperial College London; and [3]School of Surgical and Reproductive Sciences, Newcastle University, Newcastle upon Tyne, United Kingdom

ABSTRACT

The Wnt signaling pathway is aberrantly activated in many tumor types, including those of the prostate, in which β-catenin accumulates in cell nuclei and acts as a transcriptional coregulator for the androgen receptor. Because activating mutations in the β-catenin gene are rare in prostate cancer, we have looked for altered expression of other components of the Wnt signaling pathway in prostate cancer cells. Here we determined the expression levels of Wnt family genes in cultured human prostate cells and prostate cancer cell lines. We found that WNT11 expression is elevated in hormone-independent prostate cancer cell lines. Additional analysis indicated that WNT11 expression is also elevated in high-grade prostatic tumors and in hormone-independent xenografts. Growth of hormone-dependent LNCaP cells in hormone-depleted media led to increased WNT11 expression, which was repressed by the synthetic androgen R1881. This repression was inhibited by the antiandrogen bicalutamide, suggesting that androgens negatively regulate WNT11 expression through the androgen receptor. Expression of WNT11 inhibited androgen receptor transcriptional activity and cell growth in androgen-dependent cells but not in androgen-independent cells. WNT11 inhibited activation of the canonical Wnt pathway by WNT3A in HEK 293 cells and inhibited basal β-catenin/Tcf transcriptional activity in LNCaP cells. However, expression of stabilized β-catenin did not prevent the inhibition of androgen receptor transcriptional activity by WNT11. Our observations are consistent with a model in which androgen depletion activates WNT11-dependent signals that inhibit androgen-dependent but not androgen-independent cell growth.

INTRODUCTION

Most malignant prostate tumors are initially androgen dependent and androgen-ablation therapy leads to tumor regression. However, prostate cancer recurs, and no current therapy increases survival once androgen deprivation is no longer effective (1). Because the majority of androgen-independent tumors continue to express the androgen receptor (AR), aberrant activation of the AR pathway in the absence of high circulating levels of androgen is believed to contribute to the growth of such tumors. Several proteins have been described that potentiate AR transcriptional activity (see review in ref. 2). One such protein is β-catenin, which potentiates ligand-dependent AR activity and promotes AR activation by estradiol and the adrenal androgen androstenedione (3–6; for a comprehensive review see ref. 7). β-Catenin is therefore a candidate for regulating prostate cancer progression to androgen independence.

A hallmark of activation of the Wnt signaling pathway is the stabilization of β-catenin, which was first observed for the *Drosophila* homologue of β-catenin, Armadillo (see review in ref. 8). The Wnt signaling pathway is aberrantly activated in many types of cancer, most commonly in colon cancer, in which mutations in adenomatous polyposis coli or in β-catenin are frequent (see review in refs. 9 and 10). In prostate cancer, β-catenin is activated by mutation in 5% of tumors (11, 12). Mutant β-catenin accumulates in the cytoplasm and enters the nucleus, where it associates with Tcf/LEF-1 family transcription factors and activates genes involved in cell proliferation. In recent studies, 25 to 38% of metastatic androgen-independent prostate tumors were found to be comprised of tumor cells with cytoplasmic and/or nuclear β-catenin (13, 14). At present, it is not known if nuclear β-catenin promotes prostate cancer progression through its interactions with the AR or Tcf/LEF-1 family transcription factors (4).

One hypothesis is that deregulation of extracellular components of the Wnt pathway is involved in the step to androgen independence. If this proves to be the case, targeting these molecules and the genes they regulate might lead to treatments for advanced prostate cancer. As a first step in testing this hypothesis, we have used reverse transcription (RT)-PCR to examine Wnt family gene expression levels in prostate cells. There are 19 human WNT genes (15). Historically, they have been grouped into two classes, canonical and noncanonical. Canonical Wnt family members (*e.g.,* WNT1) stabilize β-catenin, thereby activating transcription of Tcf/LEF-target genes. Noncanonical Wnt family members (*e.g.,* WNT5A) activate other signaling pathways, such as the planar-cell-polarity pathway, which guides cell movements during gastrulation (16), and the Wnt/Ca^{2+} pathway (see review in ref. 17). In some instances, noncanonical Wnts antagonize the canonical pathway (18–20). Here we show that expression of WNT11, a noncanonical Wnt, is elevated in androgen-independent prostate cancer cell lines and high-grade prostate tumors. Our experiments also show that mutual inhibition of WNT11 and AR, which occurs in LNCaP cells, is lost in hormone-independent prostate cancer cells. These observations warrant further investigation into the role of WNT11 in the progression of prostate cancer to androgen-independence.

MATERIALS AND METHODS

Cell Culture. The cell lines used were obtained from American Type Culture Collection (Rockville, MD), except for LAPC-4 cells (21) obtained from Charles Sawyers (UCLA, Los Angeles, CA), CWR-R1 cells (22) obtained from Christopher Gregory (University of Carolina at Chapel Hill, Chapel Hill, NC) and LNCaP-r cells and BPH-1 cells obtained from El-Nasir Lalani (Imperial College). Fetal bovine serum (FBS), charcoal-stripped serum (CSS), antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin) and cell culture media were purchased from Invitrogen (Paisley, United Kingdom) or Sigma (Gillingham, United Kingdom), except HPC-1 medium (AthenaES, Baltimore, MD). R1881 was purchased from DuPont-NEN (Boston, MA), Casodex was kindly provided by AstraZeneca (Chesire, United Kingdom), and cycloheximide was purchased from Sigma. LAPC-4 cells were grown in Iscove's modified DMEM containing 15% FBS and 10 nmol/L R1881. CWR-R1 cells were grown in Richter's Improved MEM containing 10 mmol/L nicotinamide, 20 ng/mL epidermal growth factor, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, and 2% FBS. MDA-PCa 2b cells were grown on FNC coating mix (AthenaES) in HPC-1 medium containing 20% FBS. LNCaP-FGC, LNCaP-r, DU145, PC-3, and BPH-1 cells were grown in RPMI

Received 7/29/04; accepted 8/26/04.
Grant support: the Prostate Cancer Charity United Kingdom.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Note: Y. Kawano and M. Mazor contributed equally to this work.
Requests for reprints: Robert M. Kypta, Prostate Cancer Research Group, Department of Cancer Cell Biology, Division of Medicine, Imperial College, London W12 0NN. Phone: 0208-383-3783; Fax: 0208-383-5830; E-mail r.kypta@imperial.ac.uk.

1640 containing 10% FBS. Prostate epithelial cells (PrEC; Cambrex Cork Ltd., Cork, Ireland, United Kingdom) were grown in Prostate Epithelial Cell Growth Medium (Cambrex Bio Science, Nottingham, United Kingdom). Prostate fibroblasts and smooth muscle cells, kindly provided by Dr. Tahereh Kamalati (Imperial College), were grown in Stromal Cell Growth Medium (Cambrex). HEK 293 cells, L cells and L-WNT3A cells were grown in DMEM with 10% FBS with 0.4 mg/mL G418 (L-WNT3A only). WNT3A-conditioned medium was collected as recommended by American Type Culture Collection and used at 1:10 dilution. For drug treatments, cells were grown for 48 hours in phenol red-free medium containing 5% CSS before addition of drug. Control cultures received an equal volume of carrier that did not exceed 0.1% (v/v). Casodex (3 μmol/L) was added to cells 15 minutes before R1881. Cycloheximide was used for 24 hours at 10 μg/mL.

Prostate Tumor Tissues. Benign and malignant prostate samples from consenting patients were obtained with local ethics approval by transurethral resection and were assessed using the Gleason grading system. Xenograft tumors were generated as described previously (23). Briefly, upon castration of mice bearing androgen-dependent CWR22 tumors, prostate-specific antigen (PSA) levels fall dramatically, and the tumors regress. In some castrated animals, the tumors will relapse after a few months and are then known as androgen-independent CWR22R (24). CWR22 and CWR22R cells from Thomas Pretlow (Case Western Reserve University, Cleveland, OH) were mixed with Matrigel (BDH Chemicals, Poole, United Kingdom) to give a final volume of 0.1 mL containing approximately 5×10^5 cells/syringe. Cells were implanted by subcutaneous injection into the right flank of CD1 male nude mice or castrated male nude mice aged between 12 and 16 weeks (Charles River UK, Ltd., Margate, United Kingdom). Tumors developed approximately 2 to 3 months after injections. Once a palpable tumor developed, the animals were sacrificed by cervical dislocation; the tumor was then removed and snap frozen in liquid nitrogen. All animal work was carried out under Home Office guidelines in a dedicated animal house.

RNA Analysis. Total RNA from cell lines, cultured primary cells, and CWR22 xenograft tumor tissue was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription was done using Superscript II (Invitrogen) and 5 μg of total RNA from cultured cells or using Moloney murine leukemia virus reverse transcriptase and 2 μg of total RNA from xenograft tumor tissue. For prostate cancer tissue analysis, the one-step RT-PCR kit (Qiagen) was used to homogenize and process frozen samples. PCR amplification was carried out using increasing amounts of reverse transcribed template DNA, 10 pmol of each primer, 0.2 mmol/L dNTPs and *Taq* polymerase (Promega) in buffer provided by the manufacturer. Purified gene-specific oligonucleotides (MWG, Berlin, Germany) based on the published sequences of human Wnt genes were used. Two different pairs of oligonucleotides were used for WNT11. In general, denaturation was for 30 seconds at 94°C, annealing for 30 seconds at 58°C, and extension for 40 seconds at 72°C. Expression levels among the cell lines were estimated by comparing band intensities on 1% agarose gels containing ethidium bromide. To ensure that the results obtained were at least semi-quantitative, at least two independent preparations of RNA and two preparations of cDNA were used, and each experiment was repeated three times. In assays for actin, the amount of template and the number of cycles (26, 28, 30, and 32 cycles) were varied to determine the cycle number at which a 2-fold increase in the amount of template resulted in at least a 2-fold increase in the PCR product. Similar assays were conducted for WNT11 but using higher cycle numbers. The results presented were done at 30 cycles for actin and 40 cycles for WNT11. Note that the RT-PCR for mouse Wnt11 (Fig. 4) was not quantitative. The oligonucleotide sequences used are available on request.

To obtain an estimate of the number of WNT11 transcripts per cell, we generated a 594 bp fragment of human WNT11 (nucleotides 749-1342 of gene NM_004626) by RT-PCR from LNCaP-r cell RNA, using the primers GTAAGTGCCATGGGGTGTCT and GCTTCCAAGTGAAGGCAAAG and cloned into the pGEM-T Easy vector (Promega). Increasing dilutions of pGEM-T Easy human WNT11 and cDNA that had been generated from a specific number of LNCaP-r cells were subjected to 40 cycles of PCR using two internal WNT11 primers TGCAGGAGCTGCAGGATGTGG and AGCTCCATGGAGTGTCTCCAG to amplify a 456 bp WNT11 fragment (nucleotides 783-1259). The WNT11 primers were the same as those used for RT-PCR analysis of tissues and cell lines. The band intensities were compared and used to deduce the number of transcripts per cell. In three independent experiments, the minimum amount of WNT11 plasmid required for detection was 8×10^{-7} μg, corresponding to 2×10^5 molecules. The intensity of the PCR product at this plasmid concentration was similar to the intensity of the PCR product from cDNA derived from 2×10^4 cells, indicating that there are roughly 10 transcripts per cell in LNCaP-r cells (data not shown). A further 6-fold dilution of the cDNA yielded no detectable PCR product, suggesting that under the conditions used we could not detect WNT11 expressed at below 1.7 transcripts per cell.

DNA Reagents. pLNCX mWNT3A and pLNCX mWNT11 (mWNT11 protein is 97% identical to human WNT11) were kindly provided by Andreas Kispert (Hannover, Germany). Mouse mammary tumor virus (MMTV)-luciferase was from Charlotte Bevan (Imperial College). pOT- and pOF-luciferase were from Bert Vogelstein and Ken Kinzler (Johns Hopkins University, Baltimore, MD). The other plasmids used are described in Giannini *et al.* (25, 26).

Transient Transfections and Transcription Assays. All cells were transfected in triplicate in 6-well tissue-culture plates using Lipofectamine Plus (HEK 293, CWR-R1) or Lipofectin (LNCaP, LNCaP-r) according to manufacturers instructions (Invitrogen). For transcription assays, each well of a 6-well plate was transfected with Rous sarcoma virus (RSV) promoter-driven β-galactosidase (200 ng for prostate cells; 20 ng for HEK 293), 200 ng pOT or pOF or 200 to 400 ng MMTV-luciferase, pLNCX, pLNCX mWNT3A, or pLNCX mWNT11. In all experiments, pLNCX was used to compensate for the different amounts of pLNCX mWNT11. The stable cell lines expressing mWNT11 (Fig. 4*C*) were analyzed by transfection with 300 ng RSV-β-gal and 600 ng MMTV-luciferase without pLNCX. WNT3A- and control-conditioned medium (collected according to the directions from ATCC) was added to HEK 293 cells at 1:10 dilution after removal of DNA/lipid complexes. After transfection, cells were incubated in normal growth medium for 24 to 40 hours (for experiments using pOT and pOF) or in phenol red-free medium (for experiments using MMTV-luciferase) containing either 5% CSS (LNCaP, LNCaP-r) or 2% CSS (CWR-R1) for 16 hours. R1881 (1 nmol/L) or carrier (ethanol, EtOH) was added for 24 to 30 hours. Transfected cells were rinsed in PBS and processed for luciferase and β-galactosidase assays with the Luclite Plus (Perkin-Elmer Life Sciences) and the Galacto-Light Plus (Applied Biosystems, Foster City, CA) chemiluminescent kits. HEK 293 cell extracts were diluted 50-fold before β-galactosidase assays.

Cell Extraction and Western Blotting. For total cell extracts, we used antiandrogen-receptor (DAKO, Carpinteria, CA) at 1:1000 followed by anti-γ-tubulin (Sigma) at 1:1000. Cells were rinsed in PBS, lysed in radioimmunoprecipitation assay buffer, and subjected to Western blotting as described previously (25, 26). For analysis of soluble β-catenin, cytosolic extracts from transfected HEK 293 cells were generated by hypotonic lysis (27) and probed using anti-β-catenin mAb (BD Biosciences, Cowley, United Kingdom). To detect WNT11, a confluent 100-mm plate (Fig. 2*F*) or one well of a 6-well plate (Fig. 4*B*) of cells was rinsed three times with serum-free RPMI 1640 to remove serum proteins that interfered with detection of WNT11. Cells were then grown for 16 hours in serum-free RPMI 1640 with or without 1 nmol/L R1881 and 50 μg/mL heparin to release extracellular matrix- and cell-membrane-associated WNT11. The supernatant was centrifuged to remove cell debris and added to 50 μL StrataClean Resin (Stratagene, La Jolla, CA). Resin-bound proteins were pelleted by centrifugation, resuspended in SDS sample buffer, and heated to 95°C before Western blotting. Purified anti-WNT11 peptide antibody (28) kindly provided by Len Eisenberg (Medical University of South Carolina) was used at 1:2000.

Generation and Analysis of LNCaP Cell Lines Expressing WNT11. LNCaP cells were transfected with pLNCX or pLNCX mWNT11 in 100-mm plates using Lipofectin (Invitrogen). Two days after transfection, the cells were selected in growth medium containing 0.4 mg/mL G418. Single colonies of cells were expanded and analyzed for expression of mWNT11. For growth assays, cells (10,000 cells/well) were seeded in 96-well plates and allowed to attach for 24 hours. We measured the number of viable cells, using the cell proliferation reagent WST-1 according to manufacturers instructions (Roche, Lewes, East Sussex, United Kingdom). For Fig. 4*D*, the growth rate was calculated as the ratio between of the number of viable cells on day 4 and day 1 after plating. To measure androgen-dependent growth, the growth medium was removed on day 1; the cells were then washed in PBS and fed with androgen-depleted medium in the presence or absence of 1 nmol/L R1881.

Colony Formation Assays. LNCaP cells and LNCaP-r cells in 6-well plates were transiently transfected with either pLNCX or pLNCX mWNT11. One day after transfection, cells from each well were reseeded in 100-mm dishes and grown for 3 weeks in the presence of 0.4 mg/mL G418. Colonies were then stained using crystal violet and counted. In a representative experiment, LNCaP cells transfected with pLNCX formed 150 colonies, and those transfected with WNT11 formed 80 colonies, the latter were also smaller than those in the control. In LNCaP-r cells, the number and size of colonies were similar for cells transfected with pLNCX or WNT11, at approximately 600 colonies per plate. The higher number of colonies reflected the higher transfection and/or plating efficiency of LNCaP-r cells.

RESULTS

WNT11 Expression Increases During Prostate Cancer Progression. We used semi-quantitative RT-PCR to determine the Wnt expression in prostate cancer cell lines and cultured prostate cells. The former included cells that express AR and require androgens (or related hormones) for growth (LAPC-4, MDA-PCa 2b, and LNCaP), cells that express AR but are androgen-independent for growth (LNCaP-r and CWR-R1), and cells that do not express AR (DU145 and PC-3). We also used an immortalized epithelial cell line from a patient with benign prostate hyperplasia (BPH-1), cultured primary PrEC, primary prostate fibroblasts and prostate smooth muscle cells.

Several Wnts were expressed both in prostate epithelial and stromal cells (Fig. 1*A*; Table 1). In addition, prostate epithelial cells expressed a number of Wnts not found in stromal cells. WNT1 and WNT3A were not expressed in any prostate cell type, and WNT2 was expressed exclusively in stromal cells. The expression of WNT3 was unique to CWR-R1 cells. There were several other differences in the Wnt expression profiles of the prostate cancer cell lines, some of which might reflect their unique karyotypes.

Interestingly, when Wnt expression profiles were compared with capacity for hormone-dependent growth, WNT11 expression was found to be low or absent in hormone-dependent cell lines (Fig. 1*A*, *Lanes 3*, *8*, and *9*) and more highly expressed in hormone-independent cell lines (Fig. 1*A*, *Lanes 2*, *4*, and *7*). We calculate that androgen-independent cells contain at least 10 transcripts of WNT11 per cell whereas androgen-dependent cells contain fewer than 2 transcripts per cell (data not shown). The results suggest that progression to androgen independence correlates with increased expression of WNT11. We also found that WNT7B and WNT14 were more highly expressed in AR-negative prostate cancer cell lines (DU145 and PC3, *Lanes 2* and *4*, respectively), whereas WNT10B, like WNT11 was more highly expressed in the androgen-independent prostate cancer cell lines. Because some of these differences might be cell-line specific, we compared the Wnts expressed in androgen-dependent LNCaP cell line and an androgen-independent derivative, LNCaP-r (Fig. 1*B*). This revealed four reproducible differences. WNT5B, WNT8B, and WNT11 were more highly expressed in LNCaP-r cells, and WNT16 expression was more highly expressed in LNCaP cells. These Wnts might therefore be associated with the transition to androgen inde-

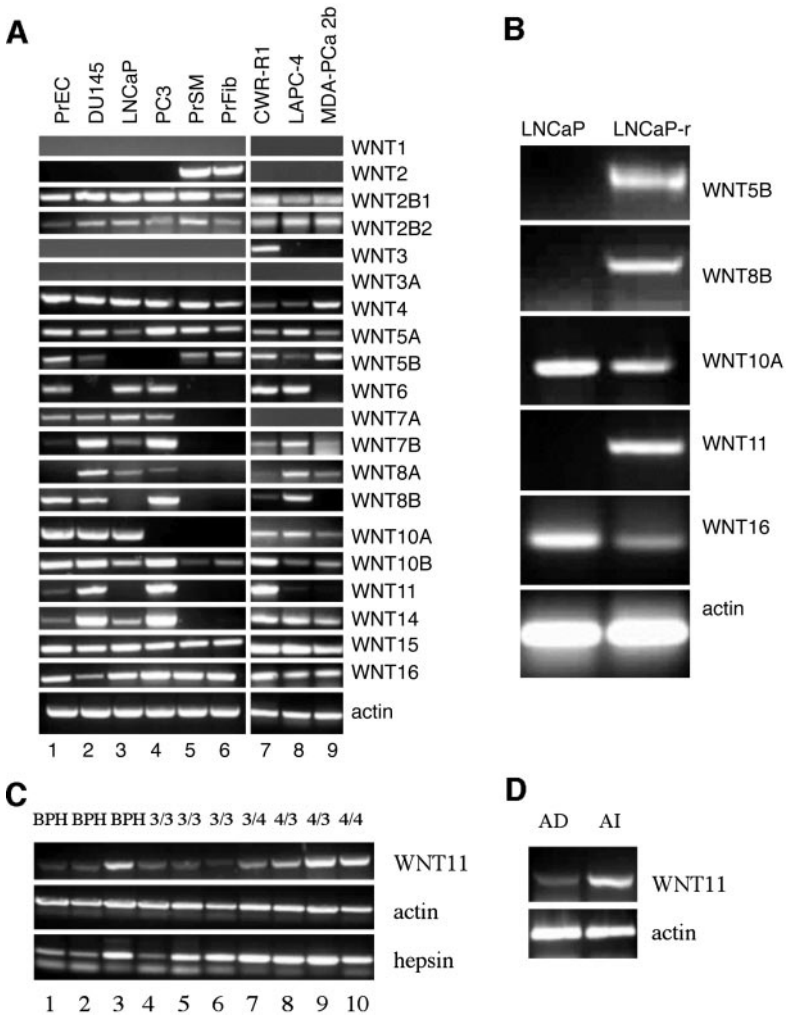

Fig. 1. Wnt family gene expression profiles of prostate cancer cells and cultured primary prostate cells. *A*, RT-PCR was used to compare expression levels of Wnt family genes in DU145 (*Lane 2*), LNCaP (*Lane 3*), PC3 (*Lane 4*), CWR-R1 (*Lane 7*), LAPC-4 (*Lane 8*), and MDA-PCa 2b (*Lane 9*) prostate cancer cell lines, in cultured primary PrEC (*Lane 1*), prostate smooth muscle cells (PrSM, *Lane 5*), and prostate fibroblasts (PrFib, *Lane 6*). *B*, RT-PCR was used to determine Wnt gene expression levels in LNCaP cells and the androgen-independent clone LNCaP-r. *C*, RT-PCR was used to determine the expression levels of WNT11, actin, and hepsin in benign prostatic hyperplasia tissue samples (BPH) and in samples from patients with prostate cancer. The *numbers on top* indicate Gleason scores. *D*, RT-PCR was used to determine the expression levels of WNT11 and actin in androgen-dependent (AD) and androgen-independent (AI) CWR22 xenograft tumor tissue.

Table 1 Comparison of Wnt family expression levels in prostate cells

	LAPC-4	MDA-PCa2b	LNCaP	LNCaP-r	CWR-R1	DU145	PC3	BPH1	PrEC	PrFib
AR	+	+	+	+	+	−	−	−	−	−
H.D.	+	+	+	−	−	−	−	−	−	−
WNT1	−	−	−	−	−	−	−	−	−	−
WNT2	−	−	−	−	−	−	−		−	+++
WNT2B1	+	+	+	+	+	++	++	+	++	+
WNT2B2	+	+	+	+	+	+	+		+	+
WNT3	−	−	−	−	+	−	−		−	−
WNT3A	−	−	−	−	−	−	−		−	−
WNT4	+	++	++	++	+	++	++		++	++
WNT5A	++	+	+	+	++	++	+++		++	++
WNT5B	+/−	+	−	+	+	+	−		++	++
WNT6	++	−	++	++	++	−	++		++	−
WNT7A	−	−	+	+	−	+	+		+	−
WNT7B	+	+/−	+	+	+/−	+++	+++	+/−	+	−
WNT8A	++	+	+	+	+/−	++	+		−	−
WNT8B	++	−	−	+/−	+−	+	+++	+	++	−
WNT10A	+	+	++	+	+	++	−		++	−
WNT10B	+	+	++	++	+++	+++	+++		+++	+
WNT11	+/−	+/−	−	+	+++	++	+++	+	+	−
WNT14	++	+	+	+	++	+++	+++		++	−
WNT15	++	++	++	++	++	++	++		++	++
WNT16	+	+	++	+	++	+	++		++	++

NOTE. The relative expression levels of Wnt family members as determined by RT-PCR is indicated by the number of +. +/− signifies weak but detectable expression. AR specifies androgen receptor status; H.D. reflects hormone-dependence for growth. WNT2B1 and WNT2B2 are alternatively spliced forms of WNT2B. PrEC, prostate epithelial cells; PrFib, prostate fibroblasts (identical data were obtained for prostate fibroblasts and smooth muscle cells).

pendence in LNCaP cells. Taken together our results indicate that the expression of WNT11 correlates well with hormone-independent growth of prostate cancer cells.

To determine whether WNT11 was expressed in prostate tumors, we used prostate tissue samples from 10 patients (Fig. 1*C*). WNT11 was expressed at low levels in two of three nonmalignant cases (*Lanes 1* and *2*) and in three of three Gleason sum score 6 cases (*Lanes 4–6*). WNT11 expression was elevated in one nonmalignant case (*Lane 3*), but this sample also had increased expression of hepsin, a gene known to be up-regulated in prostate cancer (29). This patient is on follow up; his PSA level to date has remained static. Importantly, WNT11 expression was elevated in all four cases that were Gleason grade 7 or above (*Lanes 7* to *10*). Although the number of samples examined is small, the results suggest that WNT11 expression is increased in aggressive or poorly differentiated prostate cancer.

To determine whether WNT11 expression increases during the transition from androgen-dependent to androgen-independent prostate cancer *in vivo*, we examined CWR22 xenograft tumors taken from mice (Fig. 1*D*). WNT11 was expressed in the androgen-dependent tumor, but its level of expression was higher in the androgen-independent tumor. These results support the data that used cultured prostate cancer cell lines and suggest that increased WNT11 expression accompanies the transition to androgen-independent prostate cancer.

WNT11 Expression Is Inhibited by the Androgen R1881. The LNCaP cell line is a popular tool for studies of androgen-dependent prostate cancer. We therefore chose to use LNCaP cells to examine the regulation of WNT11 expression (Fig. 2). Interestingly, growth of LNCaP cells in hormone-depleted medium induced expression of WNT11 (Fig. 2*A*, *Lane 1*). This suggested that a factor(s) in normal growth medium represses WNT11 expression, because it is not expressed in growing cells (see Fig. 1*A* and *B*). Addition of the synthetic androgen, R1881, repressed the WNT11 expression (*Lane 2*). The AR antagonist Casodex did not affect the expression of WNT11 in cells grown in hormone-depleted medium (*Lane 3*), but it prevented the repression of WNT11 expression by R1881 (*Lane 4*). These results indicate that androgens negatively regulate WNT11 expression through the AR. As controls we used DRG-1, a known androgen-dependent gene (30), and β-actin.

Because hormone depletion results in growth arrest of LNCaP cells, we wished to determine whether WNT11 expression increased in response to growth arrest *per se*. We treated LNCaP cells growing in normal medium with cycloheximide to induce nonspecific growth arrest (Fig. 2*B*). Cycloheximide treatment of LNCaP cells did not induce expression of WNT11 (*Lane 2*), suggesting WNT11 expression is not simply increased in growth-arrested cells.

To characterize further the effects of R1881 on WNT11 expression, we examined WNT11 expression at various doses of hormone and at different time points (Fig. 2*C*). Of those concentrations tested, the minimal concentration of R1881 required for repression of WNT11 expression was 1 nmol/L (Fig. 2*C*, *Lane 2*). Repression of WNT11 expression was first detectable 24 hours after addition of R1881 (Fig. 2*D*, *Lane 4*). This was in contrast to the increased expression of DRG-1, which could be detected after 4 hours (*Lane 2*) and was maximal at 24 hours (*Lane 4*). We next conducted experiments in the presence and absence of cycloheximide, an inhibitor of protein synthesis (Fig. 2*E*). The loss of WNT11 expression in the presence of R1881 (*Lane 3*) was reversed by cycloheximide (*Lane 4*), suggesting that repression of WNT11 requires *de novo* protein synthesis and is most likely an indirect AR gene target. In contrast to a previous report (30), cycloheximide prevented R1881 induction of DRG-1 (*Lane 4*). We also examined the expression of PSA, a direct gene target of AR. Although PSA was expressed in hormone-depleted cells (*Lane 1*), R1881 treatment increased PSA expression further (*Lane 3*), and cycloheximide did not affect this induction (*Lane 4*).

We wished to determine whether the changes in the expression of WNT11 mRNA correlated with changes in the level of WNT11 protein. The detection of Wnts has been hampered by low expression and/or the difficulty in generating anti-Wnt antibodies. WNT11 has been detected in conditioned medium from QCE-6 quail mesoderm cells incubated in serum-free medium in the presence of heparin, which releases WNT11 from the cell surface (31). We used this method to determine the level of WNT11 protein secreted by LNCaP cells (Fig. 2*F*). As a positive control, we used LNCaP cells transiently transfected with mWNT11 (*Lane 1*). LNCaP cells transiently transfected with empty vector (*Lane 2*) were found to secrete a small amount of WNT11 protein. This might result from the serum-free (and therefore hormone-depleted) culture conditions used to collect proteins released by heparin. The amount of WNT11 protein in conditioned-medium from LNCaP cells grown in the absence of hormone

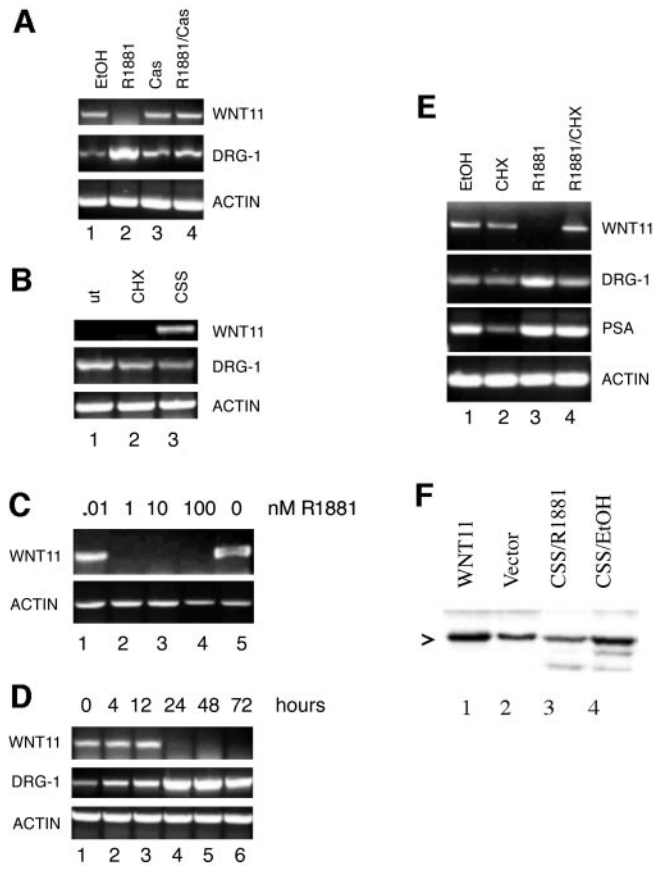

Fig. 2. *A*, Casodex blocks repression of WNT11 by R1881. RT-PCR was used to determine the expression levels of WNT11, DRG-1, and actin in LNCaP cells grown in hormone-depleted medium for 48 hours with EtOH (*Lane 1*), 1 nmol/L R1881 (*Lane 2*), 3 μmol/L Casodex (Cas; *Lane 3*), or R1881 with Casodex (*Lane 4*). *B*, cycloheximide does not induce WNT11 expression. RT-PCR was used to determine the expression levels of WNT11, DRG-1, and actin in LNCaP cells grown for 24 hours either in normal growth medium (*Lane 1*), in normal growth medium with cycloheximide (CHX; *Lane 2*), or in hormone-depleted medium (CSS; *Lane 3*). Dose-response (*C*) and time course (*D*) for the effects of R1881 on WNT11 expression. LNCaP cells were grown in hormone-depleted medium and treated for 48 hours with the indicated concentrations of R1881 (*C*) or were grown in hormone-depleted medium for 48 hours and then treated with 1 nmol/L R1881 for the indicated times (*D*), and RT-PCR was used to determine the expression levels of WNT11, DRG-1 (*D* only), and actin. *E*, repression of WNT11 expression by R1881 in LNCaP cells is inhibited by cycloheximide. RT-PCR was used to determine the expression levels of WNT11, DRG-1, PSA, and actin in LNCaP cells grown in hormone-depleted medium and treated for 48 hours with EtOH (*Lane 1*), cycloheximide (CHX; *Lane 2*), 1 nmol/L R1881 (*Lane 3*), or R1881 with CHX. *F*, detection of WNT11 protein in LNCaP cells. WNT11 protein expression was determined by Western blotting of cell-conditioned medium from heparin-treated LNCaP cells transiently transfected with mWNT11 (*Lane 1*), LNCaP cells transiently transfected with empty vector (*Lane 2*), hormone-starved LNCaP cells grown in the presence of 1 nmol/L R1881 (*Lane 3*), and hormone-starved LNCaP cells (*Lane 4*). The *arrowhead* indicates the position of WNT11.

(*Lane 4*) was greater than that from cells grown in the presence of 1 nmol/L R1881 (*Lane 3*). Thus the level of WNT11 protein, like that of WNT11 mRNA, is reduced by R1881 in LNCaP cells.

WNT11 Represses AR Transcriptional Activity in LNCaP Cells but not in Androgen-Independent Prostate Cancer Cells. The expression of WNT11 in androgen-independent prostate cancer cells and its repression by androgens in LNCaP cells suggests that there is a link between WNT11 and AR activity. Therefore, we examined the effects of expressing WNT11 on AR transcriptional activity by expressing mWNT11 and conducting transcription assays using MMTV-luciferase, a reporter containing a fragment of the MMTV promoter known to be regulated by the AR (Fig. 3). mWNT11 repressed AR transcriptional activity in a dose-dependent manner in LNCaP cells (Fig. 3*A*), but it had no effect on AR activity in the androgen-independent prostate cancer cell lines LNCaP-r and CWR-R1 (Fig. 3*B*). These results indicate that the responses of androgen-dependent and androgen-independent prostate cancer cells differ with respect to WNT11.

WNT11 Represses LNCaP Cell Growth. To determine the effects of WNT11 on cell growth, we generated stable LNCaP cell lines expressing mWNT11 or empty vector. RT-PCR analysis (which was not quantitative in this experiment; Fig. 4*A*) and Western blotting (Fig. 4*B*) indicated that several clones expressed mWNT11. As reported previously (31), WNT11 was poorly secreted into the medium in the absence of heparin (compare Fig. 4*B Lanes 3* and *4*). The presence of nonspecific bands precluded detection of WNT11 protein in cell extracts.

We next determined whether stable expression of WNT11 affected AR transcriptional activity. In agreement with the results in Fig. 3, AR transcriptional activity was significantly lower in cell lines stably

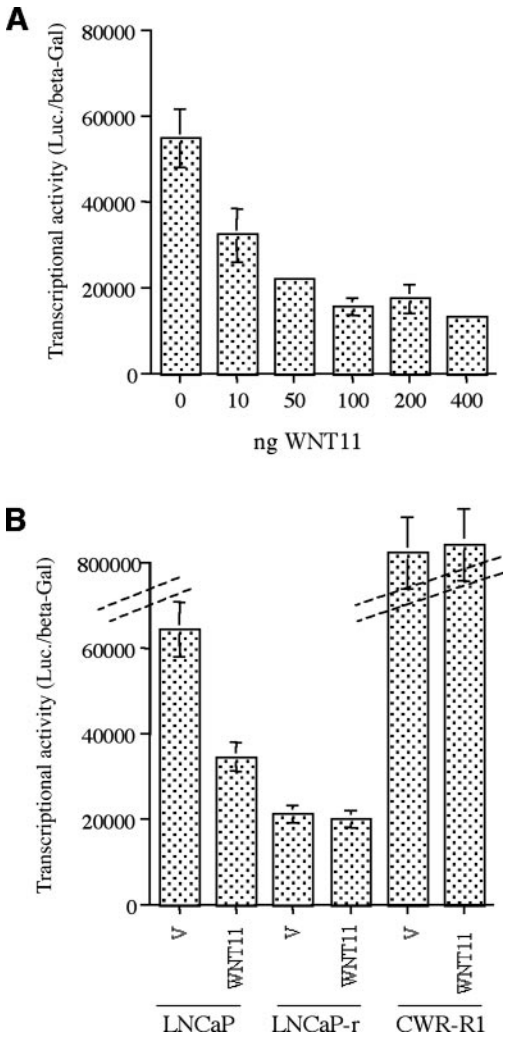

Fig. 3. WNT11 expression inhibits AR-dependent transcription. *A*, LNCaP cells were transfected with the indicated amounts of WNT11 plus pMMTV-luciferase and RSV-β-galactosidase (reporters) and grown in hormone-depleted medium for 16 hours. R1881 (1 nmol/L) was added 24 hours before measurement of luciferase and β-galactosidase activities. Values shown are luciferase activity (×1000) normalized to β-galactosidase activity and are from a representative experiment. *B*, LNCaP cells, LNCaP-r cells, and CWR-R1 cells were transfected with 50 ng pLNCX (V) or pLNCX mWNT11 plus reporters. Cells were grown in hormone-depleted medium for 16 hours. R1881 (1 nmol/L) was added for 24 hours before assay. Values shown are luciferase activity (×1000) normalized to β-galactosidase activity. All experiments were done three times in triplicate, and *error bars* represent SD (where error bars are not visible the errors are negligible). Student's *t* test indicated that the differences between the AR activities in vector and WNT11-transfected cells are significant in LNCaP cells ($P < 0.001$) but not in LNCaP-r cells ($P > 0.7$) or CWR-R1 cells ($P > 0.8$).

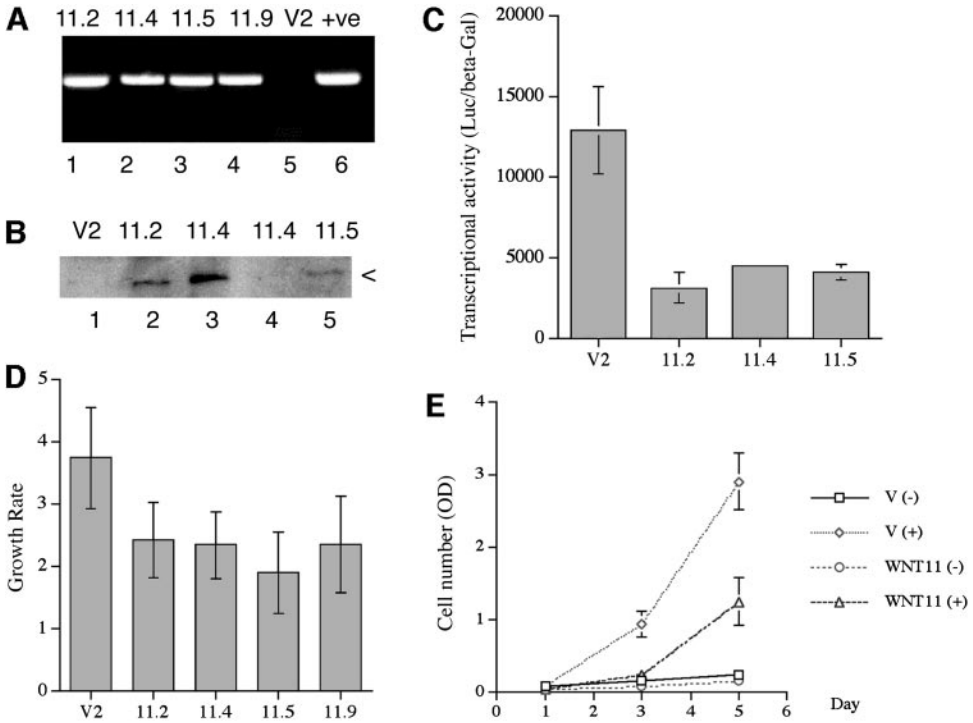

Fig. 4. WNT11 inhibits LNCaP cell growth. *A*, cell lines stably transfected with empty vector or mouse WNT11 plasmids were analyzed for expression of mouse WNT11 by RT-PCR. A control clone (V2) is shown in *Lane 5*. WNT11-expressing clones (11.2, 11.4, 11.5, 11.9) are shown in *Lanes 1* to *4*. The positive control is PCR using mouse WNT11 plasmid DNA (*Lane 6*). *B*, cell lines stably transfected with empty vector or mouse WNT11 plasmids were analyzed for WNT11 protein expression by Western blotting of cell-conditioned medium from heparin-treated cells. *Lane 1*, control clone V2; *Lane 2*, WNT11 clone 11.2; *Lane 3*, WNT11 clone 11.4; *Lane 4*, WNT11 clone 11.4 (no heparin); *Lane 5*, WNT11 clone 11.5. *C*, the indicated LNCaP clones were transfected with reporters and grown in hormone-depleted medium for 16 hours. R1881 1 (nmol/L) was added for 24 hours before assay. Values shown are luciferase activity (×100) normalized to β-galactosidase activity. The experiment was conducted twice in triplicate and *error bars* represent SD. *D*, growth rates of the indicated cell lines in normal growth medium. Experiments were done twice in sextuplet and *error bars* show SD. Student's *t* test indicated that the differences between the growth rates in empty vector control cells (V2) compared with WNT11-expressing cells are significant ($P < 0.02$, 0.01, 0.01, and 0.04 for clones 11.2, 11.4, 11.5, and 11.9, respectively). *E*, growth curves for empty vector control cells and WNT11-expressing cells in hormone-depleted medium in the absence (−) or presence (+) of 1 nmol/L R1881. The experiment was done twice in sextuplet and *error bars* show SD. Student's *t* test indicated that the difference between the growth of empty vector control cells and WNT11-expressing cells is not significant in the absence of hormone ($P > 0.2$ at day 5) but is significant in the presence of hormone ($P < 0.001$ at day 5).

expressing WNT11 than in control cells (Fig. 4*C*). There was no detectable effect of WNT11 on the level of AR protein in Western blots (data not shown), suggesting that the inhibitory effect of WNT11 on AR activity does not result from a reduction in AR expression.

When the growth rates of the LNCaP cell lines were compared (Fig. 4*D*), the growth rates (in normal growth medium) of the WNT11-expressing cell lines were significantly lower than those of the control cells. In addition, ectopic expression of WNT11 also led to a reduction in cell proliferation in LNCaP cells in colony formation assays (150 colonies per plate in vector-transfected cells and 80 colonies in WNT11-transfected cells), and this was not observed for LNCaP-r cells (600 colonies per plate for both vector- and WNT11-transfected cells; data not shown). LNCaP cell growth was also examined in hormone-depleted medium in the absence or presence of 1 nmol/L R1881 (Fig. 4*E*). WNT11-expressing cells grew more slowly than control cells in the presence of R1881. WNT11 expression did not seem to affect the growth rate of LNCaP cells grown in the absence of hormone, as might be expected given that LNCaP cells do not grow under these conditions, and endogenous WNT11 is induced by hormone-depletion. In conclusion, stable expression of WNT11 both represses AR transcriptional activity and reduces androgen-dependent growth in LNCaP cells.

WNT11 Represses the Canonical Wnt Pathway. One possible outcome of WNT11 expression is inhibition of canonical Wnt signaling, as has been observed for other noncanonical Wnts (18–20). We therefore examined the effect of WNT11 expression on the canonical Wnt signaling pathway in HEK 293 cells, which respond well to the canonical Wnt, WNT3A. Two assays were used to measure activation of the canonical pathway. In the first, the amount of cytosolic β-catenin was determined by Western blotting (Fig. 5*A*); in the second, transcription assays were conducted with a β-catenin/Tcf-responsive luciferase reporter (pOT) that contains Tcf family binding sites (Fig. 5*B*). As expected, expression of WNT3A increased the cytosolic level of β-catenin (Fig. 5*A*, *Lane 2*). In contrast, expression of WNT11 did not activate the canonical pathway (*Lane 3*). In fact, to some extent, WNT11 reduced the basal level of cytosolic β-catenin (compare *Lanes 1* and *3*). Furthermore, when WNT3A and WNT11 were coexpressed, WNT11 repressed the effects of WNT3A (*Lane 4*). Similar results were observed in transcription assays using transfected WNT11 and WNT3A (Fig. 5*B*), that is, WNT11 reduced both basal β-catenin/Tcf activity and WNT3A-induced activity. As a control, we used a luciferase reporter with mutated Tcf binding sites (pOF). WNT11 expression did not affect the activity of pOF, indicating that the effect of WNT11 on the canonical pathway is specific.

To rule out the possibility that WNT11 expression inhibited the canonical pathway by inhibiting the expression or secretion of co-transfected WNT3A, we repeated the transcription assays using conditioned medium from L cells stably expressing WNT3A (Fig. 5*C*). As expected, WNT3A-conditioned medium (but not control-conditioned medium) increased β-catenin/Tcf transcriptional activity in HEK 293 cells. In agreement with the results obtained when transfected WNT3A was used, WNT11 inhibited activation of β-catenin/Tcf transcriptional activity by WNT3A-conditioned medium. Taken together these results indicate that WNT11 can inhibit activation of the canonical Wnt signaling pathway.

β-Catenin is already present in the nuclei of LNCaP cells. The reason for this is not fully understood, but it may result from mutation of PTEN

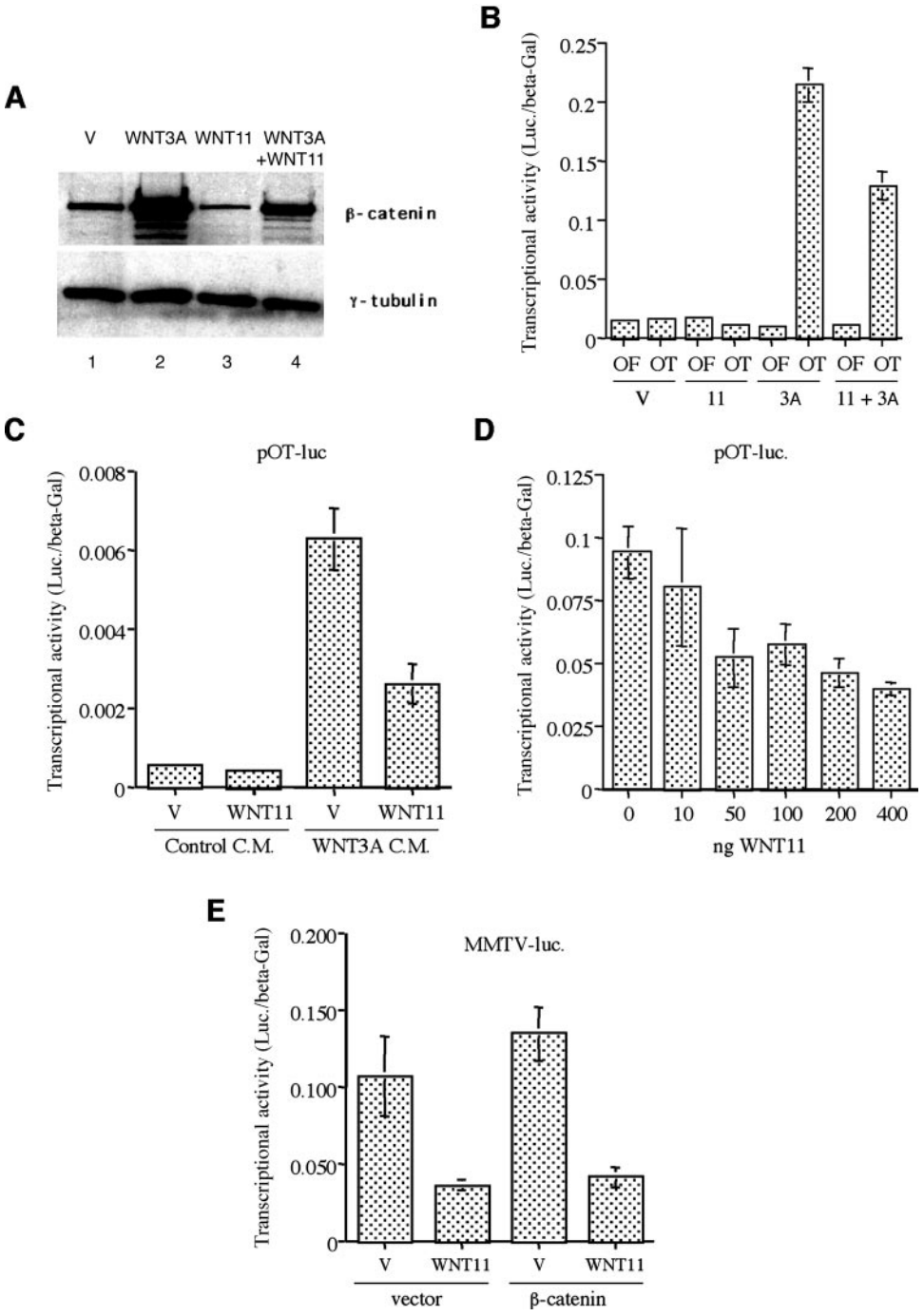

Fig. 5. WNT11 represses canonical Wnt signaling. *A*, cytosolic extracts from HEK 293 cells transfected with empty vector (*Lane 1*), vector plus WNT3A (*Lane 2*), vector plus WNT11 (*Lane 3*) or WNT3A plus WNT11 (*Lane 4*) were probed for β-catenin (top panel) and then reprobed for γ-tubulin (bottom panel). *B*, HEK 293 cells were transfected as indicated (V, empty vector; 11, WNT11; 3A, WNT3A) together with RSV-β-galactosidase and either pOT (luciferase reporter measuring β-catenin/Tcf activity) or pOF (mutated form of pOT), and extracts were assayed. *C*, HEK 293 cells were transfected with empty vector (V) or WNT11 together with RSV-β-galactosidase and pOT and then were either untreated or treated with WNT3A-conditioned medium (WNT3A) for 24 hours before assay. *D*, LNCaP cells were transfected with the indicated amounts of WNT11 (ng) and empty vector (bringing the total to 400 ng) plus pOT and RSV-β-galactosidase. Student's *t* test indicated that the differences between the transcriptional activity in empty vector transfected cells compared with WNT11-transfected cells is significant at ≥50 ng of WNT11 ($P < 0.001$ at 50 ng). *E*, LNCaP cells were transfected with 200 ng of WNT11 or pLNCX empty vector, 200 ng of pMT23 empty vector or pMT23 βAmyc (myc-tagged stabilized β-catenin; ref. 26) and MMTV-luciferase. All experiments were done three times in triplicate, values are luciferase activity normalized to β-galactosidase activity; *error bars* represent SD.

(32). Because β-catenin has been reported to activate AR-dependent transcription, we considered the possibility that WNT11 inhibited AR by reducing the level of endogenous β-catenin. However, we were unable to detect significant changes in the level of endogenous β-catenin in LNCaP cell lines expressing WNT11 (data not shown). To address this question using a more sensitive assay, we examined the effects of WNT11 on β-catenin/Tcf transcriptional activity (Fig. 5*D*). Expression of WNT11 repressed β-catenin/Tcf transcriptional activity in LNCaP cells. The effect was weaker than we observed using HEK 293 cells, possibly because β-catenin/Tcf activity is low in LNCaP cells as a result of low expression of Tcf/LEF family proteins (13).

To determine whether ectopic expression of β-catenin prevented the inhibitory effects of WNT11 on AR transcriptional activity, LNCaP cells were cotransfected with WNT11 (or empty vector) and a stabilized form of β-catenin (or empty vector) and MMTV-luciferase. As reported by others (3–5), expression of β-catenin increased AR transcriptional activity in LNCaP cells, albeit to a smaller extent in our hands. However, WNT11 similarly repressed AR transcriptional activity in the presence and absence of stabilized β-catenin. These results suggest that although WNT11 can repress the canonical Wnt pathway in HEK 293 cells by destabilizing β-catenin, this mechanism is not directly responsible for the inhibition of AR transcriptional activity. Additional studies are underway to characterize the signaling pathway leading from WNT11 to the AR.

DISCUSSION

In this report, we examined the expression profiles of members of the Wnt gene family in prostate cancer cell lines and cultured primary

prostate cells. The striking link between WNT11 expression and hormone-independent growth of prostate cancer cells prompted us to characterize further the regulation of this gene and to study the effects of WNT11 expression on AR activity and prostate cancer cell growth.

Our analysis of Wnt gene expression has led to a number of observations that provide direction for future studies. First, several Wnt genes are expressed both in normal prostate cells as well as in prostate cancer cell lines; these Wnts might therefore play "housekeeping" roles in the prostate. Second, WNT2 was specifically expressed in stromal cells and is therefore a good candidate for a stromal cell-derived factor that might promote prostate cancer progression. Third, WNT3 expression was unique to CWR-R1 cells. Interestingly, WNT3 expression is up-regulated in a number of other tumor types (33). It is not known which of these Wnts are actually secreted by the cells that we have examined. Such information requires the generation of anti-Wnt antibodies, which has until recently been hampered by difficulties purifying Wnt proteins (34).

The correlation between WNT11 expression and hormone-independent prostate cancer prompted us to focus the present work on WNT11. We found that WNT11 expression was elevated in the more aggressive or poorly differentiated tumors. It is unlikely that WNT11 is simply a marker for prostate epithelial cells because it was not expressed in several of the prostate cancer cell lines that express epithelial cell markers, and it was expressed only at a low level in normal PrECs. Moreover, WNT11 expression was elevated in hormone-refractory CWR22 tumors. Although additional studies are required using a larger number of samples, the increased expression of WNT11 in the more aggressive prostate tumors suggests that the data obtained using prostate cancer cell lines are clinically relevant.

The androgen-dependent LNCaP cell line was used to investigate the reason for the low level of expression of WNT11 in androgen-dependent cells. WNT11 expression increased after androgen depletion and this was prevented by the synthetic androgen R1881. Importantly, Casodex blocked the effects of R1881, indicating a role for AR in the response to androgens. WNT11 does not seem to be directly regulated by AR because the effects of R1881 were blocked by cycloheximide. Proteins that might regulate the expression of WNT11 include glial cell line-derived neurotrophic factor and sonic hedgehog. Glial cell line-derived neurotrophic factor, which is expressed in several tissues including the prostate (35), regulates WNT11 expression in the kidney (36, 37). Sonic hedgehog is a testosterone-regulated gene that is required for prostate development in the mouse (38) and antagonizes WNT11 expression during avian somite development (39).

We found that ectopic expression of WNT11 repressed the canonical Wnt signaling pathway. WNT5A has also been shown to inhibit the canonical Wnt pathway in several systems. The mechanism of inhibition varies, involving effects on cell-cell adhesion (18), Ca^{2+}-dependent activation of nuclear factor of activated T cells (19) or Ca^{2+}/calmodulin-dependent protein kinase II (20), and activation of SIAH-2 expression (40). Despite the high level of β-catenin in LNCaP cell nuclei, the canonical Wnt pathway is only weakly active in these cells because of their low levels of expression of Tcf/LEF family proteins (13). Therefore, the effect of WNT11 on the β-catenin/Tcf transcriptional activity *per se* might not be important for LNCaP cell growth. However, the signals from WNT11 that lead to inhibition of β-catenin/Tcf activity might also independently affect AR transcriptional activity and LNCaP cell growth.

The molecular mechanism(s) by which WNT11 represses transcriptional activity of AR is presently unclear. Because β-catenin associates with AR and can potentiate AR activity (3–6, 41), it is reasonable to hypothesize that WNT11 inhibits AR transcriptional activity by destabilizing β-catenin. This would be consistent with our observation that WNT11 represses β-catenin/Tcf transcriptional activity in LNCaP cells, which also requires β-catenin (Fig. 5*D*). However, coexpression of stabilized β-catenin with WNT11 did not rescue the inhibitory effects of WNT11 on AR transcriptional activity (Fig. 5*E*). Therefore, the signals activated by WNT11 that regulate AR transcriptional activity remain to be identified. Other signaling pathways regulated by WNT11 include those involving components of the c-Jun N-terminal kinase signaling pathway (42, 43).

There are a number of possible scenarios that might account for the increase in WNT11 expression in androgen-depleted LNCaP cells. WNT11 might be involved in the cessation of cell growth that accompanies androgen depletion. If this is the case, then a signal from WNT11 might contribute to cell growth inhibition in LNCaP cells. Indeed, ectopic expression of WNT11 inhibits both AR transcriptional activity and cell growth in LNCaP cells. An alternative possibility is that increased expression of WNT11 in androgen-depleted cells reflects a change in LNCaP cell differentiation. Androgen depletion results in the expression of neuroendocrine markers such as neurotensin (44). WNT11 can regulate hematopoietic cell fate (45) and is required for cardiac differentiation (31, 46), but it remains to be seen whether WNT11 is involved in neuroendocrine differentiation.

If WNT11 contributes significantly to the androgen-independent phenotype, the mWNT11-expressing cell lines might be predicted to grow in an androgen-independent manner. However, like control cells, these cells did not grow in hormone-depleted medium. This is perhaps not surprising given that endogenous WNT11 is expressed in LNCaP cells growing under these conditions. Our results suggest that WNT11 expression alone is not sufficient to confer androgen-independent growth and that additional changes are required for progression to androgen-independence.

In contrast to what was observed in LNCaP cells, the growth of LNCaP-r cells was not inhibited by ectopic expression of mWNT11. This might be expected because LNCaP-r cells already express endogenous WNT11, as do several other androgen-independent prostate cancer cell lines. These observations suggest that the expression level of WNT11 does not simply correlate with inhibition of cell cycle progression. Androgen-independent prostate cancer cells may grow in the presence of WNT11 because they no longer respond to the putative WNT11 growth inhibitory signal or because WNT11 expression confers an additional selective advantage. One possibility is that WNT11 is involved in metastasis; interestingly, a recent report showed that WNT11 stimulates cell migration in chicken embryo fibroblasts (47).

To summarize, our results suggest a model in which androgen depletion leads to increased expression of WNT11, which then contributes to cessation of androgen-dependent growth. The transition to androgen-independence might involve subversion of the WNT11 signal to one that promotes tumor progression, or WNT11 might confer migratory properties to prostate cancer cells that no longer respond to its growth inhibitory signal. Thus, the inhibition of signals emanating from WNT11 could lead to new treatments for advanced prostate cancer.

ACKNOWLEDGMENTS

We are grateful to Andreas Kispert, Len Eisenberg, Charlotte Bevan, Ken Kinzler, and Bert Vogelstein for reagents, and to Tahereh Kamalati, Charles Sawyers, Christopher Gregory, and El-Nasir Lalani for cells. We also thank colleagues in the Prostate Cancer Research Group for help and advice and Maria Vivanco (ICR, London) for critical review of the manuscript.

REFERENCES

1. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.

2. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocr Rev 2002;23:175–200.
3. Truica CI, Byers S, Gelmann EP. Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 2000;60:4709–13.
4. Chesire DR, Ewing CM, Gage WR, Isaacs WB. In vitro evidence for complex modes of nuclear beta-catenin signaling during prostate growth and tumorigenesis. Oncogene 2002;21:2679–94.
5. Yang F, Li X, Sharma M, et al. Linking beta-catenin to androgen-signaling pathway. J Biol Chem 2002;277:11336–44.
6. Mulholland DJ, Cheng H, Reid K, Rennie PS, Nelson CC. The androgen receptor can promote beta-catenin nuclear translocation independently of adenomatous polyposis coli. J Biol Chem 2002;277:17933–43.
7. Cheshire DR, Isaacs WB. beta-Catenin signaling in prostate cancer: an early perspective. Endocr Relat Cancer 2003;10:537–60.
8. Miller JR, Moon RT. Signal transduction through beta-catenin and specification of cell fate during embryogenesis. Genes Dev 1996;10:2527–39.
9. Polakis P. Wnt signaling and cancer. Genes Dev 2000;14:1837–51.
10. Barker N, Clevers H. Catenins, Wnt signaling and cancer. Bioessays 2000;22:961–5.
11. Voeller HJ, Truica CI, Gelmann EP. beta-Catenin mutations in human prostate cancer. Cancer Res 1998;58:2520–3.
12. Chesire DR, Ewing CM, Sauvageot J, Bova GS, Isaacs WB. Detection and analysis of beta-catenin mutations in prostate cancer. Prostate 2000;45:323–34.
13. Chesire DR, Isaacs WB. Ligand-dependent inhibition of beta-catenin/TCF signaling by androgen receptor. Oncogene 2002;21:8453–69.
14. De La Taille A, Rubin MA, Chen MW, et al. beta-Catenin-related anomalies in apoptosis-resistant and hormone-refractory prostate cancer cells. Clin Cancer Res 2003;9:1801–7.
15. Miller JR. The Wnts. Genome Biol 2002;3:REVIEWS3001.
16. Heisenberg CP, Tada M, Rauch GJ, et al. Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature (Lond) 2000;405:76–81.
17. Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT. The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape. Trends Genet 2000;16:279–83.
18. Torres MA, Yang-Snyder JA, Purcell SM, DeMarais AA, McGrew LL, Moon RT. Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. J Cell Biol 1996;133:1123–37.
19. Saneyoshi T, Kume S, Amasaki Y, Mikoshiba K. The Wnt/calcium pathway activates NF-AT and promotes ventral cell fate in Xenopus embryos. Nature (Lond) 2002;417:295–9.
20. Ishitani T, Kishida S, Hyodo-Miura J, et al. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. Mol Cell Biol 2003;23:131–9.
21. Klein KA, Reiter RE, Redula J, et al. Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. Nat Med 1997;3:402–8.
22. Gregory CW, Johnson RT Jr, Mohler JL, French FS, Wilson EM. Androgen receptor stabilization in recurrent prostate cancer is associated with hypersensitivity to low androgen. Cancer Res 2001;61:2892–8.
23. Gnanapragasam VJ, Robson CN, Neal DE, Leung HY. Regulation of FGF8 expression by the androgen receptor in human prostate cancer. Oncogene 2002;21:5069–80.
24. Nagabhushan M, Miller CM, Pretlow TP, et al. CWR22: the first human prostate cancer xenograft with strongly androgen-dependent and relapsed strains both in vivo and in soft agar. Cancer Res 1996;56:3042–6.
25. Giannini AL, Vivanco M, Kypta RM. alpha-Catenin inhibits beta-catenin signaling by preventing formation of a beta-catenin*T-cell factor*DNA complex. J Biol Chem 2000;275:21883–8.
26. Giannini AL, Vivanco MM, Kypta RM. Analysis of beta-catenin aggregation and localization using GFP fusion proteins: nuclear import of alpha-catenin by the beta-catenin/Tcf complex. Exp Cell Res 2000;255:207–20.
27. Orford K, Crockett C, Jensen JP, Weissman AM, Byers SW. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem 1997;272:24735–8.
28. Eisenberg CA, Gourdie RG, Eisenberg LM. Wnt-11 is expressed in early avian mesoderm and required for the differentiation of the quail mesoderm cell line QCE-6. Development 1997;124:525–36.
29. Magee JA, Araki T, Patil S, et al. Expression profiling reveals hepsin overexpression in prostate cancer. Cancer Res 2001;61:5692–6.
30. Ulrix W, Swinnen JV, Heyns W, Verhoeven G. The differentiation-related gene 1, Drg1, is markedly upregulated by androgens in LNCaP prostatic adenocarcinoma cells. FEBS Lett 1999;455:23–6.
31. Eisenberg CA, Eisenberg LM. WNT11 promotes cardiac tissue formation of early mesoderm. Dev Dyn 1999;216:45–58.
32. Persad S, Troussard AA, McPhee TR, Mulholland DJ, Dedhar S. Tumor suppressor PTEN inhibits nuclear accumulation of beta-catenin and T cell/lymphoid enhancer factor 1-mediated transcriptional activation. J Cell Biol 2001;153:1161–74.
33. Katoh M. Molecular cloning and characterization of human WNT3. Int J Oncol 2001;19:977–82.
34. Willert K, Brown JD, Danenberg E, et al. Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature (Lond) 2003;423:448–52.
35. Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson EM Jr. Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp Neurol 1999;158:504–28.
36. Pepicelli CV, Kispert A, Rowitch DH, McMahon AP. GDNF induces branching and increased cell proliferation in the ureter of the mouse. Dev Biol 1997;192:193–8.
37. Ehrenfels CW, Carmillo PJ, Orozco O, Cate RL, Sanicola M. Perturbation of RET signaling in the embryonic kidney. Dev Genet 1999;24:263–72.
38. Podlasek CA, Barnett DH, Clemens JQ, Bak PM, Bushman W. Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium. Dev Biol 1999;209:28–39.
39. Marcelle C, Stark MR, Bronner-Fraser M. Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. Development 1997;124:3955–63.
40. Topol L, Jiang X, Choi H, Garrett-Beal L, Carolan PJ, Yang Y. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent beta-catenin degradation. J Cell Biol 2003;162:899–908.
41. Pawlowski JE, Ertel JR, Allen MP, et al. Liganded androgen receptor interaction with beta-catenin: nuclear co-localization and modulation of transcriptional activity in neuronal cells. J Biol Chem 2002;277:20702–10.
42. Pandur P, Lasche M, Eisenberg LM, Kuhl M. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. Nature (Lond) 2002;418:636–41.
43. Yamanaka H, Moriguchi T, Masuyama N, et al. JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. EMBO Rep 2002;3:69–75.
44. Sehgal I, Powers S, Huntley B, Powis G, Pittelkow M, Maihle NJ. Neurotensin is an autocrine trophic factor stimulated by androgen withdrawal in human prostate cancer. Proc Natl Acad Sci USA 1994;91:4673–7.
45. Brandon C, Eisenberg LM, Eisenberg CA. WNT signaling modulates the diversification of hematopoietic cells. Blood 2000;96:4132–41.
46. Chang CH, Jiang TX, Lin CM, Burrus LW, Chuong CM, Widelitz R. Distinct Wnt members regulate the hierarchical morphogenesis of skin regions (spinal tract) and individual feathers. Mech Dev 2004;121:157–71.

Wnt Genes and Prostate Cancer

In the article on Wnt genes and prostate cancer in the November 1, 2004 issue of *Cancer Research* (1), the oligonucleotide primers used to detect Wnt15 by RT-PCR are actually designed to detect Wnt2B. The oligonucleotide primer sequences used were taken from the paper by Bergstein et al. (*Genomics* 1997;46:450–8) and their correct identity was not checked. Therefore, the data on the expression of Wnt15 in prostate cells in Fig. 1*A* are incorrect and the expression level of Wnt15 in prostate cells remains to be determined.

1. Zhu H, Mazor M, Kawano Y, Walker MM, Leung HY, Armstrong K, Waxman J, Kypta RM. Analysis of Wnt gene expression in prostate cancer: mutual inhibition by WNT11 and the androgen receptor. Cancer Res 2004;64:7918–26.