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Human Cancer Biology

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Activation of β-Catenin Signaling in Androgen Receptor–Negative Prostate Cancer Cells

Xinhai Wan[1], Jie Liu[1], Jing-Fang Lu[1], Vassiliki Tzelepi[1,4], Jun Yang[1], Michael W. Starbuck[1], Lixia Diao[2], Jing Wang[2], Eleni Efstathiou[1,5], Elba S. Vazquez[6], Patricia Troncoso[3], Sankar N. Maity[1], and Nora M. Navone[1]

Abstract

Purpose: To study Wnt/β-catenin in castrate-resistant prostate cancer (CRPC) and understand its function independently of the β-catenin–androgen receptor (AR) interaction.

Experimental Design: We carried out β-catenin immunocytochemical analysis, evaluated TOP-flash reporter activity (a reporter of β-catenin–mediated transcription), and sequenced the β-catenin gene in MDA prostate cancer 118a, MDA prostate cancer 118b, MDA prostate cancer 2b, and PC-3 prostate cancer cells. We knocked down β-catenin in AR-negative MDA prostate cancer 118b cells and carried out comparative gene-array analysis. We also immunohistochemically analyzed β-catenin and AR in 27 bone metastases of human CRPCs.

Results: β-Catenin nuclear accumulation and TOP-flash reporter activity were high in MDA prostate cancer 118b but not in MDA prostate cancer 2b or PC-3 cells. MDA prostate cancer 118a and MDA prostate cancer 118b cells carry a mutated β-catenin at codon 32 (D32G). Ten genes were expressed differently (false discovery rate, 0.05) in MDA prostate cancer 118b cells with downregulated β-catenin. One such gene, hyaluronan synthase 2 (*HAS2*), synthesizes hyaluronan, a core component of the extracellular matrix. We confirmed HAS2 upregulation in PC-3 cells transfected with D32G-mutant β-catenin. Finally, we found nuclear localization of β-catenin in 10 of 27 human tissue specimens; this localization was inversely associated with AR expression ($P = 0.056$, Fisher's exact test), suggesting that reduced AR expression enables Wnt/β-catenin signaling.

Conclusion: We identified a previously unknown downstream target of β-catenin, HAS2, in prostate cancer, and found that high β-catenin nuclear localization and low or no AR expression may define a subpopulation of men with bone metastatic prostate cancer. These findings may guide physicians in managing these patients. *Clin Cancer Res; 18(3); 726–36. ©2011 AACR.*

Introduction

Recent reports have indicated that androgen receptor (AR)–mediated mechanisms are central to the castrate-resistant progression of prostate cancer from localized to bone-metastatic disease (1–3), although multiple pathways likely cooperate to drive its growth at this advanced stage.

We recently developed 2 xenograft models, MDA prostate cancer 118a and MDA prostate cancer 118b, that were derived from 2 bone metastases in a man who had castrate-resistant prostate cancer (CRPC) with widespread osteoblastic metastases (4). The cancer cells in these xenografts, like those in the human tumors of origin, do not express AR and can grow in castrated male mice. As such, they can be used to identify AR-independent mechanisms of castrate-resistant progression that likely would define a subtype of prostate cancers. It is also likely that the same mechanisms play an important, albeit more modest, role when the AR pathway is active. The results of our initial studies using MDA prostate cancer 118a and MDA prostate cancer 118b cells suggested that they might have an active, highly upregulated Wnt/β-catenin signaling pathway

Authors' Affiliations: Departments of [1]Genitourinary Medical Oncology; [2]Bioinformatics and Computational Biology; [3]Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas; [4]Department of Pathology, University of Patras, Patras; [5]Department of Clinical Therapeutics, University of Athens, Athens, Greece; and [6]Departament de Biological Chemistry, School of Sciences, University of Buenos Aires, CONICET, Buenos Aires, Argentina

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Current address for J. Liu: Saint Mary's Hospital, 56 Franklin Street, Waterbury, CT 06706.

N.M. Navone and S.N. Maity contributed equally to this work.

Corresponding Author: Nora M. Navone, Department of Genitourinary Medical Oncology–Research, Unit 18-6, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: 713-563-7273; Fax: 713-745-9880; E-mail: nnavone@mdanderson.org

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Translational Relevance

Presently, there is no curative therapy for castrate-resistant prostate cancer. We confirm here that β-catenin nuclear accumulation occurs in about 37% of castrate-resistant prostate cancer bone metastases, and we report for the first time that HAS2 is a downstream target of β-catenin. HAS2 expression and the β-catenin:androgen receptor ratio may help define a subpopulation of men with prostate cancer for individualized management.

compared with that in PC-3 and MDA prostate cancer 2b cells, which are 2 other bone-derived prostate cancer cell lines. Briefly, in our original comparative gene-array study (Human Genome U133A microarray, Affymetrix) of MDA prostate cancer 118a and MDA prostate cancer 118b, MDA prostate cancer 2b, and PC-3 cells, we found that the fibroblast growth factor (FGF) axis mediated the osteoblastic progression of these cells in bone (4). This comparison also revealed greater expression of several genes downstream of the canonical Wnt/β-catenin pathway, including *FGF18*, immunoglobulin transcription factor-2 (*ITF2*; also called *TCF4*), neuronal cell adhesion molecule (*NrCAM*), SRY-related HMG-box 2 (*SOX2*), and bone morphogenetic protein 4 (*BMP4*), in the MDA prostate cancer 118a and MDA prostate cancer 118b cells [Wnt-canonical target genes were from "The Wnt homepage" (5) and from a recent publication (6)].

The protein β-catenin has at least 2 functions of interest in prostate cancer: it participates in cadherin-mediated adhesion, and it is the "molecular node" of the Wnt canonical signaling pathway. In the absence of Wnt signals (i.e., when the pathway is inactive), the serine/threonine kinase glycogen synthase kinase 3b (GSK3b) forms complexes with adenomatous polyposis coli, axin/conductin, and casein kinase 1 proteins, which in turn bind to soluble β-catenin and facilitate its phosphorylation at codons 29, 33, and 37 (containing serine residues) and codon 41 (containing threonine residues). Phosphorylated β-catenin then binds the E3 ubiquitin ligase β-TrCP and undergoes proteosomal degradation, preventing the accumulation and transcriptional activity of β-catenin. When Wnt ligands bind their receptor complex, the resulting activation of the cytoplasmic protein disheveled inactivates GSK3b, thereby preventing degradation of soluble β-catenin and stabilizing it in the cytoplasm (7). Cytoplasmic β-catenin then translocates to the nucleus, where it heterodimerizes with transcription factors of the T-cell factor/lymphoid enhancer–binding factor (TCF/LEF) family (8–10). Those heterodimers then bind to DNA and activate the expression of specific genes (11, 12). Accumulation of soluble β-catenin is therefore critical for activation of Wnt transcription in the pathway.

Previous studies implicated β-catenin in the pathogenesis of prostate cancer because it localizes in tumor-cell nuclei in 20% to 40% of CRPC specimens (13–15). More recently, another group reported that activation of Wnt/β-catenin signaling is involved in prostate cancer initiation and progression in a mouse model (16, 17). Together, these findings imply that the Wnt canonical pathway is involved in the pathogenesis of a subgroup of advanced prostate cancers.

β-catenin may also act as a coactivator of the AR (18), and it has been proposed that the AR competes with TCF/LEF transcription factors for β-catenin, thus interfering with β-catenin–TCF/LEF signaling (19). However, because the MDA prostate cancer 118b tumor line does not express AR (4), the activation of β-catenin in this model cannot involve that interaction. Therefore, the purpose of this study was to use the AR-null MDA prostate cancer 118b cells to identify downstream target genes of Wnt/β-catenin signaling in prostate cancer.

Materials and Methods

Cell cultures, mutant β-catenin generation, and Western blot analysis

Human MDA prostate cancer 2b cells (20) were propagated in BRFF-HPC1 medium (Athena Enzyme Systems) with 20% FBS (Sigma-Aldrich). The prostate cancer cell lines PC-3 and LNCaP, obtained from the American Type Culture Collection, were maintained in RPMI 1640 medium (GIBCO Invitrogen) with 10% FBS. Short-term cultures of MDA prostate cancer 118b prostate cancer cells (4) were isolated from subcutaneous tumors developed in severe combined immunodeficient (SCID) mice (Charles River Laboratories). Briefly, the tumors were digested in Accumax cell-disaggregation solution (Innovative Cell Technologies), filtered through a 70-micron cell strainer, and then separated with a Ficoll gradient solution. These cells were then cultured in CnT52 medium (Millipore) in petri dishes or in 6-well plates coated with FNC coating mix (Athena Enzyme Systems).

D32G-mutant β-catenin was generated by nucleotide substitution in wild-type (wt) human β-catenin (a gift from Dr. P. McCrea, Department of Biochemistry and Molecular Biology, The University of Texas MD Anderson Cancer Center) with the use of a QuikChange site-directed mutagenesis kit (Stratagene Agilent). The mutant was confirmed by direct sequencing. wt and D32G-mutant human β-catenin were subcloned into p3XFLAG-CMV-7.1 expression vector (Sigma-Aldrich).

Western blotting was carried out following standard procedures. The Flag epitope of p3XFLAG-CMV-7.1 expression vector and β-catenin were detected after incubation with anti-Flag (monoclonal anti-Flag M2-peroxidase; Sigma-Aldrich) or mouse anti–β-catenin (BD Transduction Laboratories) antibodies.

Immunocytochemical and immunohistochemical analyses

For immunocytochemical analysis, MDA prostate cancer 118b, PC-3, and MDA prostate cancer 2b cells grown on

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tissue culture slides were immunostained with antibodies to β-catenin (BD Biosciences) and then with an anti-mouse secondary antibody labeled with Alexa 594 dye; counterstaining was with 4′,6-diamidino-2-phenylindole (DAPI).

Using a Leica TCS Sp5 spectral confocal microscope, we captured immunofluorescent images in which β-catenin was detected at 590-nm excitation and 618-nm emission, and DAPI, at 360-nm excitation and 460-nm emission.

For immunohistochemical analysis, tissue sections (4 μm thick) were treated with Target Retrieval Solution (Dako) and stained with anti–β-catenin (1:100 dilution; Zymed Laboratories) or anti-AR (1:50 dilution; Dako) antibodies. The slides were counterstained with Mayer's hematoxylin (Poly Scientific R & D).

Real-time reverse transcription PCR, β-catenin knockdown, and gene expression analyses

The relative mRNA level for each gene was quantified by using real-time reverse transcription (RT)-PCR with SYBR Green (Applied Biosystems Life Technologies) and a Stratagene Mx3000P quantitative PCR (qPCR) system (Applied Biosystems). cDNA was prepared by TaqMan RT reagents (Roche Applied Biosystems). Gene-specific primers used for cDNA amplification are listed in Supplementary Table S1.

To knock down β-catenin in MDA prostate cancer 118b cells, the cells were transiently transfected with a validated β-catenin–specific siRNA (b-cat-si; s438, Ambion Applied Biosystems) and, as a control, a nontargeted siRNA (control-si), by a Nucleofector kit (Amaxa Lonza). Forty-eight hours after their transfection, cells were collected to isolate total RNA by an RNeasy mini kit (QIAGEN). cDNA was subsequently prepared for comparative gene-array analysis (HuGene 1.0 ST; Affymetrix). The analysis was conducted in quadruplicate on all preparations.

Animals, intrabone injections, and bone tissue sample processing

All animal experiments were conducted in accordance with accepted standards of humane animal care. Six- to 8-week-old intact male CB17 SCID mice (Charles River Laboratories) were given injections into the distal end of 1 femur with control-si–transfected MDA prostate cancer 118b cells and 6 mice, with b-cat-si–transfected MDA prostate cancer 118b cells (1×10^6) according to procedures described elsewhere (4).

Two weeks later, 6 mice were killed (3 from each group: control-si–transfected and b-cat-si–transfected MDA prostate cancer 118b cells), and the femurs bearing MDA prostate cancer 118b tumors were resected, fixed, and embedded in methylmethacrylate by the Bone Histomorphometry Core Facility at MD Anderson (M.W.S.). Tissue sections were stained with von Kossa stain, and Osteo II software, version 8.40.20 (Bioquant), was used to measure the ratio of bone volume to tissue volume.

Six weeks after the intrabone cell injections, the tumor burden of another 6 mice (3 from each group) was monitored indirectly by X-ray analysis. Then the marrow area shown in the X-ray film was outlined in the mice's femurs with the NIH ImageJ software. The relative bone marrow cavity area was calculated by dividing the area in the tumor-bearing bone by the area in the noninjected bone (i.e., contralateral femur).

TCF luciferase reporter assay and transfection

β-catenin–mediated transcription was evaluated by 2 different promoter–reporter constructs. We used a TOP-flash reporter gene construct containing 4 consensus TCF-binding sites (21). A FOP-flash construct with a mutated TCF-binding site was used as a negative control.

MDA prostate cancer 118b, PC-3, and MDA prostate cancer 2b xenograft tumors were harvested, made into single-cell suspensions, and plated to grow to 60% confluence. After 24 hours, cells were transfected with Lipofectamine 2000 (Invitrogen) plus 2 μg of the TOP-flash reporter or the FOP-flash control construct. Transfected cells were harvested 24 hours later. *Renilla* was used as a coreporter vector to normalize transfection efficiency. Reporter assays were done by a luciferase reporter system (Promega).

Human prostate cancer bone metastasis specimens

We tested 27 archived samples from prostate cancer bone metastases selected from a tissue bank supported by the NIH prostate cancer SPORE grant at MD Anderson. All specimens had been obtained after the patients had provided written informed consent for the use of their tissues, according to an Institutional Review Board–approved protocol. All sections were from formalin-fixed, paraffin-embedded tissue specimens; specimens had been decalcified in formic acid. For β-catenin immunostaining, sections were classified according to the percentages of cells with positive nuclear, cytoplasmic, and membranous staining. For AR, we scored the percentage of cells showing positive nuclear immunostaining. Slides were read independently by 2 investigators (V.T. and N.M.N.); evaluations were concordant in 90% of the readings. Differences were resolved by consensus after joint review.

Statistical analyses

Correlations between β-catenin nuclear localization and AR expression were analyzed by the Fisher exact test. Two-sample t tests for equal variance were used to identify differences between the means of the different treatment groups. Statistical significance was set at $P < 0.05$.

Results

Comparative gene expression and immunohistochemical analyses reveal active β-catenin/TCF signaling in MDA prostate cancer 118b cells

Real-time RT-PCR analysis showed that *FGF9*, *BMP4*, *ITF2*, *NrCAM*, and *SOX2* expression was greater in MDA prostate cancer 118b than it was in PC-3 xenografts. The expression patterns of these genes were similarly higher in MDA prostate cancer 118b than in MDA prostate cancer 2b xenografts. AR was highly expressed in only the MDA prostate cancer 2b xenografts. Collectively, these findings

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Table 1. Relative mRNA levels ($\times 10^{-4}$) in various genes in different subcutaneous prostate tumor cell lines compared with the levels in glyceraldehyde-3-phosphate dehydrogenase

Gene	Tumor cell line		
	MDA prostate cancer 118b	PC-3	MDA prostate cancer 2b
FGF9	863 ± 121 ⇑	3 ± 0.2	Undetectable
BMP4	1331 ± 190 ⇑	19 ± 4	Undetectable
ITF2	2783 ± 197 ⇑	46 ± 6	Undetectable
NrCAM	3581 ± 275 ⇑	39 ± 12	41 ± 5
SOX2	7904 ± 121 ⇑	59 ± 10	24 ± 2
OCT4	66 ± 6	62 ± 11	158 ± 31
AR	Undetectable	7 ± 2	2870 ± 820
Cyclin D1	63 ± 27	505 ± 193	823 ± 115
c-Myc	424 ± 57	1500 ± 479	1552 ± 336

NOTE: For each gene, data from 3 independent reactions were used to calculate the means and SDs shown. If no value in the linear range of PCR cycles of a specific RNA was detected, probably because little or no mRNA was expressed in that particular tumor sample, it was considered undetectable. Primers were human specific, and these were tested by mRNA isolated from primary mouse osteoblasts and PC-3 cells grown *in vitro*. Arrows indicate the genes that are upregulated in MDA prostate cancer 118b relative to those in both PC-3 and MDA prostate cancer 2b cells.

(summarized in Table 1) suggest that β-catenin–Wnt signaling is upregulated in MDA prostate cancer 118b cells.

β-catenin was localized in both the cytoplasm and nucleus of the MDA prostate cancer 118b cells but was present in only the membrane of the MDA prostate cancer 2b and PC-3 cells, as assessed by immunohistochemical staining of the cells growing subcutaneously in SCID mice (Fig. 1A). We confirmed the nuclear localization of β-catenin in MDA prostate cancer 118b cells on confocal microscopy (Fig. 1B).

We found high basal levels of TOP-flash reporter transactivation in MDA prostate cancer 118b cells but not in MDA prostate cancer 2b or PC-3 cells (Fig. 1C). Thus, these findings, together with those showing β-catenin nuclear localization in MDA prostate cancer 118b cells, suggest that the Wnt canonical pathway is active and highly upregulated in these cells.

MDA prostate cancer 118b and MDA prostate cancer 118a cells contain mutant β-catenin

We analyzed the sequence of the β-catenin gene and found that MDA prostate cancer 118b cells have a mutation in codon 32, with GAC changed to GGC, that is, aspartic acid (D) substituted for glycine (G). Codon 32 lies within the consensus recognition motif for ubiquitination of β-catenin, so mutations at codon 32 alter ubiquitination and result in transformation of cells expressing this mutation (22, 23). We also analyzed the status of β-catenin in MDA prostate cancer 118a cells, which are derived from a different bone metastasis from the same man. We found that MDA prostate cancer 118a and MDA prostate cancer 118b cells bear the same β-catenin mutation, but no such mutations were found in the MDA prostate cancer 2a, MDA prostate cancer 2b, LNCaP, and PC-3 lines (data not shown).

The *D32G* mutation has been reported to be associated with β-catenin nuclear accumulation in prostate cancer (24), suggesting pathway activation. We subsequently sequenced the entire coding frame sequence of β-catenin in MDA prostate cancer 118b cells and confirmed that D32G is the only β-catenin mutation. Furthermore, we obtained evidence suggesting that MDA prostate cancer 118b cells are heterozygous for the D32G β-catenin mutant (Fig. 2A). These results suggest that mutation leads to stabilization of β-catenin in MDA prostate cancer 118b cells.

D32G-mutant β-catenin is a potent transcription activator

We transiently transfected MDA prostate cancer 2b and PC-3 cells with empty vector or with wt or D32G-mutant β-catenin and assessed TOP-flash reporter transactivation. The D32G mutant significantly induced TCF/LEF-mediated transactivation in both prostate cancer cell lines (Fig. 2B).

Collectively, these results show that the D32G mutant can upregulate β-catenin–TCF signaling in prostate cancer cells.

D32G-mutant β-catenin induces activation of β-catenin/TCF target genes in prostate cancer cells

We next transiently transfected PC-3 cells with empty vector or with Flag–wt or Flag–D32G-mutant β-catenin. Gene expression analysis showed that D32G-mutant β-catenin stimulated increased expression of *BMP4*, *WISP*1, and *FGF9* (known downstream target genes in the β-catenin–TCF pathway) at higher levels than wt β-catenin (Fig. 2C), suggesting that the D32G mutant is a potent transcriptional

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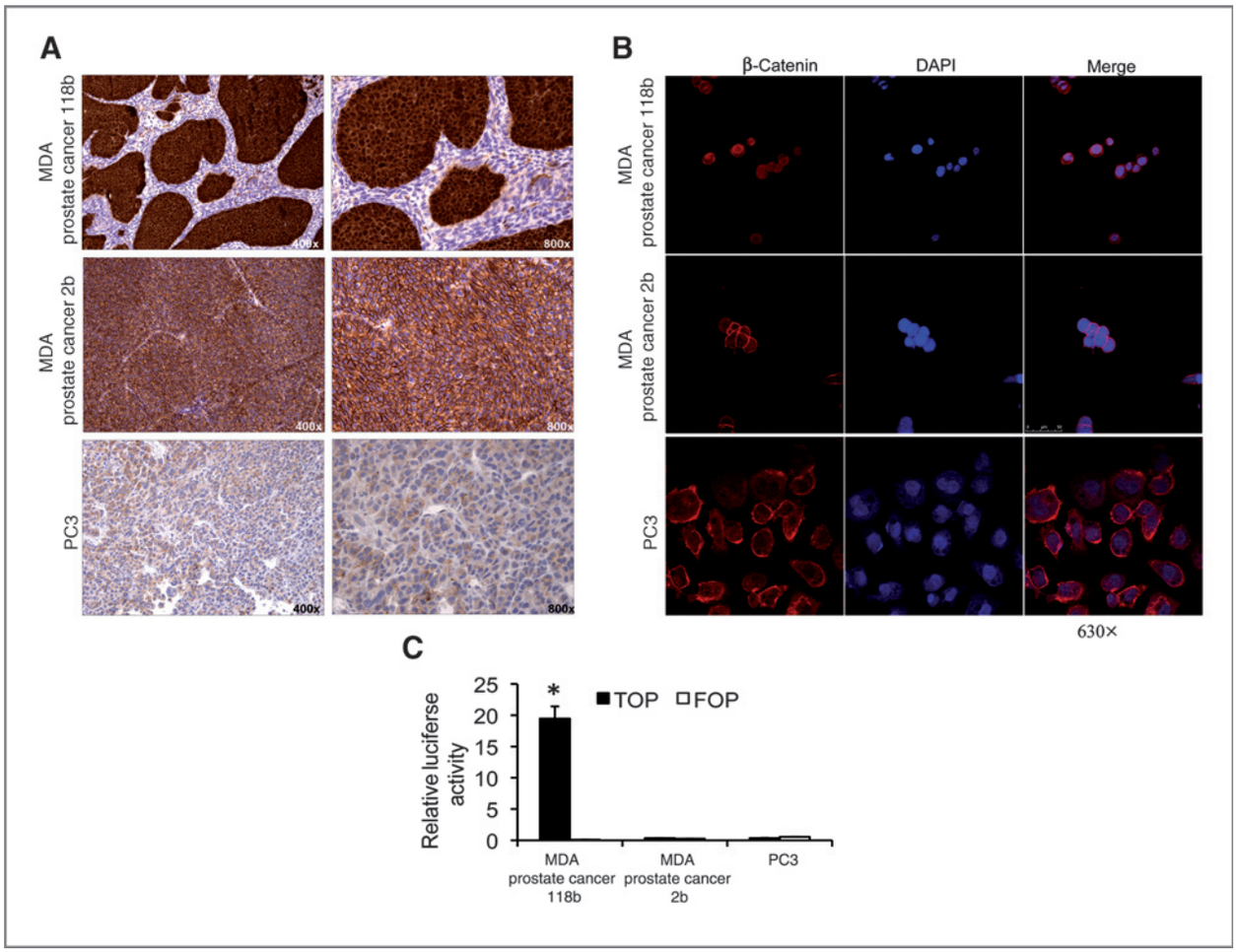

Figure 1. MDA prostate cancer 118b prostate cancer cells have an activated Wnt canonical signaling pathway. A, β-catenin immunohistochemical staining of subcutaneous xenografts with anti–β-catenin antibody showing strong nuclear and cytoplasmic staining on MDA prostate cancer 118b, strong membranous staining on MDA prostate cancer 2b, and diffuse staining on PC-3 cells. Original magnification, 400× and 800× on left and right panels, respectively. B, photomicrographs of those same cell lines immunostained with anti–β-catenin antibody and visualized on confocal microscopy. Original magnification, 630×. C, transcriptional activation of TCF reporter in MDA prostate cancer 118b, MDA prostate cancer 2b, and PC-3 prostate cancer cells. Cells were transfected with the TOP-flash reporter or the FOP-flash control construct. *Renilla* was used as a coreporter vector to normalize transfection efficiency. Reporter assays were conducted with a luciferase reporter system. *, $P < 0.001$ versus MDA prostate cancer 118b cells transfected with the FOP-flash control construct.

activator. In contrast, neither D32G-mutant nor wt β-catenin significantly changed the expression of c-Myc and cyclin D1.

We next assessed the effect of β-catenin silencing in MDA prostate cancer 118b cells. Transient transfection with siRNA (b-cat-si) to knock down β-catenin in MDA prostate cancer 118b cells significantly reduced the β-catenin mRNA level ($P < 0.05$; Fig. 3A) as well as BMP4, WISP1, and cyclin D1 ($P < 0.01$, 0.005, and 0.005, respectively) and modestly reduced the expression of FGF9 ($P < 0.05$). These results suggest that the expression of BMP4, WISP1, and FGF9 in MDA prostate cancer 118b cells is partially regulated by D32G-mutant β-catenin expression because their expression was also activated in PC-3 cells after expression of D32G mutant but not wt β-catenin.

To test the effect of β-catenin knockdown *in vivo*, we injected b-cat-si- and control-si–transfected MDA prostate cancer 118b cells intrafemorally into SCID mice. Two weeks after those injections, the bone volume in the distal end of the femur injected with control-si–MDA prostate cancer 118b cells was significantly lower than it was in the femurs injected with b-cat-si–MDA prostate cancer 118b cells ($P = 0.0268$; Fig. 3B). At 6 weeks after the injections, X-ray analysis revealed a greater bone marrow cavity area in the femurs injected with control-si–MDA prostate cancer 118b cells than that in the femurs injected with b-cat-si–MDA prostate cancer 118b cells ($P = 0.036$; Fig. 3C). Together, the 2- and 6-week results indicate that the tumor burden was reduced in the bones injected with b-cat-si–MDA prostate cancer 118b cells, suggesting that β-catenin silencing reduces the tumorigenicity of MDA prostate cancer 118b

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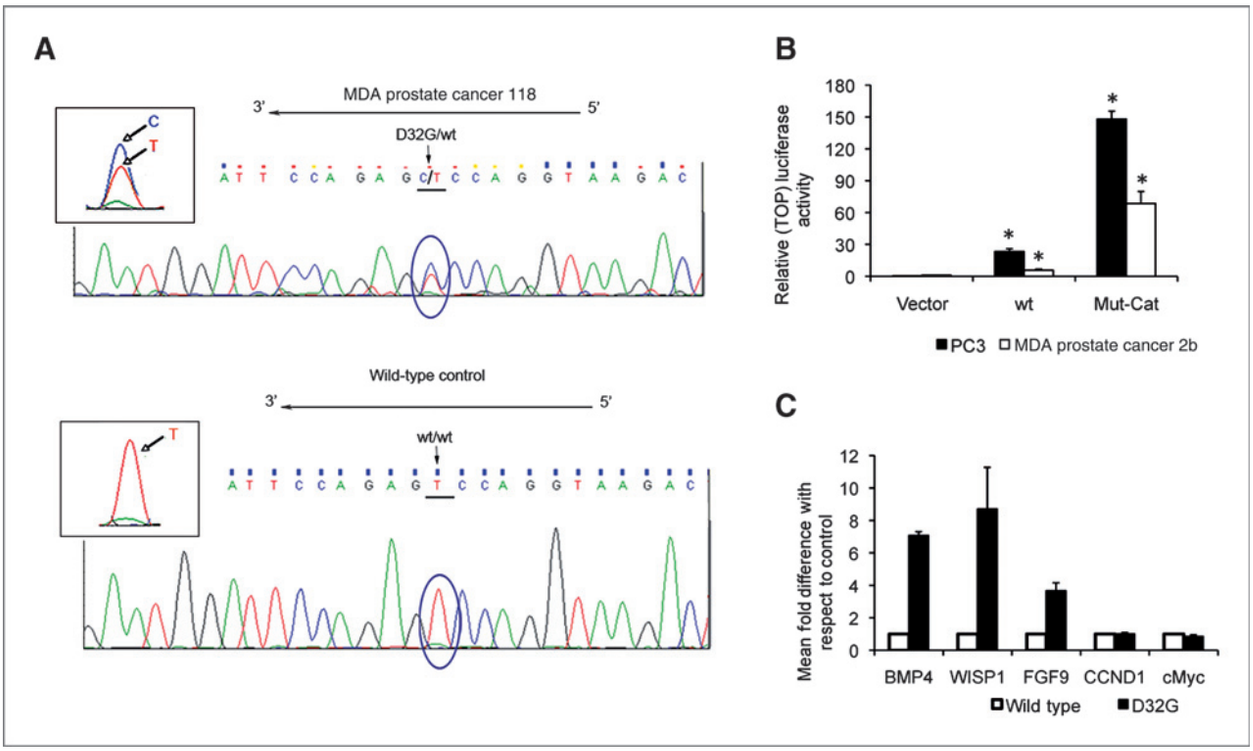

Figure 2. A, β-catenin mutation in MDA prostate cancer 118b cells. The cDNA sequence of exon 3 in MDA prostate cancer 118b cells growing subcutaneously in SCID mice (top) shows the heterozygous T-to-C (D32G) nucleotide mutation. MDA prostate cancer 2b cells were used as the wild-type (wt) control (bottom). Similar results were obtained from sequencing of exon 3 of DNA extracted from the MDA prostate cancer 118b and MDA prostate cancer 2b cells. B, transcriptional activation of TOP-flash reporter in prostate cancer cells transfected with empty vector (vector) or with wt or D32G-mutant β-catenin (Mut-Cat). *, $P < 0.001$ versus empty vector. C, relative mRNA level of various genes 48 hours after transient transfection of PC-3 cells with wt or D32G-mutant β-catenin. The mean mRNA levels and SDs from 3 independent reactions with each gene are expressed relative to those in cells transfected with wt control.

cells. This was as expected, given the known oncogenic properties of β-catenin. Our results also indicate that our strategy to downregulate D32G expression in MDA prostate cancer 118b cells was successful.

To identify novel β-catenin downstream target genes, we subsequently carried out a comparative gene array analysis (HuGene 1.0 ST; Affymetrix) between b-cat-si- and control-si–transfected MDA prostate cancer 118b cells. Initially, the array results appeared to be highly consistent between replicates both within and between treatments. Paired *t* testing identified no genes that were expressed differently between the test and control groups. However, using mixed linear model analysis, we identified 10 genes that were expressed differently between the control-si- and b-cat-si–transfected groups, at the false discovery rate of 0.05 (Fig. 3D). Seven of the 10 were downregulated: *CTNNB1* [intracellular β-catenin complex; ref. 25)]; *Axin2* (a downstream target and negative pathway regulator); *HAS3* and *HAS2* [hyaluronan synthases 3 and 2; HAS2 synthesizes hyaluronan, or hyaluronic acid (HA), a core component of the extracellular matrix (ECM; ref. 26)]; *APCDD1* (adenomatosis polyposis coli downregulated 1); *IRF3* (interferon regulatory factor 3); and *FAM178B* (family with sequence similarity 178 member B). The other 3 were upregulated: *CCDC80* (coiled-coil domain containing 80), *AMIGO2* (adhesion molecule with Ig-like domain 2), and *SLC17A5* (solute carrier family 17, an anion/sugar transporter).

In an independent assay, we also conducted gene expression analysis by using real-time qRT-PCR and confirmed that *CTNNB1* and *Axin2* expression was downregulated in MDA prostate cancer 118b cells transfected with b-cat-si relative to their expression in control-si–transfected cells (by 3.8 and 2.3 times, respectively; data not shown). *Axin2* is a widespread Wnt-axis downstream target gene (27, 28). These results thus confirmed the successful reduction of Wnt canonical pathway activity.

D32G-mutant β-catenin regulates HAS2 expression

Because HA has been shown to predict biochemical recurrence of human prostate cancer (29) and to induce tumorigenesis and metastases in experimental systems (30, 31), we selected HAS2 and HAS3 to further test the role of β-catenin on regulation of their expression. First, we confirmed that the expression of HAS2 and HAS3 is 2.3 times lower in MDA prostate cancer 118b cells transfected with b-cat-si relative to their expression in cells transfected with control-si (data not shown).

To further assess the effect of wt and D32G-mutant β-catenin in HAS2 and HAS3 expression, we transiently transfected PC-3 cells with empty vector or with Flag-wt

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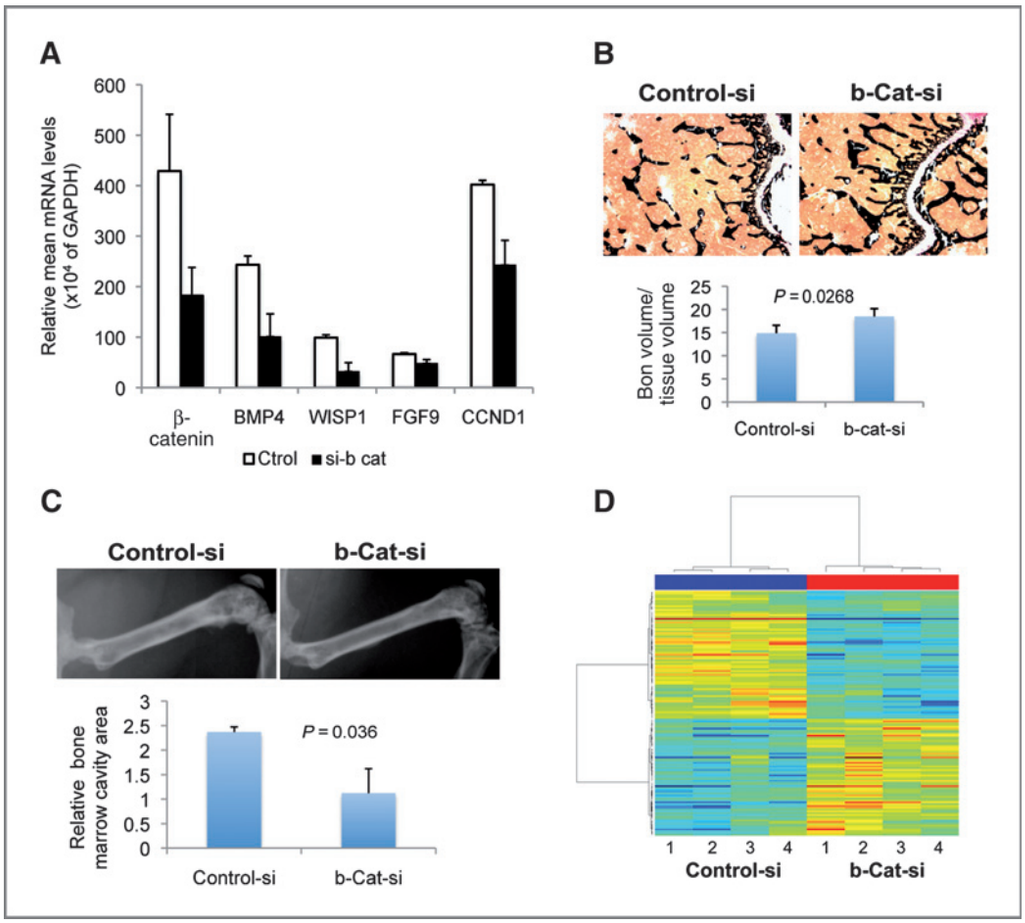

Figure 3. A, the expression of selected genes in MDA prostate cancer 118b cells with knocked down β-catenin. β-catenin was knocked down by transiently transfecting the cells with siRNA (si-b cat). As a control, the cells were transfected with nontargeted siRNA (ctrol). Gene expression was determined by quantitative RT-PCR using total RNA isolated 48 hours after transfection. B, top, representative images of sections from the distal end of femurs 2 weeks after intrabone injections with MDA prostate cancer 118b cells transfected with control siRNA (control-si) or β-catenin siRNA (b-Cat-si): cross sections of undecalcified bone stained with von Kossa (black). Original magnification, 400×. Bottom, ratio of bone volume to tissue volume, as assessed by bone histomorphometric analysis of the undecalcified bone sections. C, top, X-ray images of rear limbs of representative mice 6 weeks after intrafemoral injection of control-si- or b-cat-si–transfected MDA prostate cancer 118b cells. Bottom, relative bone marrow cavity area measured with NIH-ImageJ. D, heat map of the genes expressed differently between control-si- and b-cat-si–transfected MDA prostate cancer 118b cells. The "lanes" labeled 1 through 4 for each siRNA are experimental replicates.

or Flag–D32G-mutant β-catenin to establish whether that would result in activation of the Wnt/β-catenin pathway. For that, we assessed β-catenin protein levels and transcriptional activation of *Axin2*, a well-recognized Wnt–β-catenin–regulated gene (27, 28). We found similar levels of β-catenin expression in PC-3 cells transfected with wt and D32G-mutant β-catenin, indicating the effectiveness of the transfection (Fig. 4A). Axin2 expression was significantly induced by both wt and D32G-mutant β-catenin ($P = 0.003$ and 0.005, respectively; Fig. 4B), indicating that the Wnt canonical signaling pathway was upregulated. However, D32G-mutant β-catenin increased the expression of Axin2 18-fold, whereas wt β-catenin increased it by only 1.8-fold, which suggests that the D32G mutant is a more potent transactivator than the wt is or that it accumulates more efficiently in the cells' nuclei than the wt does.

When we assessed HAS2 and HAS3 expression in those transfected PC-3 cells, we found higher HAS2 expression in the PC-3 cells transfected with the D32G-mutant β-catenin than in those transfected with wt β-catenin or empty vector ($P = 0.007$; Fig. 4C). No HAS3 induction was observed with either mutant or wt β-catenin (Fig. 4D). Together, these results suggest that HAS2 is a downstream target of β-catenin in prostate cancer cells.

β-catenin and AR nuclear accumulation are inversely correlated in prostate cancer bone metastases

To establish whether β-catenin's accumulation in the cytoplasm and nuclei of AR-negative human prostate cancer cells was an isolated (or low-incidence) occurrence in MDA prostate cancer 118b cells, we stained sections from 27 archived CRPC bone metastases (excluding the case of origin of the MDA prostate cancer 118 xenograft) with

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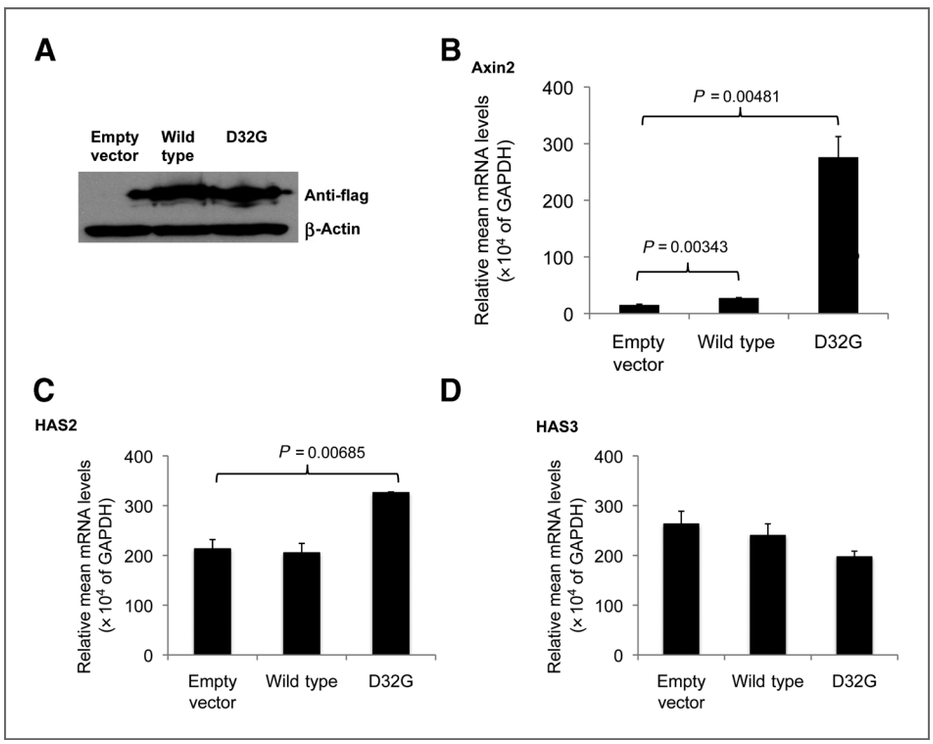

Figure 4. A, Western blot analysis of lysates from PC-3 cells transiently transfected with empty vector or with wild-type or D32G-mutant β-catenin with anti-Flag or β-actin antibodies. Relative mRNA levels (vs. those in GAPDH) of Axin2 (B), HAS2 (C), and HAS3 (D) in PC-3 cells 48 hours after transient expression with empty vector or with wild-type or D32G-mutant β-catenin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

antibodies against AR and β-catenin in consecutive sections. In accordance with previously reported results (13–15), β-catenin nuclear staining was found in about 40% of the specimens. In addition, we found that the sample with the highest expression of β-catenin was negative for AR expression, a pattern similar to the one we found in MDA prostate cancer 118b cells (Fig. 5). Furthermore, we found a marginally statistically significant inverse association between β-catenin nuclear localization and AR nuclear staining ($P = 0.056$, Fisher exact test; Fig. 5).

These results suggest that the β-catenin:AR ratio defines a subpopulation of men with prostate cancer bone metastases.

Discussion

The results of these studies showed that the AR-negative prostate cancer cell line MDA prostate cancer 118b expresses D32G-mutant β-catenin, which results at least partially in nuclear β-catenin accumulation and active β-catenin–TCF signaling. We also showed—for the first time, to our knowledge—that D32G-mutant β-catenin induces expression of HAS2, a membrane-bound synthase of HA, a major constituent of the ECM (32). Our results concur with those previously published that β-catenin stimulates HA production and also lend support to previous observations that the transforming effects of β-catenin depend on HA production and HA–cell interactions (33). Earlier studies also showed that increased HA production promotes anchorage-independent growth and cell invasiveness and that HA expression in prostate cancer biopsy specimens correlates with biochemical recurrence after radical prostatectomy (29, 33). Our findings add to these by suggesting that β-catenin–induced HAS2 plays an important role in the progression of prostate cancer.

Abundant evidence also exists showing that activation of downstream target genes by β-catenin–TCF complex leads to increased proliferation and decreased differentiation of epithelial cells (34). Exon 3 is the hot spot for mutation in various malignant human tumors because it contains not only the consensus sequences for multiple kinases but also a ubiquitination consensus sequence. At that exon, the most common mutations occur at serine and threonine residues Ser37, Thr41, and Ser45. In addition, the nonphosphorylatable residues Asp32 and Gly34 rank within the top 6 mutations identified in human tumors: β-catenin residues in the destruction motif Asp32, Ser33, Gly34, and Ser37 directly bind β-TrCP1 (35), and mutation of Asp32 greatly reduces the ability of β-catenin to be ubiquitinated by β-TrCP1 without altering the ability to act as a substrate for GSK3b (23). Mutated Asp32 also mediates a transforming phenotype for growth and migration in the stable nontransformed epithelial cell line MDCK (23).

Furthermore, the Wnt signaling pathway has previously been implicated in the progression of prostate cancer (19). Various of the amino acid substitution mutations located at exon 3 of β-catenin have been found in about 5% of prostate cancer specimens tested by several groups of investigators (24, 36, 37). For example, in 1 previous study of a codon-32

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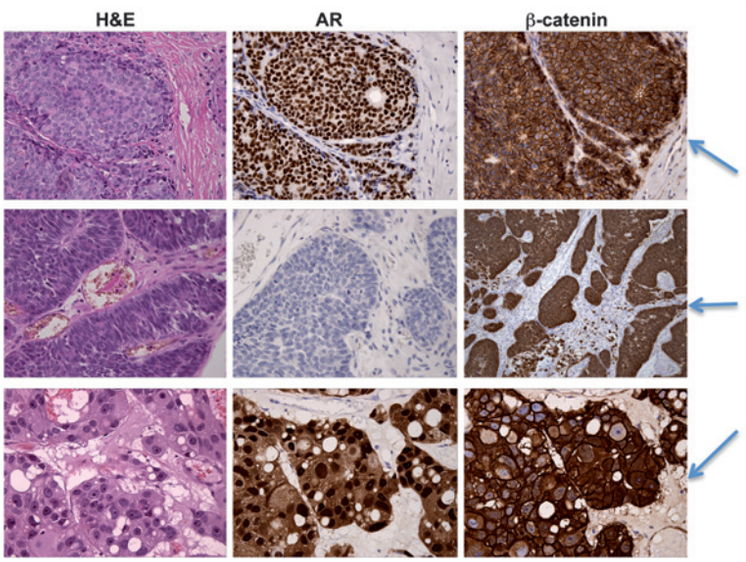

Summary of β-catenin and AR expression in 27 human prostate cancer bone metastases

Number of cases	β-catenin nuclear	β-catenin cytopl	β-catenin membrane	AR
15	0	60–100	100	60–100
1		30	100	60–100
1	10	90	90	0
1	20	100	0	0
8	5–50	60–100	60–100	60–100
1	5	60	70	20

β-catenin nuclear localization and AR expression

	AR >70	AR < 20
β-catenin nuclear 0	16	0
β-catenin nuclear 5-30	8	3

$P = 0.056$, Fisher' exact test

Figure 5. Immunohistochemical analysis of β-catenin and AR in human prostate cancer bone metastases. The photomicrographs illustrate different groups of prostate cancers characterized by β-catenin staining in the cell membrane and positive AR nuclei staining (top), nuclear/cytoplasmic β-catenin staining and no or low AR nuclear staining (middle), and nuclear/cytoplasmic and membranous β-catenin staining and AR nuclear staining (bottom). Original magnification, 400×. The table on the right summarizes β-catenin and AR expression in 27 human prostate cancer bone metastases, grouped according to the percentage of cells with positive staining of the markers indicated. The table beneath the photomicrographs summarizes the number of cases with AR nuclear staining in more than 70% (AR > 70) or less than 20% (AR < 20) of the cells that also have no β-catenin nuclear staining (β-catenin nuclear 0) and the number of those that have β-catenin nuclear staining in 5% to 30% of cells (β-catenin nuclear 5–30). $P = 0.056$, Fisher exact test. H&E, hematoxylin and eosin staining; Cytopl, cytoplasm.

mutant of β-catenin, D32A, in which aspartate is mutated to alanine, expression of the D32A mutant in a nontransformed epithelial cell line increased oncogenic cellular transformation. In addition, a slightly different codon-32 mutant of β-catenin, D32G, in which aspartate is mutated to glycine, was identified in MDA prostate cancer 118b cells and, as our results have confirmed, that mutation is associated with β-catenin nuclear accumulation in prostate cancer (24), suggesting that the Wnt canonical pathway is activated. Thus, although mutations in β-catenin have been reported in only 5% of cases of prostate cancer, the high transcription activity that results from the D32G mutant is an important tool for identifying downstream target genes in prostate cancer.

Moreover, β-catenin may act as a coactivator of the AR (18), and it has been proposed that the AR competes with TCF/LEF for β-catenin, thus interfering with β-catenin–TCF/LEF signaling (19). Our finding an inverse association between β-catenin nuclear localization and AR expression in human prostate cancer bone metastases supports the concept that the β-catenin:AR ratio may help to identify different subpopulations of patients with prostate cancer who may require different management of the disease.

It has also been reported that β-catenin–LEF-1 signaling is activated after epithelial–mesenchymal transition (EMT; thought to mediate invasive and metastatic behavior during cancer progression) and that β-catenin signaling contributes to the maintenance of EMT (38, 39). Recent reports indicate that androgens induce EMT in prostate cancer epithelial cells and that expression levels of AR correlate inversely with androgen-mediated EMT in those cells (40). Thus, low AR content may be required for the EMT phenotype by enabling β-catenin–LEF-1 signaling. Together, this evidence and our results suggest

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that activation of β-catenin in the presence of low AR levels serves as an alternative mechanism of androgen-induced EMT in prostate cancer epithelial cells.

We conclude that our identification of a previously unknown downstream target gene of activated Wnt–β-catenin, *HAS2*, combined with high nuclear localization of β-catenin in prostate cancer cells with little or no AR expression may define a subpopulation of men with bone metastatic prostate cancer whose disease requires a different form of treatment than those used in other men with prostate cancers. This pattern of findings may thus help clinicians provide individualized therapy for some men with advanced prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Burd CJ, Morey LM, Knudsen KE. Androgen receptor corepressors and prostate cancer. Endocr Relat Cancer 2006;13:979–94.
2. Sun S, Sprenger CC, Vessella RL, Haugk K, Soriano K, Mostaghel EA, et al. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J Clin Invest 2010;120:2715–30.
3. Watson PA, Chen YF, Balbas MD, Wongvipat J, Socci ND, Viale A, et al. Constitutively active androgen receptor splice variants expressed in castration-resistant prostate cancer require full-length androgen receptor. Proc Natl Acad Sci U S A 2010;107:16759–65.
4. Li ZG, Mathew P, Yang J, Starbuck MW, Zurita AJ, Liu J, et al. Androgen receptor-negative human prostate cancer cells induce osteogenesis in mice through FGF9-mediated mechanisms. J Clin Invest 2008;118:2697–710.
5. The Wnt home page. Wnt target genes. Stanford: 1997–2010 Roel Nusse. [cited 2010 Oct]. Available from: http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes.
6. Yochum GS, McWeeney S, Rajaraman V, Cleland R, Peters S, Goodman RH. Serial analysis of chromatin occupancy identifies beta-catenin target genes in colorectal carcinoma cells. Proc Natl Acad Sci U S A 2007;104:3324–9.
7. van Es JH, Barker N, Clevers H. You Wnt some, you lose some: oncogenes in the Wnt signaling pathway. Curr Opin Genet Dev 2003;13:28–33.
8. Molenaar M, van de Wetering M, Oosterwegel M, Peterson-Maduro J, Godsave S, Korinek V, et al. XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos. Cell 1996;86:391–9.
9. van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Es J, Loureiro J, et al. Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTCF*. Cell 1997;88:789–99.
10. Behrens J, von Kries JP, Kühl M, Bruhn L, Wedlich D, Grosschedl R, et al. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 1996;382:638–42.
11. He T-C, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-*MYC* as a target of the APC pathway. Science 1998;281:1509–12.
12. Tetsu O, McCormick F. β-Catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–6.
13. Chesire DR, Ewing CM, Gage WR, Isaacs WB. *In vitro* evidence for complex modes of nuclear β-catenin signaling during prostate growth and tumorigenesis. Oncogene 2002;21:2679–94.
14. de la Taille A, Rubin MA, Chen M-W, Vacherot F, de Medina SG, Burchardt M, et al. β-Catenin-related anomalies in apoptosis-resistant and hormone-refractory prostate cancer cells. Clin Cancer Res 2003;9:1801–7.
15. Yardy GW, Brewster SF. Wnt signalling and prostate cancer. Prostate Cancer Prostatic Dis 2005;8:119–26.
16. Yu X, Wang Y, DeGraff DJ, Wills ML, Matusik RJ. Wnt/β-catenin activation promotes prostate tumor progression in a mouse model. Oncogene 2011;30:1868–79.
17. Yu X, Wang Y, Jiang M, Bierie B, Roy-Burman P, Shen MM, et al. Activation of β-catenin in mouse prostate causes HGPIN and continuous prostate growth after castration. Prostate 2009;69:249–62.
18. Terry S, Yang X, Chen M-W, Vacherot F, Buttyan R. Multifaceted interaction between the androgen and Wnt signaling pathways and the implication for prostate cancer. J Cell Biochem 2006;99:402–10.
19. Verras M, Sun Z. Roles and regulation of Wnt signaling and β-catenin in prostate cancer. Cancer Lett 2006;237:22–32.
20. Navone NM, Olive M, Ozen M, Davis R, Troncoso P, Tu SM, et al. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clin Cancer Res 1997;3:2493–500.
21. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, et al. Constitutive transcriptional activation by a β-catenin–Tcf complex in $APC^{-/-}$ colon carcinoma. Science 1997;275:1784–7.
22. Al-Fageeh M, Li Q, Dashwood WM, Myzak MC, Dashwood RH. Phosphorylation and ubiquitination of oncogenic mutants of β-catenin containing substitutions at Asp32. Oncogene 2004;23:4839–46.
23. Provost E, McCabe A, Stern J, Lizardi I, D'Aquila TG, Rimm DL. Functional correlates of mutation of the Asp32 and Gly34 residues of beta-catenin. Oncogene 2005;24:2667–76.
24. Chesire DR, Ewing CM, Sauvageot J, Bova GS, Isaacs WB. Detection and analysis of β-catenin mutations in prostate cancer. Prostate 2000;45:323–34.
25. Fuerer C, Nusse R, Ten Berge D. Wnt signalling in development and disease. Max Delbrück Center for Molecular Medicine meeting on Wnt Signaling in Development and Disease. EMBO Rep 2008;9:134–8.
26. Viola M, Vigetti D, Genasetti A, Rizzi M, Karousou E, Moretto P, et al. Molecular control of the hyaluronan biosynthesis. Connect Tissue Res 2008;49:111–4.
27. Jho E-H, Zhang T, Domon C, Joo C-K, Freund J-N, Costantini F. Wnt/β-Catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002;22:1172–83.
28. Lustig B, Jerchow B, Sachs M, Weiler S, Pietsch T, Karsten U, et al. Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol 2002;22:1184–93.

DOI:10.1158/1078-0432.CCR-11-2521

29. Gomez CS, Gomez P, Knapp J, Jorda M, Soloway MS, Lokeshwar VB. Hyaluronic acid and HYAL-1 in prostate biopsy specimens: predictors of biochemical recurrence. J Urol 2009;182:1350–6.
30. Bharadwaj AG, Kovar JL, Loughman E, Elowsky C, Oakley GG, Simpson MA. Spontaneous metastasis of prostate cancer is promoted by excess hyaluronan synthesis and processing. Am J Pathol 2009;174:1027–36.
31. Simpson MA. Concurrent expression of hyaluronan biosynthetic and processing enzymes promotes growth and vascularization of prostate tumors in mice. Am J Pathol 2006;169:247–57.
32. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23:435–61.
33. Zoltan-Jones A, Huang L, Ghatak S, Toole BP. Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. J Biol Chem 2003;278:45801–10.
34. Clevers H. Wnt/β-Catenin signaling in development and disease. Cell 2006;127:469–80.
35. Wu G, Xu G, Schulman BA, Jeffrey PD, Harper JW, Pavletich NP. Structure of a β-TrCP1-Skp1-β-catenin complex: destruction motif binding and lysine specificity of the SCFβ-TrCP1 ubiquitin ligase. Mol Cell 2003;11:1445–56.
36. Voeller HJ, Truica CI, Gelmann EP. β-Catenin mutations in human prostate cancer. Cancer Res 1998;58:2520–3.
37. Gerstein AV, Almeida TA, Zhao G, Chess E, Shih I-M, Buhler K, et al. *APC/CTNNB1* (β-catenin) pathway alterations in human prostate cancers. Genes Chromosomes Cancer 2002;34:9–16.
38. Eger A, Stockinger A, Schaffhauser B, Beug H, Foisner R. Epithelial mesenchymal transition by c-Fos estrogen receptor activation involves nuclear translocation of β-catenin and upregulation of β-catenin/lymphoid enhancer binding factor-1 transcriptional activity. J Cell Biol 2000;148:173–88.
39. Li J, Zhou BP. Activation of β-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. BMC Cancer 2011;11:49.
40. Zhu ML, Kyprianou N. Role of androgens and the androgen receptor in epithelial-mesenchymal transition and invasion of prostate cancer cells. FASEB J 2010;24:769–77.