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Wnt3a Growth Factor Induces Androgen Receptor-Mediated Transcription and Enhances Cell Growth in Human Prostate Cancer Cells

Meletios Verras,[1,2] Jeffrey Brown,[3] Xiaomeng Li,[1,2] Roel Nusse,[3] and Zijie Sun[1,2]

Departments of [1]Urology, [2]Genetics, and [3]Developmental Biology and Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California

ABSTRACT

The Wnt signaling pathway plays a critical role in embryogenesis and tumorigenesis. However, biological roles of Wnt growth factors have not been fully characterized in prostate development and the pathogenesis of prostate cancer. In this study, we used Wnt3a-conditioned medium (Wnt3a-CM) and purified Wnt3a proteins to investigate whether there is a direct effect of Wnt3a on androgen receptor (AR)-mediated transcription and to determine its role in the growth of prostate cancer cells. We demonstrated that Wnt3a-CM either induces AR activity in the absence of androgens or enhances AR activity in the presence of low concentrations of androgens, whereas purified Wnt3a showed a pronounced effect in the presence of low concentrations of ligands. We also showed that Wnt3a-CM and the purified Wnt3a enhance the level of cytosolic and nuclear β-catenin, suggesting an involvement of β-catenin in this regulation. Moreover, treatment of LNCaP cells with Wnt3a-CM and purified Wnt3a significantly enhances cell growth in the absence of androgens. Our findings demonstrate that Wnt3a plays an important role in androgen-mediated transcription and cell growth. These results suggest a novel mechanism for the progression of prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer deaths in the United States (1). The androgen signaling pathway, which is mainly mediated through the androgen receptor (AR), is important for the normal and neoplastic development of prostate cells (2, 3). Androgen ablation is an effective treatment for the majority of patients with advanced prostate cancer (4). However, most of the patients develop androgen-insensitive prostate cancer within 2 years, for which there is currently no effective treatment. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (3, 5). Recently, several lines of evidence have led to an increased interest in defining the possible role of Wnt signaling in the development and progression of prostate cancer [please see the review by Chesire and Isaacs (6)].

The Wnt ligands, of which there are more than 19 closely related but distinct secreted cysteine-rich glycoproteins, have been characterized according to their roles in early development and tumorigenesis.[4] Evidence from recent studies suggests critical roles for the Wnt ligands in controlling cell proliferation, adhesion, survival, movement, and polarity (7, 8). Receptors for the Wnt proteins are members of the Frizzled family. In vertebrates, Wnt proteins activate different intracellular signaling cascades either through the "canonical" or "non-canonical" pathways (9). In the canonical pathway, secreted Wnt ligands bind to Frizzled and regulate the stability of β-catenin, a key component of Wnt signaling. In the absence of a Wnt signal, β-catenin is constitutively down-regulated by the multicomponent destruction complex containing glycogen synthase kinase 3β, axin, and APC, which promotes phosphorylation on the serine and threonine residues in the NH_2-terminal region of β-catenin and thereby targets it for degradation through the ubiquitin proteasome pathway (10). Wnt signaling inhibits this process, which leads to accumulation of β-catenin in the nucleus and promotes the formation of transcriptionally active complexes with lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) transcription factors (11, 12).

Wnt signaling and its key component, β-catenin, have been implicated in human malignancy (13, 14). The link between stabilized β-catenin and tumorigenesis was considerably strengthened by discoveries of mutations in the destruction complex and in β-catenin itself in a variety of human tumors (15). Loss of control of intracellular β-catenin levels through mutation in β-catenin itself and/or other components of the protein degradation complex has been reported in prostate cancer samples (16, 17). However, only a small proportion of prostate cancer samples possessed these mutations, suggesting that other possible mechanisms may be involved in the regulation. It has been shown that loss of E-cadherin can result in an increase of the cellular β-catenin in prostate cancer cells (18). Overexpression of E-cadherin in E-cadherin–negative tumor cells decreases cellular β-catenin levels and reduces AR-mediated transcription (19).

A protein–protein interaction between the AR and β-catenin has been identified (19–21). Through this interaction, β-catenin acts as an AR coactivator, augmenting AR-mediated transcription (19). These data provided an additional line of evidence linking Wnt/β-catenin to the androgen signaling pathway in the growth and progression of prostate cancer.

Potential roles for Wnt in tumorigenesis were suggested previously (22, 23). However, the molecular mechanisms by which Wnt signaling regulates the growth and progression of tumor cells are unclear. Knowledge regarding Wnt signaling in the pathogenesis of prostate cancer is lacking. In this study, we examine the role of Wnt 3A in the regulation of androgen signaling in prostate cancer cells. Intriguingly, we demonstrated that Wnt3a induces AR-mediated transcription and cell growth in a ligand-independent manner. These findings provide the first line of evidence that the Wnt growth factor can regulate and interact with the androgen signaling pathway in prostate cancer cells, which suggests a novel mechanism for the development of androgen-insensitive prostate cancer.

MATERIALS AND METHODS

Cell Culture and Conditioned Medium Production. The monkey kidney cell line CV-1 and human prostate cell lines DU145 and PC3 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS; HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T medium (Invitrogen, Carlsbad, CA) with 5% FCS. Wnt3a-conditioned medium (Wnt3a-CM) and L cell control medium (L-CM) were prepared as described previously (24). Briefly, mouse L cells stably transfected with a Wnt3a cDNA driven by the rat phosphoglycerokinase gene promoter were cultured in DMEM supplemented with 10% charcoal-stripped FCS (CS-FCS) for 4 days. The Wnt3a-CM was then harvested, centrifuged at 1,000 × *g* for 15 minutes, and filtered using 0.45 μm cellulose

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Requests for reprints: Zijie Sun, Departments of Urology and Genetics, R135, Edwards Building, Stanford University School of Medicine, Stanford, CA 94305-5328. E-mail: zsun@stanford.edu.

[4] http://www.stanford.edu/~rnusse/wntwindow.html.

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acetate bottle top filters (Corning, New York, NY). L-CM was prepared under the same conditions from L cells stably transfected with the pGKneo vector alone (24).

Plasmid Construction. The pGL3-OT and pGL3-OF constructs were the gifts of Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). The pcDNA-TCF-1 construct was provided by Dr. H. C. Clevers (Center for Biomedical Genetics, Utrecht, the Netherlands). The pPSA7kb-luc plasmid was obtained from Dr. Jan Trapman (Department of Pathology, Erasmus University, Rotterdam, The Netherlands) (25). A cytomegalovirus-driven β-galactosidase (β-gal) reporter was generated by cloning the lacZ gene into the pcDNA3 vector (19). A double-stranded oligonucleotide corresponding to the human AR cDNA sequence (5′-GGT-GTCACTATGGAGCTCTCA-3′, amino acids 568–575) was synthesized and cloned into the pBS/U6 vector, provided by Dr. Yang Shi (Harvard Medical School, Boston, MA) to make the short hairpin RNA (shRNA) construct (26).

Transfection, Luciferase, and β-gal Assays. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen). Cells were incubated with Wnt3a-CM or L-CM in the presence or absence of dihydrotestosterone (DHT) 24 hours after transfection. After an 18- to 24-hour incubation, cells were harvested, and the luciferase and β-gal activities were measured. The relative light units (RLU) from individual transfections were normalized using β-gal activity in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as mean RLU/β-gal ($\pm$SD).

Preparation of Cell Fractions. LNCaP cells treated with Wnt3a-CM or the control L-CM were grown to confluence in 6-well plates, washed once with PBS, and harvested by scraping. Cells were then centrifuged at $750 \times g$ for 2 minutes, resuspended in a hypotonic buffer [10 mmol/L Tris-HCl (pH 7.8), 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin] and incubated on ice for 10 minutes. The cells were lysed by Dounce homogenization and then centrifuged at $2,000 \times g$ for 30 minutes to pellet unlysed cells and nuclei. The cytosolic fraction was obtained by further fractionation at $100,000 \times g$ for 1 hour.

Northern Blotting. Total RNA from LNCaP cells treated with Wnt3a-CM or L-CM in the presence or absence of DHT was isolated using a RNAwiz kit (Ambion, Austin, TX). Six micrograms of RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ), and hybridized with a DNA fragment (amino acids 1–261) derived from the human prostate-specific antigen (PSA) gene. Hybridization was performed overnight at 65°C in 0.5 mol/L sodium phosphate (pH 7.2), 1% bovine serum albumin, and 7% SDS. The blots were stripped and rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase probe (27).

Immunoprecipitation and Western Blotting. Coimmunoprecipitation assays were carried out essentially as described previously (28). Proteins were eluted by boiling in SDS-sample buffer, resolved by 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membranes were then probed with a 1:500 dilution of a polyclonal antibody against the NH_2 terminus of AR (Upstate, Charlottesville, VA) or an anti–β-catenin monoclonal antibody. Proteins were detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The anti-Wnt3a polyclonal antibody was generated and used in the study.

Immunofluorescence. Cells were cultured in 8-well Lab Tek chambered cover slides (Nalge Nunc International, Naperville, IL), fixed in 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were then incubated with anti–β-catenin monoclonal antibody (Signaling Transduction Laboratories, Lexington, KY) for 1 hours and labeled with anti-mouse 594 Alexa secondary antibody (Molecular Probes, Eugene, OR). The nuclei were counterstained with 10 μg/mL Hoechst (Molecular Probes). Samples were analyzed with a Zeis LSM confocal laser scanning microscope.

Cell Proliferation and Colony Formation. Approximately 2,000 cells per well were plated and cultured in the presence of either Wnt3a-CM or L-CM and then harvested at different time points. Proliferation assays were carried out using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) kit (Promega, Madison, WI). Cell numbers were determined by absorbance at 490 nm as suggested by the manufacturer. For colony formation assay, LNCaP cells were plated in 6-well plates (500–1,000 cells per well) for 24 hours and then maintained in Wnt3a-CM or L-CM or in DMEM containing purified Wnt3a proteins for 10 to 12 days. The cells were stained with crystal violet (Sigma, St. Louis, MO), and colonies containing more than 50 cells were counted. Colony assays were performed a minimum of three times, and the results are reported as a mean of three experiments.

Purification of Wnt3a. Purified Wnt3a proteins were isolated as described previously (29). A detailed protocol can also be found on-line.[4]

RESULTS

Wnt3a-CM Enhances AR-Mediated Transcription in a Ligand-Independent Manner. The Wnt pathway has been implicated in the growth and differentiation of various tissues and organs. Recent data showing that β-catenin, a key player in the Wnt pathway, interacts with the AR provided a direct link between Wnt signaling and the pathogenesis of prostate cancer (19–21). In this study, we first used Wnt3a-CM to directly investigate the role of Wnt signaling in prostate cancer cells. Using a specific antibody, we verified the expression of Wnt3a in the Wnt3a-CM prepared from the mouse L cells (Fig. 1*A*). We found that treatment with Wnt3a-CM of both mouse L and DU145 cells increased the level of cytosolic β-catenin (Fig. 1*B*). Moreover, the Wnt3a-CM induced β-catenin–mediated TCF-1 transcription (Fig. 1*C*). These results are consistent with a previous report and confirmed the properties of the Wnt3a-CM (24).

To evaluate the effect of Wnt3a on AR-mediated transcription, we transfected a luciferase reporter driven by the 7-kb PSA gene promoter, an AR-regulated target gene (30), into LNCaP cells. The cells were cultured in the presence or absence of DHT with or without Wnt3a-CM. As shown in Fig. 2*A*, Wnt3a-CM significantly increased endogenous AR-mediated transcription from the PSA promoter. Intriguingly, cells treated with the Wnt3a-CM showed an approximately

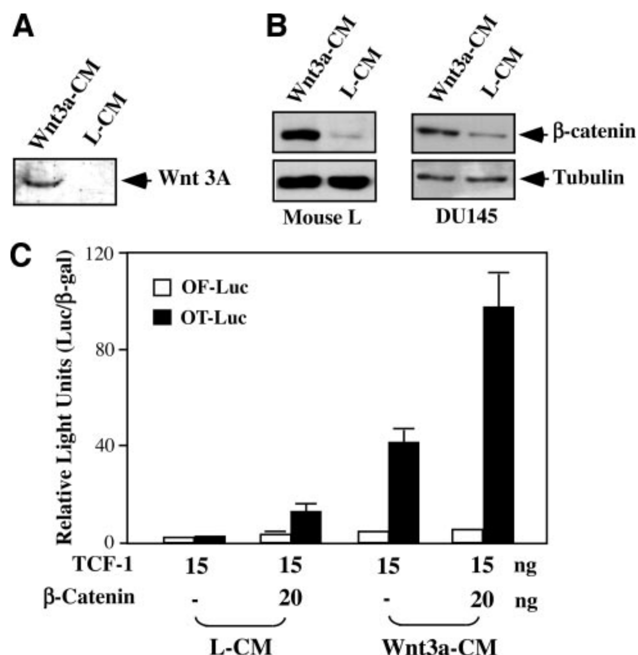

Fig. 1. Wnt3a-CM stabilizes β-catenin and stimulates TCF-mediated transcription. *A*. Ten microliters of Wnt3a-CM and control medium were analyzed by Western blot with a rabbit polyclonal antibody against Wnt3a. *B*. Mouse L and DU145 (a human prostate cancer cell line) cells were cultured with either Wnt3a-CM or L-CM for 20 hours. Whole cell lysates were prepared and analyzed by Western blot with an anti–β-catenin antibody. The samples were also probed with an anti-tubulin antibody to verify equal loading. *C*. CV-1 cells were transiently transfected with 100 ng of pGL3-OT (*OT-Luc*) or the inactive mutant pGL3-OF (*OF-Luc*), 25 ng of pcDNA3-β-gal (β-*gal*), and other plasmids as identified in the figure. The transfected cells were incubated in DMEM with 5% FCS for 24 hours, washed, and then cultured with either Wnt3a-CM or L-CM for another 24 hours. The cells were harvested, and the luciferase and β-gal activities were measured. Luciferase activity is reported as RLU (luciferase/β-gal) and represented as mean $\pm$ SD.

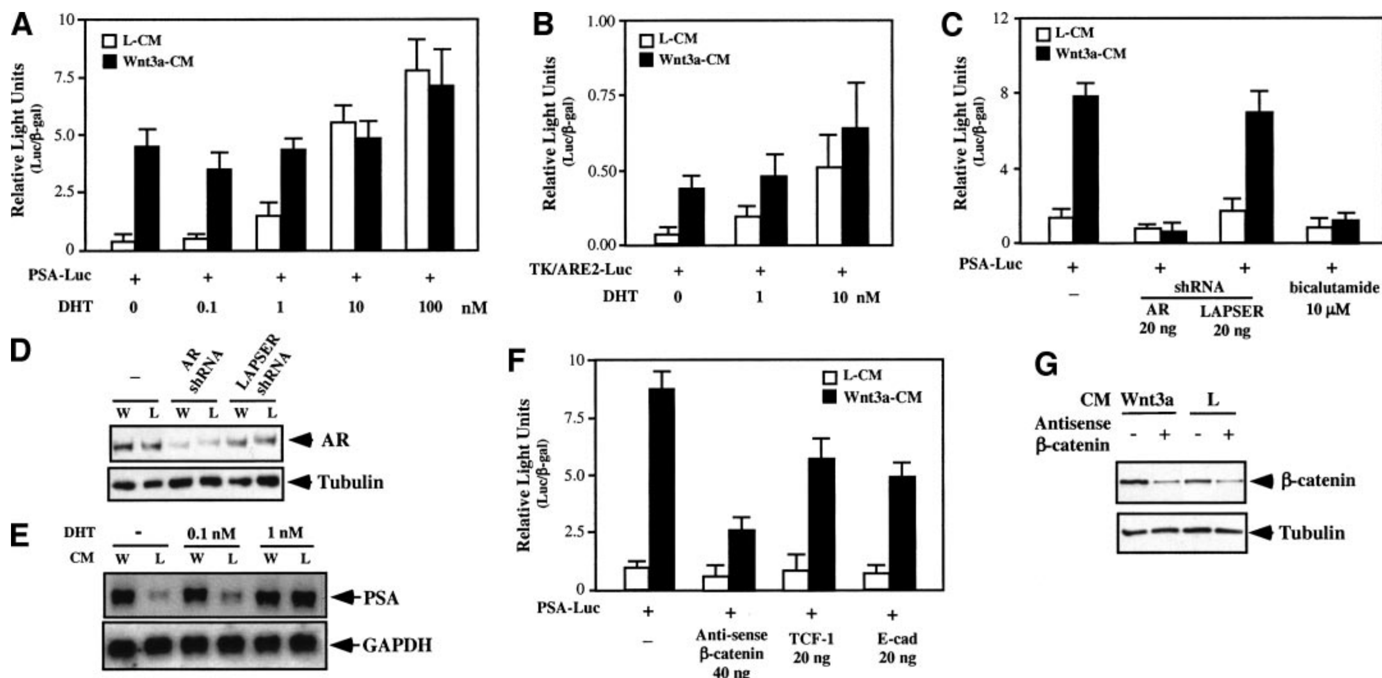

Fig. 2. Wnt3a-CM induces AR-mediated transcription. *A*. LNCaP cells were transiently transfected with 100 ng of PSA7kb-luc reporter (*PSA-Luc*) and 25 ng of pcDNA3-β-gal and incubated in T-medium with 5% CS-FCS for 16 hours. The cells were washed and incubated with Wnt3a-CM or L-CM in the presence of different amounts of DHT for another 24 hours. Luciferase and β-Gal activities were measured and reported as RLU. *B*. LNCaP cells were transfected with 100 ng of TK/ARE2-luc and 25 ng of pcDNA3-β-Gal and treated as described above. Luciferase and β-gal activities were measured. *C*. Transient transfections were carried out as described above. Twenty ng of AR shRNA plasmid or LAPSER shRNA vector (45), as the control, and other plasmids as marked in the figure were transfected into LNCaP cells. Cells were cultured with either Wnt3a-CM or L-CM. An AR antagonist, bicalutamide, was added into cells 24 hours after transfection. Luciferase and β-gal activities were measured. *D*. The cytosolic fractions were prepared from the above-mentioned LNCaP cells transfected with AR or LAPSER shRNA constructs and analyzed by Western blot with AR and tubulin antibodies. *E*. LNCaP cells were cultured with Wnt3a-CM or L-CM, in the absence of DHT or in the presence of 0.1 or 1 nmol/L DHT, for 24 hours. Total RNA was isolated. Six micrograms of RNA were analyzed by Northern blot with a DNA fragment (amino acids 1–261) derived from the human PSA gene. A human glyceraldehyde-3-phosphate dehydrogenase probe (*GAPDH*; amino acids 104–168) was used to verify equal loading. *F*. LNCaP cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3-β-gal, and 40 ng of antisense β-catenin vector or 20 ng of TCF-1 or E-cadherin expression vector. Transfected cells were incubated in T-medium with 5% CS-FCS for 24 hours and then treated as described in *A*. *G*. Cytosolic fractions were isolated from the above-mentioned cells and analyzed by Western blot with β-catenin and tubulin antibodies.

11-fold induction of AR activity compared with cells treated with the control medium in the absence of DHT. These results provide the first evidence that the Wnt growth factor can independently activate AR-mediated transcription. In addition, Wnt3a-CM also showed an induction of AR-mediated transcription in the presence of 0.1 and 1 nmol/L DHT.

To verify that induction of the PSA promoter by Wnt3a-CM is a specific effect, we repeated the experiments in LNCaP cells with a luciferase reporter driven by a minimal promoter with two androgen response elements (AREs). A similar induction of AR-mediated transcription was observed in the cells treated with Wnt3a-CM (Fig. 2*B*). To further ensure that the induction by Wnt3a-CM is directly through the AR protein, we tested the effect of Wnt3a-CM on cells that were cotransfected with a shRNA construct of AR to knock down the AR protein. As shown in Fig. 2*C*, reduction of AR protein expression can abolish the AR-mediated transcription. This was correlated with a decreased level of cytosolic AR proteins in the cells (Fig. 2*D*). In addition, an AR antagonist, bicalutamide, can also block the activity of AR in cells treated with Wnt3a-CM (Fig. 2*C*). Taken together, these data indicate that the effect of Wnt3A is mediated through AR.

To evaluate the effect of Wnt3a-CM in a biologically relevant setting, we tested whether the conditioned medium regulates expression of the endogenous PSA gene. We measured transcripts of PSA in LNCaP cells treated with different amounts of DHT in the presence of Wnt3a-CM or control medium. As shown in Fig. 2*E*, Wnt3a-CM induces an approximately 5-fold increase in the expression of PSA in the absence of DHT. In addition, in the presence of 0.1 nmol/L DHT, the expression of PSA is about 2.5-fold higher in cells treated with Wnt3a-CM than in cells treated with L-CM (Fig. 2*E*). These results provide an additional line of evidence that Wnt3a-CM can activate AR-mediated transcription. Taken together, we have demonstrated that Wnt3a-CM can activate AR-mediated transcription in the absence of ligand and augment AR activity in the presence of a low concentration of androgens.

Given that β-catenin is a key downstream effector of the Wnt pathway and acts as an AR coactivator, we further investigated whether β-catenin is involved in the Wnt3A-induced AR activity. We repeated the transient transfection experiments with an antisense construct of β-catenin. As observed previously (19), it specially reduces the level of cellular β-catenin proteins (Fig. 2*G*), and the induction of AR activity by Wnt3a-CM (Fig. 2*F*). It has been shown that overexpression of TCF/LEF and E-cadherin can compete for β-catenin binding to AR and reduce AR-mediated transactivation (31, 32). We therefore tested whether coexpression of TCF-1 and E-cadherin can affect the induction of AR activity mediated by Wnt3a-CM. As shown in Fig. 2*F*, PSA promoter/reporter activities were reduced approximately 35% to 45%, relative to the controls, in cells transfected with the TCF-1 and E-cadherin expression vectors. These data suggest an involvement of β-catenin in the induction of AR activity mediated by Wnt3a-CM.

Wnt3a-CM Increases the Level of Cytosolic and Nuclear β-Catenin in Prostate Cancer Cells. Wnt3a-CM has been shown to increase accumulation of cytosolic free β-catenin (24). Cotransfections of β-catenin antisense and TCF-1 and E-cadherin constructs suggested that β-catenin is involved in Wnt3a-CM–mediated AR activity. To evaluate whether Wnt3a-CM affects the cytosolic pool of β-catenin in LNCaP cells, we examined the levels of β-catenin in the different cellular fractions prepared from cells treated with the

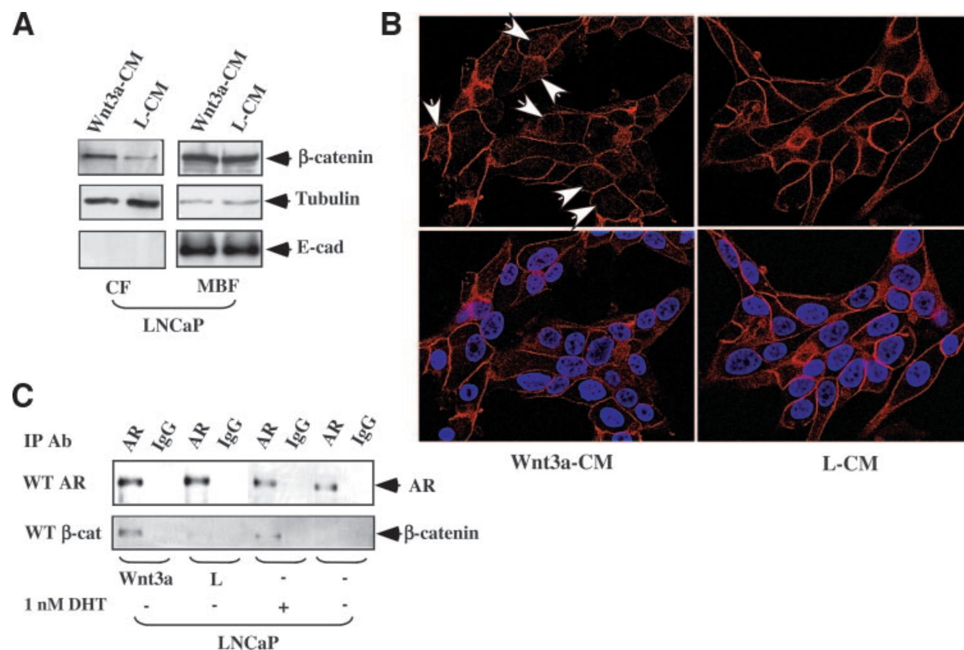

Fig. 3. Wnt3a-CM enhances the level of cytosolic and nuclear β-catenin. *A*. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours and then harvested. A cytosolic fraction (*CF*) and membrane-associated fraction (*MBF*) were prepared and analyzed by Western blotting (see Materials and Methods). The expression of E-cadherin was examined to evaluate the purity of the above-mentioned fractions. *B*. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were stained with the anti–β-catenin antibody followed by a secondary antibody conjugated with rhodamine (*red*). The nuclei were counterstained with Hoechst, and the ones with a high level of β-catenin are marked with *arrows*. *C*. Equal amounts of nuclear fractions isolated from LNCaP cells incubated with Wnt3a-CM (*Wnt3a*) or L-CM (*L*) were subjected to immunoprecipitation with normal mouse IgG or anti-AR monoclonal antibody. The nuclear fractions isolated from LNCaP cells cultured in the presence or absence of 1 nmol/L DHT were also included in experiments as controls. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using anti–β-catenin or anti-AR antibodies (*WT*).

Wnt3a-CM and control medium. The cytosolic fraction and the membrane-associated fraction were prepared, representing the free cytosolic pool and membrane bound β-catenin, respectively (33). As shown in Fig. 3*A*, there was no significant change in the amount of β-catenin protein in the membrane-associated fraction isolated from the cells treated with Wnt3a-CM in comparison with untreated cells. However, there was a significant increase of cytosolic β-catenin in the cells treated with Wnt3a-CM compared with the controls. In addition, the level of tubulin, as a control, was similar in cells treated with Wnt3a-CM and L-CM in both the cytosolic fraction and membrane-associated fractions. The results indicate that Wnt3a-CM increased the levels of cytosolic β-catenin in LNCaP cells.

Next, we examined the effects of Wnt3a-CM on the cellular localization of β-catenin in LNCaP cells by immunofluorescence staining. As shown in Fig. 3*B*, clear cell membrane staining with the β-catenin antibody was observed in LNCaP cells. However, there is also an increase in nuclear β-catenin in cells treated with Wnt3a-CM. These data are consistent with the results from the Western blot (Fig. 3*A*) and suggest that Wnt3a-CM can stimulate nuclear translocation of β-catenin.

It has been shown that β-catenin forms a protein complex with AR and enhances AR-mediated transcription in LNCaP cells. Given that Wnt3a-CM enhances cytosolic free β-catenin and nuclear translocation of β-catenin, we next examined whether Wnt3a-CM enhances the formation of the β-catenin–AR protein complex in nuclei. Using the nuclear fraction of LNCaP cells treated with Wnt3a-CM, we assessed the levels of β-catenin in the protein complex with AR by coimmunoprecipitation. We observed more β-catenin proteins interacting with AR in cells treated with Wnt3a-CM than in cells treated with the control medium in the absence of 1 nmol/L DHT (Fig. 3*C*). As described previously, we also observed that β-catenin forms a protein complex with the AR in LNCaP cells in the presence of androgens. The data provide another line of evidence indicating that Wnt3a-CM induces the formation of AR–β-catenin protein complexes in the nucleus.

Wnt3a-CM Promotes Cell Growth and Colony Formation in the Absence of Androgens. Next, we investigated the role of Wnt3a-CM in the regulation of LNCaP cell growth. In particular, we addressed whether Wnt3a-CM can function as a growth factor to promote LNCaP cell growth in a ligand-independent manner. LNCaP cells were cultured with Wnt3a-CM that was prepared in RPMI 1640 with 10% CS-FCS (see Materials and Methods). The growth of LNCaP cells was first assessed by the MTS assay. In the presence of Wnt3a-CM, the cell numbers were 20% and 35% higher after 4 and 6 days compared with controls (Fig. 4*A*). We then assessed the growth-promoting effect of Wnt3a-CM using a colony formation assay. Approximately 500 LNCaP cells were seeded in each well and incubated with Wnt3a-CM. After 12 days, cells were fixed and stained with crystal violet. There are more and larger colonies in the samples incubated with Wnt3a-CM than in the ones treated with control medium (Fig. 4*B*). The number of colonies containing >50 cells is significantly higher in the samples treated with Wnt3a-CM than in the controls ($P < 0.001$; Fig. 4*C*). The above-mentioned data demonstrate that Wnt3a-CM increases the growth of prostate cancer cells in the absence of androgens. We also performed the above experiments in the presence of 0.1 nmol/L DHT and observed a clear effect of Wnt3a-CM in enhancing the growth of prostate cancer cells (data not shown).

Purified Wnt3a Proteins Enhance AR-Mediated Transcription and Cell Growth. Recently, Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated (29) and appear active in inducing self-renewal of hematopoietic stem cells. We found that, like Wnt3a-CM, purified Wnt3a proteins can enhance the level of cytosolic β-catenin in two prostate cancer cell lines, PC3 and LNCaP (Fig. 5*A*). Then, we tested the activity of purified Wnt3a proteins in transient transfection assays. The PSA-luc plasmids were transfected with or without a wild-type AR expression vector into PC3 cells, which are AR negative. In the presence of 0.1 nmol/L DHT, Wnt3a-CM induces approximately 35% to 40% of AR-mediated transcription (Fig. 5*B*). Intriguingly, under a similar experimental condition, purified Wnt3a proteins show a potent and dosage-dependent enhancement of AR transactivation. To confirm this finding, we repeated transient transfection experiments in LNCaP cells. As shown in Fig. 5*C*, purified Wnt3a proteins show a similar induction of AR-mediated transcription in the presence or absence of 0.1 nmol/L DHT. However, the effect is more pronounced in the cells treated with the ligand. To further assess the growth-promoting effect of purified Wnt3a proteins, we repeated the colony formation assays. We ob-

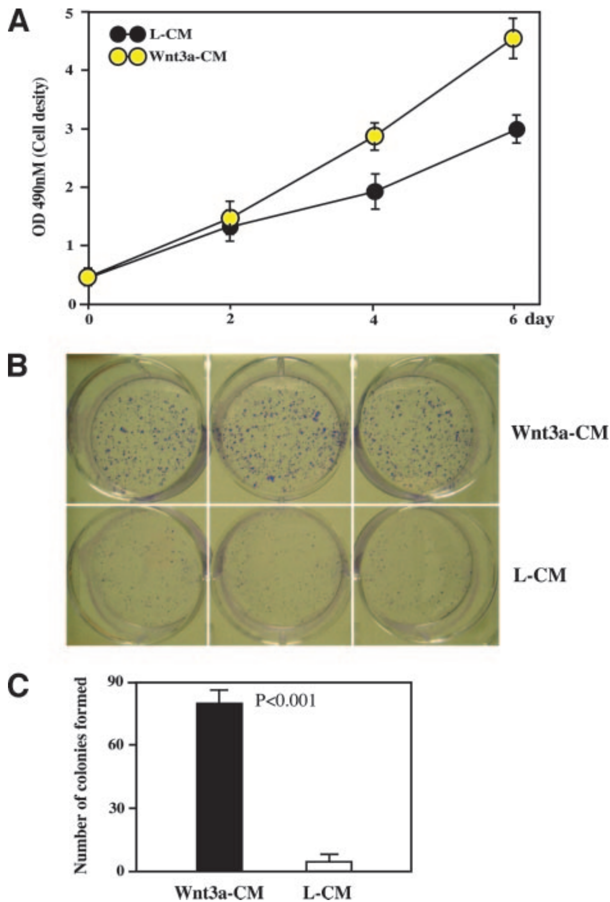

Fig. 4. Wnt3a-CM promotes the growth of LNCaP cells in a ligand-independent manner. *A*. LNCaP cells were cultured with Wnt3a-CM or L-CM in the absence of DHT. At the indicated time points, cells were harvested and analyzed by the MTS assay. The data represent the mean ± SD of three independent experiments. *B*. For the colony formation assay, 500 LNCaP cells were seeded in 6-well plates and cultured in Wnt3a-CM or L-CM in the absence of DHT. Cells were fixed and stained with crystal violet after a 12-day incubation. *C*. Colonies containing >50 cells were counted and analyzed. The results are from three separate transfection experiments.

served an increase of colony size and number in the samples treated with purified Wnt3a proteins compared with the ones treated with buffer only (Fig. 5*D*). The number of colonies containing >50 cells is significantly higher in the samples treated with purified Wnt3a proteins than in the controls ($P < 0.001$; data not shown). Taken together, the above results confirm an important role of the Wnt3a proteins in AR-mediated transcription and prostate cell growth.

DISCUSSION

Wnt signaling pathways regulate a variety of processes including cell growth, development, and oncogenesis (13, 34). However, the biological roles of the Wnt growth factors serving as the upstream signaling of β-catenin have not been fully characterized in prostate cancer cells. In this study, we investigate whether there is a direct effect of the Wnt growth factor on AR-mediated transcription and its role in the growth of prostate cancer cells.

Wnt3a-CM prepared from mouse L cells stably transfected with mouse Wnt3a cDNA has been well characterized (24). It has become a great resource and is used frequently to study the Wnt signaling pathway (8, 35). Previous studies have shown that Wnt3a-CM increases the cytosolic and nuclear levels of β-catenin (8, 24). Microarray data demonstrated that treatment of human embryonic carcinoma with Wnt3a-CM up-regulated the expression of β-catenin, downstream target genes of TCF/LEF, and other factors involved in the regulation of β-catenin (35). These multiple lines of evidence confirm a specific signaling pathway mediated by Wnt3a-CM in cells.

In this study, we showed that Wnt3a-CM stimulates AR-mediated transcription. We demonstrated that Wnt3a-CM is capable of inducing AR-mediated transcription from the PSA promoter/reporter and the expression of endogenous PSA transcripts in a ligand-independent manner. In LNCaP cells, the stimulation by Wnt3a-CM of AR is very effective and is almost as great as the effect achieved by adding 1 nmol/L DHT. In addition, our data also showed that Wnt3a-CM is able to increase AR-mediated transcription in the presence of low concentrations of DHT. These data provide the first line of evidence showing a unique and important role of Wnt3a in the regulation of the androgen signaling pathway in a ligand-dependent manner.

To understand the molecular mechanism by which Wnt3a augments AR-mediated transcription, we performed several experiments to confirm the involvement of the AR in the regulation. We showed that Wnt3a-CM induces transcription of the 7-kb PSA promoter-luc and endogenous PSA gene. Moreover, Wnt3a-CM also affects a minimal promoter containing only two AREs. Furthermore, we demonstrated that induction of AR-dependent promoters by Wnt3a-CM can be completely abolished by an AR shRNA construct and an AR antagonist, bicalutamide. These data implicate that induction by Wnt3a-CM is mediated through the AR.

β-Catenin plays a central role in the Wnt signaling pathway. As reported previously, we observed an increase in free cytosolic and nuclear β-catenin in both prostate and nonprostate cancer cells that were treated with Wnt3a-CM (Fig. 1*A* and *B*). Previous studies by us and others have shown that β-catenin is a coactivator of AR (19–21). Therefore, we examined whether β-catenin is a downstream effector of Wnt3a, augmenting AR-mediated transcription. Using an antisense construct of β-catenin, we were able to partially block the effect of Wnt3a-CM on AR-mediated activity. Moreover, overexpression of TCF or E-cadherin, β-catenin–binding proteins, also reduced the Wnt3a-CM–mediated AR activity on the PSA promoter. These data suggest an involvement of β-catenin in Wnt3a-CM–induced AR transcription, which is in agreement with the previous finding that β-catenin acts as an AR coactivator (19–21). However, given the fact that expression of the antisense β-catenin and TCF and E-cadherin constructs only partially blocks the effect of Wnt3a-CM, it appears that other factors and/or pathways may also be involved in the regulation. It has been shown that Wnt growth factors/ligands can stimulate both canonical and non-canonical pathways. In this regard, the molecular mechanism(s) by which Wnt growth factors regulate the androgen signaling in prostate cells must be explored further.

Although the mechanisms by which prostate cancer cells develop into the androgen-insensitive stage are currently unclear, it is believed that the tumor cells must either bypass or adapt the androgen signaling pathway to survive in a low-androgen environment during progression. AR mutations have been identified in some androgen-insensitive prostate cancers (36–38). Amplification of the *AR* gene has been observed in some biopsy samples during androgen ablation therapy (39). Recent studies showed that the modulation of the AR protein by phosphorylation, acetylation, and sumoylation also regulates AR activity (40–43). In particular, it has been shown that phosphatidylinositol 3′-kinase/Akt and PTEN regulate AR-mediated transcription through either direct phosphorylation of AR proteins (44) or modification of AR cofactors, such as β-catenin (27). In the current study, we provide several lines of evidence demonstrating that Wnt3a is able to stimulate AR-mediated transcription in the absence of ligand or the presence of a low level of ligand in prostate cancer cells. The above-mentioned data suggest that signals delivered through the AR are still essential in androgen-insensitive prostate cancer cells. Intriguingly,

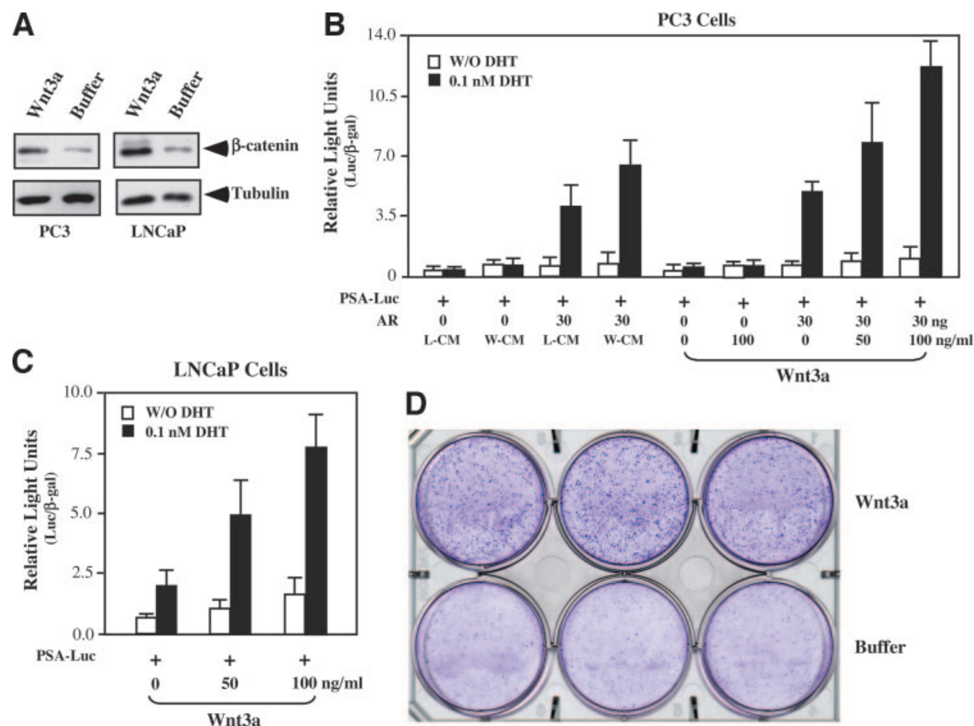

Fig. 5. Purified Wnt3a proteins enhance AR-mediated transcription and cell growth. *A*. Both PC3 and LNCaP cells were cultured in the presence of purified Wnt-3a (100 ng/mL) or the elution buffer (*Buffer*) as a control for 24 hours. Cytosolic fractions were isolated and analyzed by Western blot. *B*. PC3 cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3-β-Gal, and 30 ng of pcDNA3-AR; washed after 24 hours; and then incubated in Wnt-3a-CM or L-CM or treated with the indicated amounts of purified Wnt-3a proteins in the presence or absence of 0.1 nmol/L DHT. Luciferase and β-Gal activities were measured as described previously. *C*. LNCaP cells were transiently transfected with 100 ng of PSA-Luc and 25 ng of pCMV-β-Gal. After 24 hours, transfected cells were washed and incubated with the indicated amounts of purified Wnt-3a. *D*. Approximately 1,000 LNCaP cells were plated in triplicate in DMEM containing 10% CS-FCS in the presence of 100 ng/mL Wnt-3a. Ten days after incubation, the cells were stained with crystal violet. A representative plate is shown. Colonies containing >50 cells were counted and analyzed as described in Fig. 4*C*.

we also show that Wnt3a-CM is able to promote the growth of prostate cancer cells in a ligand-independent manner. The fact that Wnt3a-CM can promote cell growth and induce AR-mediated transcription suggests a unique role of the Wnt growth factor in the progression of prostate cancer cells from the androgen-sensitive to -insensitive stages. It is possible that aberrant expression of Wnt growth factors and/or their receptors in prostate cancer tissues may play a critical role in the progression of prostate cancer.

Although attempts to purify Wnt proteins have been hampered by several technical difficulties, including their high degree of insolubility, active Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated recently (29). In this study, we first showed that, like Wnt3a-CM, purified Wnt3a proteins increase the cytosolic level of β-catenin in prostate cancer cells. Then we confirmed the role of purified Wnt3a proteins on AR-mediated transcription and cell growth in prostate cancer cells. In PC3 cells, Wnt3a proteins enhance AR-mediated transcription in a dosage-dependent manner. A similar effect by purified Wnt3a proteins was also observed in LNCaP cells. These data provide a direct line of evidence demonstrating a true effect of Wnt3a on AR-mediated transcription in prostate cells. However, we observed that purified Wnt3a proteins only slightly affect AR activity in the absence of DHT. This is different from the results that we observed in the experiments when Wnt3a-CM was used. Currently, we do not know the exact reason(s) why purified Wnt3a proteins have less effect on AR-mediated transcription than Wnt3a-CM in the absence of androgens. Further characterization of different protein fractions during purification processes may lead to the identification of additional factors or cofactors that contribute to Wnt3a-mediated augmentation of AR activity. Additional studies of the expression profiles of Wnt ligands and receptors in prostate tissues and prostate cancer cells will also help us to fully understand the signaling pathway(s) regulated by Wnt growth factors in prostate cancer cells.

In this study, we provide several lines of evidence that Wnt3a acts as an upstream signal to induce the transcriptional activity of AR and the growth of prostate cancer cells, possibly through β-catenin. In particular, using purified Wnt3a proteins, we confirm the important role of the Wnt3a growth factor in inducing AR-mediated transcription and cell growth. The effect of Wnt3a may play a critical role in maintaining or increasing AR activity in the setting of decreased androgen levels during androgen ablation therapy. Therefore, further study of the molecular mechanisms by which Wnt growth factors modulate androgen signaling should provide fresh insight into the progression of prostate cancer, which may help us to identify new steps that can be targeted for prostate cancer treatment.

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