Research Article

Cancer Prevention Research

Inactivation of AR/TMPRSS2-ERG/Wnt Signaling Networks Attenuates the Aggressive Behavior of Prostate Cancer Cells

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Abstract

The development of prostate cancer and its progression to castrate-resistant prostate cancer (CRPC) after antiandrogen ablation therapy are driven by persistent biological activity of androgen receptor (AR) signaling. Moreover, studies have shown that more than 50% of human prostate cancers overexpress *ERG* (v-ets avian erythroblastosis virus E26 oncogene related gene) due to AR-regulated *TMPRSS2-ERG* fusion gene. However, the reported roles of *TMPRSS2-ERG* fusion in cancer progression are not clear. In this study, we investigated the signal transduction in the AR/TMPRSS2-ERG/Wnt signaling network for studying the aggressive behavior of prostate cancer cells and further assessed the effects of BR-DIM and CDF [natural agents-derived synthetic formulation and analogue of 3,3′-diindolylmethane (DIM) and curcumin, respectively, with improved bioavailability] on the regulation of AR/TMPRSS2-ERG/Wnt signaling. We found that activation of AR resulted in the induction of *ERG* expression through *TMPRSS2-ERG* fusion. Moreover, we found that *ERG* overexpression and nuclear translocation activated the activity of Wnt signaling. Furthermore, forced overexpression of *ERG* promoted invasive capacity of prostate cancer cells. More important, we found that BR-DIM and CDF inhibited the signal transduction in the AR/TMPRSS2-ERG/Wnt signaling network, leading to the inactivation of Wnt signaling consistent with inhibition of prostate cancer cell invasion. In addition, BR-DIM and CDF inhibited proliferation of prostate cancer cells and induced apoptotic cell death. On the basis of our findings, we conclude that because BR-DIM and CDF downregulate multiple signaling pathways including AR/TMPRSS2-ERG/Wnt signaling, these agents could be useful for designing novel strategies for the prevention and/or treatment of prostate cancer. *Cancer Prev Res; 4(9); 1495–506. ©2011 AACR.*

Introduction

Despite significant effort made in the fight against cancers, prostate cancer is still the most common cancer in men and the second leading cause of cancer-related deaths in the United States with an estimated 217,730 new cases and 32,050 deaths in 2010 (1). Tumor invasion and metastasis after failure of androgen ablation therapy and the emergence of castrate-resistant prostate cancer (CRPC) contribute to high mortality of patients with a diagnosis with prostate cancer. Emerging evidence suggests that the mechanisms involved in the progression of prostate cancer include the deregulation of androgen receptor (AR), Akt, Wnt, and Hedgehog signaling (2–5). Among them, AR signaling is more critical for the development of prostate cancer and the progression of prostate cancer to CRPC (5). It is believed that because of AR overexpression and androgen hypersensitivity found in CRPC, AR is easily activated by low concentration of androgen present in the prostates of men treated with antiandrogen therapy (6, 7).

Moreover, studies have shown that a significant fraction (>50%) of human prostate cancers expresses a fusion gene product, especially the AR-regulated overexpression of *ERG* due to *TMPRSS2-ERG* gene fusion (8, 9). *ERG* (v-ets avian erythroblastosis virus E26 oncogene related gene) is a transcription factor and has been known as an oncogene. ERG regulates cellular growth, differentiation, and organism development; therefore, alteration of *ERG* gene and its products may cause deregulation of cell growth and differentiation, resulting in the development of cancer. It has been shown that overexpression of ERG transcript is sufficient for the initiation of prostate cancer (10). Importantly, the patients with *TMPRSS2-ERG*–mediated overexpression of ERG show a significantly higher risk of recurrence (58.4% at 5 years) than the patients lacking *TMPRSS2-ERG* fusion gene (8.1%, $P < 0.0001$; ref. 9). The frequency

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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doi: 10.1158/1940-6207.CAPR-11-0077

of the expression of AR-regulated ERG due to *TMPRSS2-ERG* fusion was found to be increased in moderate to poorly differentiated prostate cancer (11). Importantly, *ERG* overexpression could cooperate with the loss of *PTEN*, promoting tumor progression of prostate cancer (12, 13). Moreover, *TMPRSS2-ERG* fusion has been associated with activated AR and Wnt signaling in CRPC (14, 15). Furthermore, the cross-talk between *ERG* and *Wnt* or *EZH2* in epithelial-to-mesenthymal transition phenotypic cells or prostate lineage–specific differentiation has been documented (16, 17). Although controversies exist about the role of *TMPRSS2-ERG* fusion in prostate cancer (9, 18), it is generally believed that AR-regulated overexpression of ERG due to *TMPRSS2-ERG* fusion together with the activation of AR and Wnt signaling is likely to form a signaling network that contributes to the development and progression of prostate cancer. However, the precise molecular mechanism(s) underlying the regulation of this signal network and finding novel ways to attenuate these signaling networks have not been fully investigated.

Interestingly, activation of AR could also be due to the upregulation of several signaling pathways including Wnt signaling pathway (19). Increased transcriptional activity of β-catenin resulting from the activated Wnt signaling has been detected in many types of human cancer including prostate cancer and thus β-catenin is believed to play important roles in the progression of prostate cancer (20, 21). Moreover, experimental studies have shown that β-catenin modulates AR signaling at multiple levels including transactivation of AR (22), suggesting the novelty and importance of a signaling network between AR-regulated overexpression of ERG due to *TMPRSS2-ERG* fusion and the activation of Wnt signaling in prostate cancer. Therefore, it is important to investigate the regulation of AR/TMPRSS2-ERG/Wnt signaling in prostate cancer progression and design novel strategies by which one could attenuate these signaling networks for the prevention or treatment of prostate cancer more effectively with better survival outcome.

We have previously shown that 3,3′-diindolylmethane [(DIM), a "natural product" and its formulated product (BR-DIM; BioResponse) with enhanced bioavailability] could downregulate the expression of AR (23); therefore, BR-DIM could, in turn, inhibit the expression of ERG in prostate cancer cells that are positive for *TMPRSS2-ERG* fusion because ERG expression could be downregulated because of AR inactivation by BR-DIM and subsequent inactivation of *TMPRSS2*, which is an AR target gene. In addition, studies have shown that curcumin (another "natural product") could inhibit the activation of AR and Wnt signaling (24, 25); however, curcumin shows rapid metabolism and lacks systemic and target tissue bioavailability (26, 27). To overcome such a problem, we have recently reported the development of a synthetic analogue of curcumin (CDF) that showed much superior bioavailability (28), suggesting that CDF could be useful for the prevention of prostate cancer progression. In this study, we investigated the mechanistic role of AR/TMPRSS2-ERG/Wnt signaling network in the aggressive behavior of prostate cancer cells and further investigated the effects of BR-DIM and CDF on the regulation of AR/TMPRSS2-ERG/Wnt signaling in prostate cancer cells.

Materials and Methods

Cell lines, reagents, and antibodies

VCaP [American Type Culture Collection (ATCC)], LNCaP (ATCC), C4-2B, and ARCaP (Novicure) prostate cancer cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. The cell lines have been tested and authenticated through the core facility Applied Genomics Technology Center at Wayne State University. The method used for testing was short tandem repeat profiling by using the PowerPlex 16 System from Promega. BR-DIM (BioResponse; formulated-DIM with higher bioavailability *in vivo*; ref. 29) was generously provided by Dr. Michael Zeligs and was dissolved in dimethylsulfoxide (DMSO) to make a 50 mmol/L stock solution. CDF (3,4-difluro-benzo-curcumin or simply difluorinated curcumin; ref. 28) was dissolved in DMSO to make a 5 mmol/L stock solution. Anti-AR (Santa Cruz Biotechnology), anti-ERG (Santa Cruz Biotechnology), anti-ERG (Epitomics), anti-PSA (Santa Cruz Biotechnology), anti-CBP (Santa Cruz Biotechnology), anti-Wnt-16 (Santa Cruz Biotechnology), anti-β-catenin (Cell Signaling Technology), anti-Wnt-3a (Cell Signaling Technology), anti-LRP6 (Cell Signaling Technology), anti-Naked2 (Cell Signaling Technology), anti-Axin1 (Cell Signaling Technology), anti-GAK-3β (Cell Signaling Technology), anti-β-actin (Sigma) and anti-GAPDH (Sigma) primary antibodies were used for Western blot analysis and immunoprecipitation.

Preparation of cytoplasmic, nuclear, or total lysates

VCaP, LNCaP, and C4-2B prostate cancer cells were treated with 25 μmol/L BR-DIM or 2.5 to 5 μmol/L CDF for 24 and 48 hours. Some samples were followed by 1 nmol/L dihydrotestosterone (DHT) or 10 nmol/L testosterone treatment for 24 hours. After treatment and harvesting, the cells were resuspended in lysis buffer [0.08 mol/L KCl/35 mmol/L HEPES pH 7.4/5 mmol/L K-phosphate pH 7.4/5 mmol/L $MgCl_2$/25 mmol/L $CaCl_2$/0.15mol/L sucrose/2mmol/L phenylmethylsulfonylfluoride/8mmol/L dithiothreitol (DTT)] and frozen at 80°C overnight. The cell suspension was thawed and passed through a 28-gauge needle 3 times. A small aliquot of the cells were checked for cell membrane breakage by using Trypan Blue. Then, the cell suspension was centrifuged and the supernatant was saved as cytoplasmic lysate. The pellet was suspended in lysis buffer, and the nuclei were lysed by sonication. After centrifugation, supernatant was saved as nuclear lysate. The protein concentration in the lysates was measured using the Coomassie Plus Protein Assay Kit (Pierce). For total protein extraction, BR-DIM- or CDF-treated VCaP, LNCaP, and C4-2B prostate cancer cells were

lysed in radioimmunoprecipitation assay (RIPA) buffer. After centrifugation, the concentration of total protein was measured using BCA protein assay (Pierce).

Immunoprecipitation

Nuclear lysate (500 μg) were subjected to immunoprecipitation by adding 5 μg of anti-CBP antibody and incubation overnight at 4°C. After adding 50 μL of Protein G Agarose (Santa Cruz Biotechnology) and incubation for 1 hour, the samples were centrifuged. The agarose pellet was then washed 3 times, resuspended in Laemmli buffer, and boiled for 5 minutes. The boiled samples were centrifuged and the supernatant was used for Western blot analysis.

Western blot analysis

Immunoprecipitates, whole-cell lysates, and cytoplasmic or nuclear proteins were subjected to standard Western blot analysis as described previously (30). The signal was then detected using the chemiluminescent detection system (Pierce) and quantified using AlphaEaseFC (Alpha Innotech). The ratios of targets against β-actin or GAPDH were calculated by standardizing the ratios of each control to the unit value.

Transient transfection with ERG cDNA constructs

A cytomegalovirus (CMV)-driven N-terminally truncated ERG cDNA expression construct (10) was transiently transfected into LNCaP and C4-2B cells by using ExGen 500 (Fermentas). After 5 hours, the transfected cells were washed and incubated with complete RPMI 1640 medium overnight followed by treatment with 25 μmol/L BR-DIM or 5 μmol/L CDF for 48 hours. Subsequently, the total proteins from transfected and untransfected cells with or without BR-DIM and CDF treatments were extracted and subjected to Western blot analysis by using specific antibodies as shown under the figure legend. In another set of experiment, the effect of ERG cDNA on the activity of cell invasion was assessed by invasion assay.

siRNA transfection

VCaP cells were transfected with ERG siRNA (Santa Cruz Biotechnology) or control RNA duplex (Santa Cruz Biotechnology) by DharmaFECT (ThermoScientific) for 48 hours followed by 1 nmol/L DHT treatment for 24 hours or followed by 25 μmol/L BR-DIM and 5 μmol/L CDF treatment for 48 hours. The total cellular proteins from each sample were extracted. The level of ERG and Wnt-16 expression was detected by Western blot analysis. In another set of experiment, the effect of ERG siRNA on the activity of cell invasion was measured by invasion assay.

Invasion assay

The invasive activity of VCaP, LNCaP, and C4-2B cells with ERG cDNA or siRNA transfection was measured using the BD BioCoat Tumor Invasion Assay System (BD Biosciences) according to the manufacturer's protocol with minor modification. Briefly, ERG cDNA or siRNA transfected VCaP, LNCaP, or C4-2B cells (5×10^4) with serum-free medium supplemented with or without 25 μmol/L BR-DIM or 2.5 μmol/L CDF were seeded into the top chamber of the system. Bottom wells in the system were filled with complete medium and same reagent treatment as the top chamber. After 24 hours of incubation, the cells in the top chamber were removed, and the cells, which invaded through Matrigel matrix membrane, were stained with 4 μg/mL Calcein AM in Hank's buffered saline at 37°C for 1 hour. Then, fluorescence of the invaded cells was read in a ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590 nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

TOPflash assay for measuring activity of Wnt signaling

TOPflash assay was carried out according to the manufacture's instruction (Millipore). TOPflash contains 6 copies of the TCF/LEF-binding site and luciferase reporter. FOPflash contains a mutated TCF/LEF-binding site. The TOPflash or FOPflash reporter plasmid was transiently cotransfected with ERG cDNA construct into LNCaP and C4-2B cells by using ExGen 500 (Fermentas) with or without 25 μmol/L BR-DIM or 2.5 μmol/L CDF treatment. In addition, the TOPflash or FOPflash reporter plasmid was also transiently cotransfected with ERG siRNA into VCaP cells by using the TurboFect Transfection Reagent (Fermentas) with or without BR-DIM or CDF treatment. After 48 hours of transfection, luciferase activity in each sample was measured. The β-catenin-TCF–mediated gene transcription was determined by the ratio of TOPflash/FOPflash luciferase activity.

Cell proliferation studies by MTT and WST-1 assays

VCaP, LNCaP, and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 hours, the cells were treated with 25 μmol/L BR-DIM or 5 μmol/L CDF for 48 hours. Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were subjected to MTT assay as described previously (30) and WST-1 assay (Roche) according to the manufacturer's protocol. The growth inhibition of VCaP, LNCaP, and C4-2B cells after BR-DIM and CDF treatment was statistically evaluated by Student's *t* test using GraphPad StatMate software (GraphPad Software Inc.).

Histone/DNA ELISA for detection of apoptosis

VCaP, LNCaP, and C4-2B prostate cancer cells were seeded in 6-well plates. After 24 hours, the cells were treated with 25 μmol/L BR-DIM or 5 μmol/L CDF for 48 hours. Control cells were treated with 0.1% DMSO (vehicle control). After treatment, the cells were subjected to quantitative measurement of apoptotic cells in control, BR-DIM-, or CDF-treated cells by using the Cell Death Detection ELISA Kit (Roche) according to the manufacturer's protocol as described previously (30). The induction of apoptosis by BR-DIM or CDF treatment in VCaP, LNCaP, and C4-2B prostate cancer cells was statistically evaluated by Student's

t test using GraphPad StatMate software (GraphPad Software Inc.).

Results

AR induced ERG expression through *TMPRSS2-ERG* fusion

Because VCaP cells harbor *TMPRSS2-ERG* fusion gene whereas C4-2B and LNCaP cells have no such fusion gene, we first tested the specificity of 5 ERG antibodies from different companies. We observed a strong ERG signal in VCaP cells even under very short exposure time and no signal was detected in LNCaP cells under short exposure except with the antibody from Abcam (Supplementary Fig. S1). However, after long exposure, we observed ERG signal in LNCaP cells by all 5 antibody staining (Supplementary Fig. S1), suggesting that LNCaP cells have a very low level of ERG protein expression. The size of ERG signal in LNCaP cells was little different from the size in VCaP cells by different antibodies, reflecting the difference between wild-type ERG in LNCaP cells and *TMPRSS2-ERG* fusion in VCaP cells. We chose the ERG antibodies from Santa Cruz Biotechnology and Epitomics for our subsequent studies because clear-cut ERG signal was observed using these antibodies.

To confirm the signal transduction between AR and ERG under *TMPRSS2-ERG* fusion status, we treated VCaP, C4-2B, and LNCaP cells with androgens. As expected, we observed that DHT or testosterone treatment resulted in the induction of AR and prostate-specific antigen (PSA) and caused AR nuclear translocation in VCaP, C4-2B, and LNCaP cells (Fig. 1A and B; Supplementary Fig. S2); however, ERG induction and nuclear translocation after DHT treatment were observed only in VCaP cells (Fig. 1A; Supplementary Fig. S2), suggesting that DHT could induce AR activation, which, in turn, stimulates ERG expression only under the status of *TMPRSS2-ERG* fusion.

ERG expression activated Wnt signaling

To investigate the relationship between *TMPRRS2-ERG* fusion and Wnt signaling, we treated VCaP cells with DHT for 24 hours. We found the induction of AR and ERG expression as we expected (Fig. 1A and C). Importantly, we also found that Wnt signaling was activated after DHT treatment. Several Wnt ligands and coregulators including β-catenin (Fig. 1C), Wnt-3a, Wnt-16, and LRP6 (Fig. 1D) were upregulated, whereas Naked2 and Axin1, which are known to inhibit Wnt signaling, were downregulated (Fig. 1D) after the activation of AR and ERG stimulated by DHT. However, we did not find such effects of DHT on Wnt signaling in C4-2B and LNCaP cells (Fig. 1B), which have no *TMPRSS2-ERG* fusion. These results suggest that there is a cross-talk between AR-regulated *TMPRSS2-ERG* fusion and Wnt signaling.

It has been known that transcriptional coactivator CBP plays important roles in the regulation of transcription (31) because the CBP could bind to transcription factors to form protein–protein complex and regulate transcription of

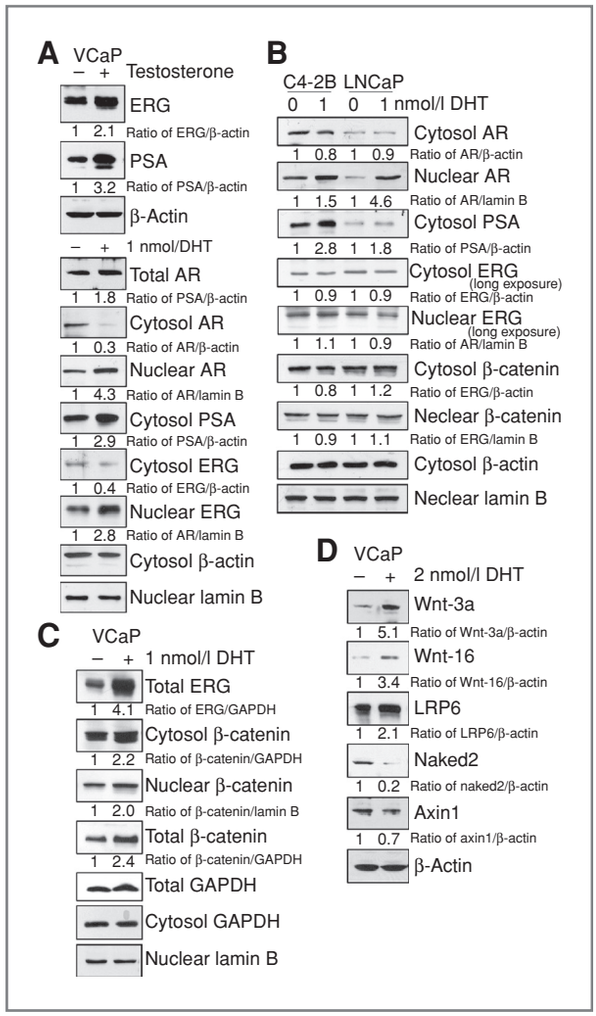

Figure 1. AR/TMPRSS2-ERG/Wnt signaling was upregulated by testosterone or DHT treatment. A, VCaP cells were treated with 10 nmol/L testosterone or 1 nmol/L DHT for 24 hours. B, C4-2B and LNCaP cells were treated with 1 nmol/L DHT for 24 hours. C, VCaP cells were treated with 1 nmol/L DHT for 24 hours. D, VCaP cells were treated with 2 nmol/L DHT for 24 hours. Western blot analysis was conducted to measure the expression of molecules in AR, ERG, and Wnt signaling.

genes. It has been found that CBP regulates the activation of Wnt signaling by interacting with β-catenin (32). Therefore, we conducted immunoprecipitation experiments by using nuclear protein extracts to examine the interaction of CBP with transcription factor ERG and other proteins related to Wnt signaling. We found that CBP could form protein complex with ERG, AR, GSK-3β, or β-catenin in the nucleus of VCaP cells (Fig. 2A). Importantly, we observed that the activity of CBP binding to ERG, AR, GSK-3β, or β-catenin was increased after DHT treatment in VCaP cells, suggesting the increased activity of AR and ERG in the transcriptional regulation and Wnt signaling activation. These results clearly suggest that AR-regulated ERG due to *TMPRSS2-ERG* fusion could participate in the activation of Wnt signaling.

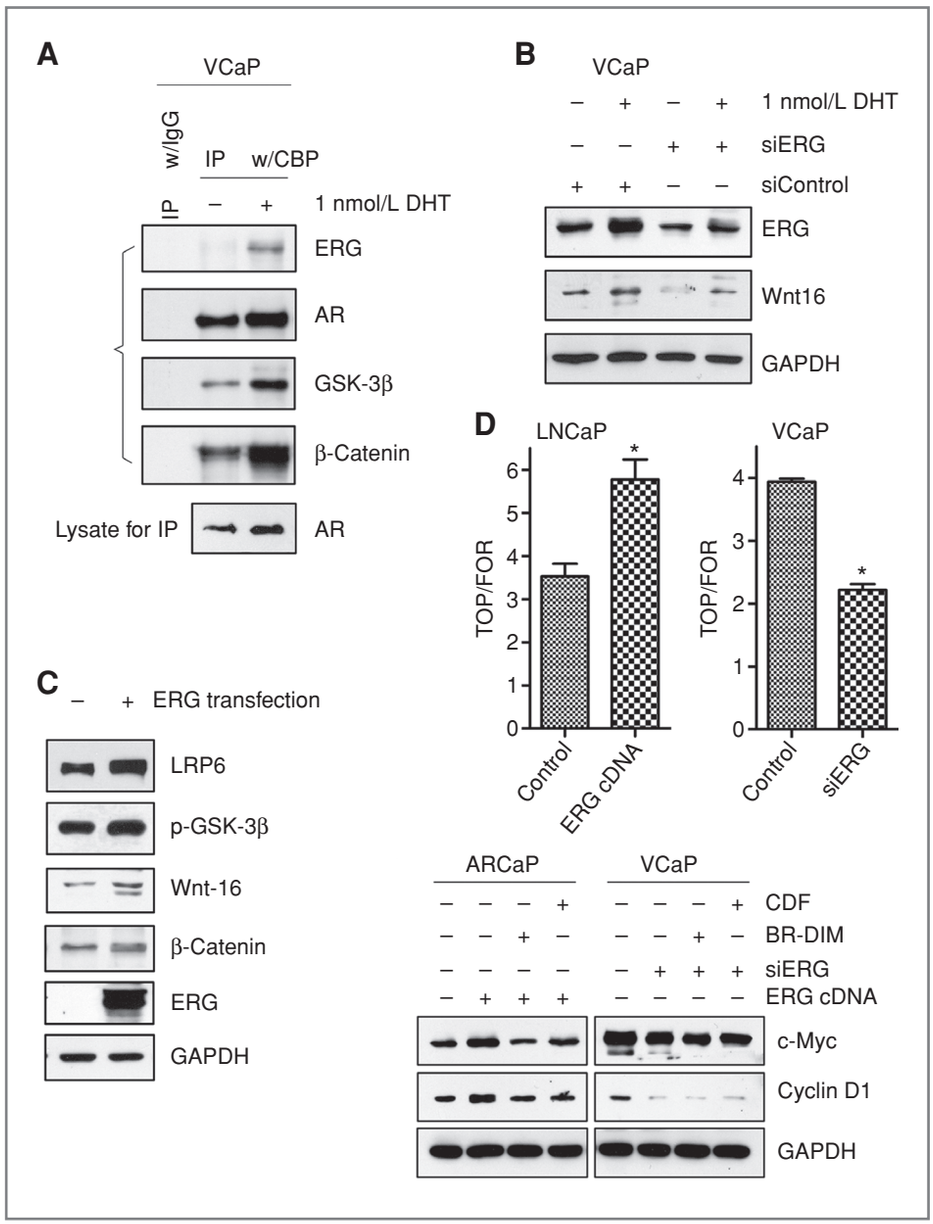

Figure 2. Overexpression of ERG activated Wnt signaling. A, VCaP cells were treated with 1 nmol/L DHT for 24 hours. Immunoprecipitation (IP) was conducted using anti-CBP and anti-IgG (control) antibodies. Western blot analysis was conducted to measure the binding activity of ERG, AR, GSK-3β, and β-catenin to CBP. B, VCaP cells were transfected with ERG siRNA (siERG) or control siRNA (siControl) for 48 hours followed by 1 nmol/L DHT treatment of 24 hours. Western blot analysis was conducted. C, ARCaP cells were transfected with ERG cDNA for 48 hours. Western blot analysis was conducted. D, TOPflash assay showed that Wnt signaling activity was upregulated by ERG cDNA transfection and downregulated by ERG siRNA (siERG) transfection. Western blot analysis showed that the expression of Wnt targets, c-Myc and cyclin D1, was upregulated by ERG cDNA transfection and downregulated by ERG siRNA, BR-DIM, and CDF. *, $P < 0.05$ compared with control; $n = 3$.

To further verify whether the activation of Wnt signaling that we have observed was mediated through AR-regulated ERG, we transfected ERG siRNA into VCaP cells and treated the transfected cells with DHT. We found that the expression of Wnt-16 was inhibited after ERG siRNA transfection and that the DHT induced upregulation of Wnt-16 in control cells was abrogated by ERG siRNA (Fig. 2B). These results suggest that the activation of Wnt signaling is mediated through AR-regulated ERG under the status of *TMPRSS2-ERG* fusion. We also transfected ERG cDNA into ARCaP cells that have very low expression of ERG and found that ERG cDNA transfection upregulated the level of Wnt signaling molecules such as Wnt-16, LRP6, p-GSK-3β, and β-catenin (Fig. 2C), further confirming the cross-talk between ERG and Wnt signaling.

To access the activity of Wnt signaling after ERG cDNA or siRNA transfection, we conducted TOPflash assay to measure TCF-mediated gene transcription that commonly reflects the activity of Wnt signaling. We found that ERG cDNA transfection increased the activity of Wnt signaling whereas ERG siRNA transfection downregulated the activity of Wnt signaling (Fig. 2D). Western blot analysis showed that the forced overexpression of ERG by cDNA transfection upregulated the expression of *c-Myc* and *cyclin D1* (Fig. 2D), 2 important target genes of Wnt signaling, and that ERG siRNA transfection decreased

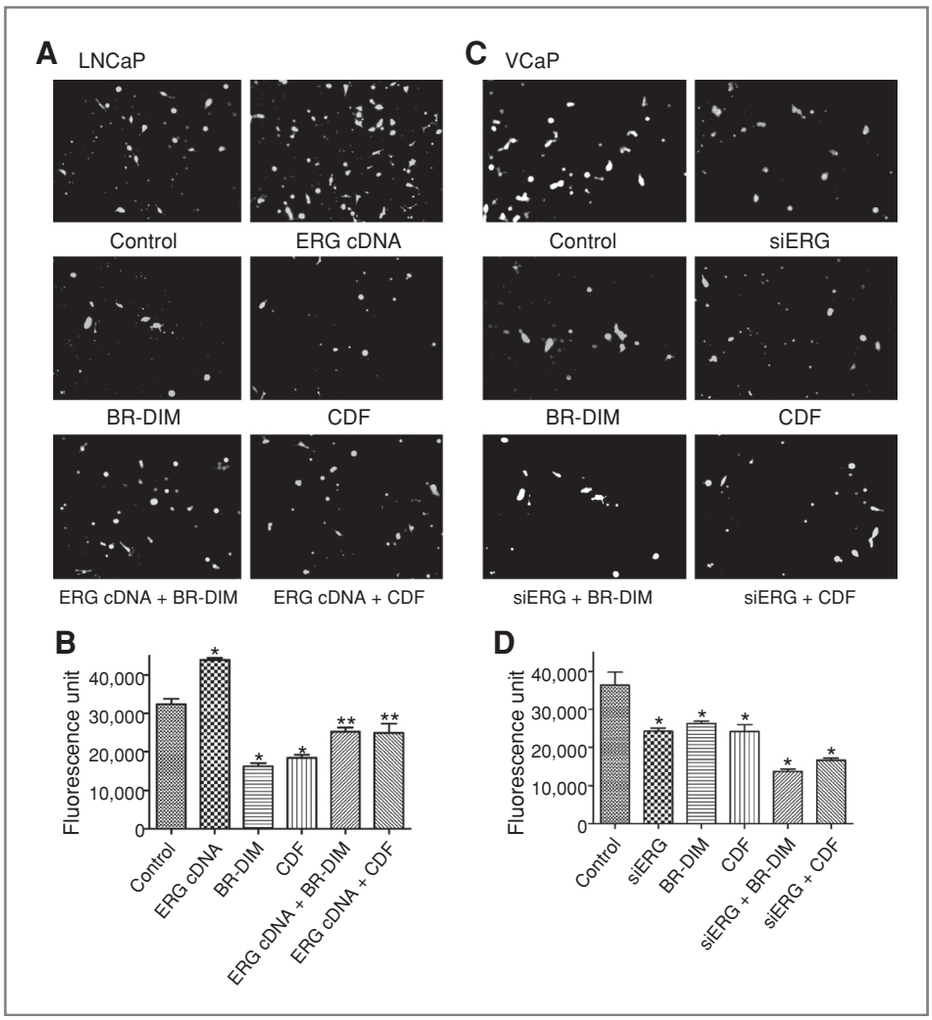

Figure 3. ERG overexpression promoted the invasive activity of prostate cancer cells whereas ERG siRNA, BR-DIM, and CDF inhibited the invasion of prostate cancer cells. A, invasion assay was conducted to test the invasive activity of LNCaP cells transfected ERG cDNA and/or treated with 25 μmol/L BR-DIM or 2.5 μmol/L CDF. B, fluorescence from the invaded LNCaP cells. The value indicated the comparative amount of invaded LNCaP cells. C, invasion assay was conducted to test the invasive activity of VCaP cells transfected ERG siRNA and/or treated with 25 μmol/L BR-DIM or 2.5 μmol/L CDF. D, fluorescence from the invaded VCaP cells. The value indicated the comparative amount of invaded VCaP cells. *, $P < 0.05$ compared with control; **, $P < 0.05$ compared with ERG cDNA; $n = 3$.

the expression level of *c-Myc* and *cyclin D1* (Fig. 2D). These results clearly suggest that ERG expression is mechanistically associated with the activation of Wnt signaling.

High expression of ERG promoted invasion of prostate cancer cells

Because high activity of Wnt signaling has been associated with cancer invasion, we conducted invasion assay to measure invasive capacity of LNCaP cells transfected with or without ERG cDNA constructs. We found that ERG cDNA transfection induced the invasive capacity of LNCaP cells (Fig. 3A and B), suggesting that high expression of ERG could promote invasion of prostate cancer cells through activation of Wnt signaling. To further confirm this observation, we transfected ERG siRNA into VCaP cells to inactivate ERG expression, and carried out invasion assay. We found that the inhibition of ERG decreased the invasive capacity of VCaP cells (Fig. 3C and D), which is consistent with the data obtained from ERG cDNA transfection studies.

BR-DIM and CDF inhibited the signal transduction in AR/TMPRSS2-ERG/Wnt signaling network, leading to the inhibition of Wnt signaling

We have previously reported that DIM could decrease the expression of AR, leading to the inhibition of prostate cancer cell growth (33). In this study, we tested the effect of BR-DIM and CDF on AR expression and found that both of them could downregulate the expression of AR (Fig. 4A; Supplementary Fig. S3). To investigate whether the inhibition of AR could lead to the downregulation of AR target gene *PSA* or *ERG* due to *TMPRSS2-ERG* fusion, we tested the effects of testosterone, DHT, BR-DIM, and CDF on the expression of AR, PSA, and ERG in VCaP cells that harbor *TMPRSS2-ERG* fusion gene. We found that testosterone or DHT treatment significantly upregulated the expression of ERG and PSA as expected and increased nuclear translocation of AR and ERG (Fig. 4B and C; Supplementary Fig. S3), suggesting that the expression of ERG in VCaP cells is regulated by AR. Importantly, we found that BR-DIM or CDF pretreatment abrogated the upregulation of ERG and PSA and inhibited the nuclear

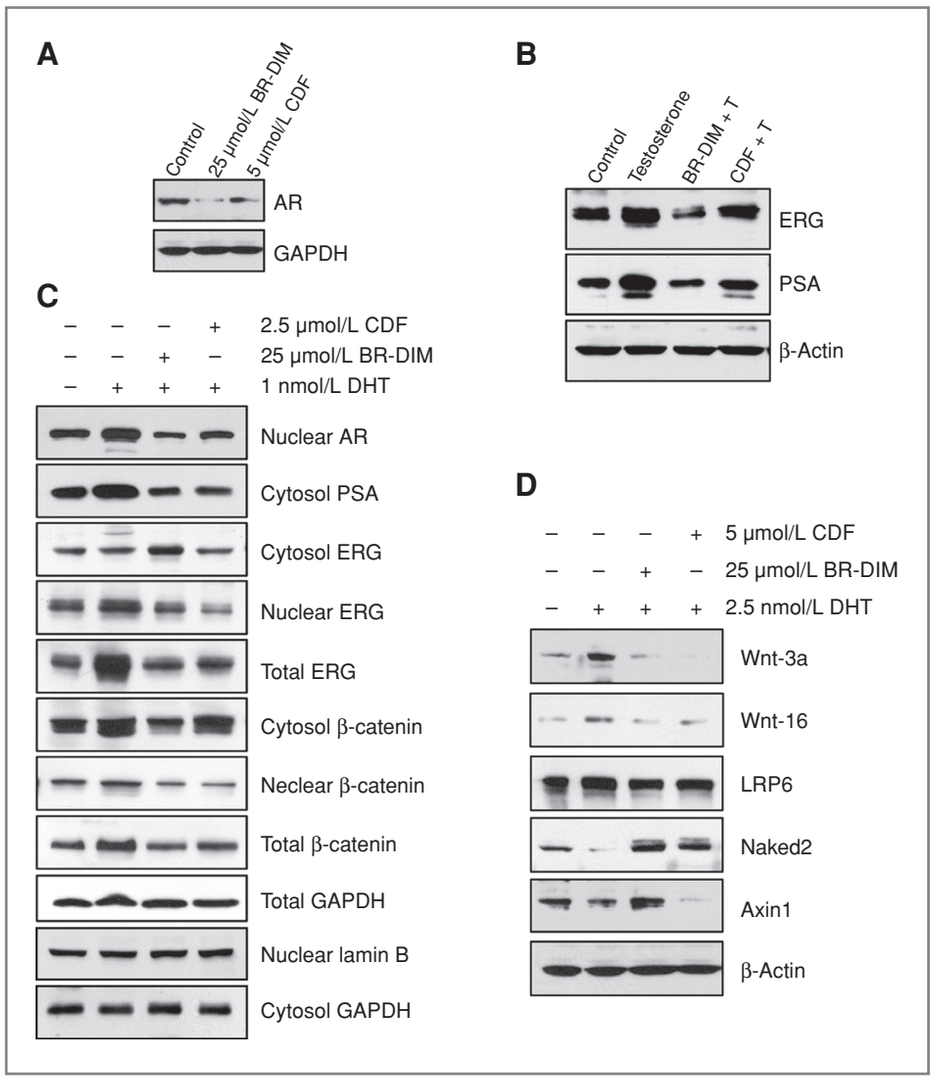

Figure 4. AR/TMPRSS2-ERG/Wnt signaling was downregulated by BR-DIM and CDF. A, VCaP cells were treated with 25 μmol/L BR-DIM or 5 μmol/L CDF for 48 hours. B, VCaP cells were treated with 25 μmol/L BR-DIM or 5 μmol/L CDF for 48 hours followed by 10 nmol/L testosterone for 24 hours. C, VCaP cells were treated with 25 μmol/L BR-DIM or 2.5 μmol/L CDF for 48 hours followed by 1 nmol/L DHT treatment of 24 hours. D, VCaP cells were treated with 25 μmol/L BR-DIM or 5 μmol/L CDF for 48 hours followed by 2.5 nmol/L DHT treatment of 24 hours. Western blot analysis was conducted to measure the expression of molecules in AR, ERG, and Wnt signaling.

translocation of AR and ERG stimulated by testosterone or DHT. These results show that BR-DIM and CDF could inhibit AR-regulated expression of ERG in cells containing *TMPRSS2-ERG* fusion gene.

More important, we also found that BR-DIM and CDF treatment, which inhibits AR and ERG activation, abrogated the activation of Wnt signaling stimulated by DHT under the status of *TMPRSS2-ERG* fusion, showing downregulation of Wnt-3a, Wnt-16, and LRP6 by BR-DIM or CDF, and upregulation of Naked2 and Axin1 by BR-DIM (Fig. 4D). These results suggest that the antitumor effects of BR-DIM and CDF in part could be mediated through the regulation of AR/TMPRSS2-ERG/Wnt signaling.

We also tested the effect of BR-DIM and CDF on the ERG transfection–mediated activation of Wnt signaling. We found that BR-DIM and CDF treatment attenuated the ERG-mediated upregulation in the expression of Wnt signaling molecules such as Wnt-16, LRP6, β-catenin, and p-GSK-3β (Fig. 5A). Moreover, BR-DIM and CDF treatment resulted in the inhibition of the expression of Wnt-16 in ERG siRNA–transfected cells (Fig. 5B), suggesting the inhibitory effects of BR-DIM and CDF on Wnt signaling.

By immunoprecipitation studies, we found that BR-DIM could abrogate upregulation of CBP binding to ERG, AR, GSK-3β, or β-catenin stimulated by DHT (Fig. 5C), suggesting that BR-DIM could inhibit the activation of Wnt signaling and ERG-regulated transcription. Indeed, by TOPflash assay, we found that BR-DIM and CDF could abrogate the upregulation of TCF-mediated gene transcription stimulated by ERG cDNA transfection (Fig. 5D). We also observed that BR-DIM and CDF could further inhibit the TCF-mediated gene transcription in ERG siRNA–transfected VCaP cells (Fig. 5D). Moreover, we found that CDF significantly inhibited the expression of AR, PSA, β-catenin, and Wnt signaling target c-Myc in both VCaP (Fig. 6A) and C4-2B

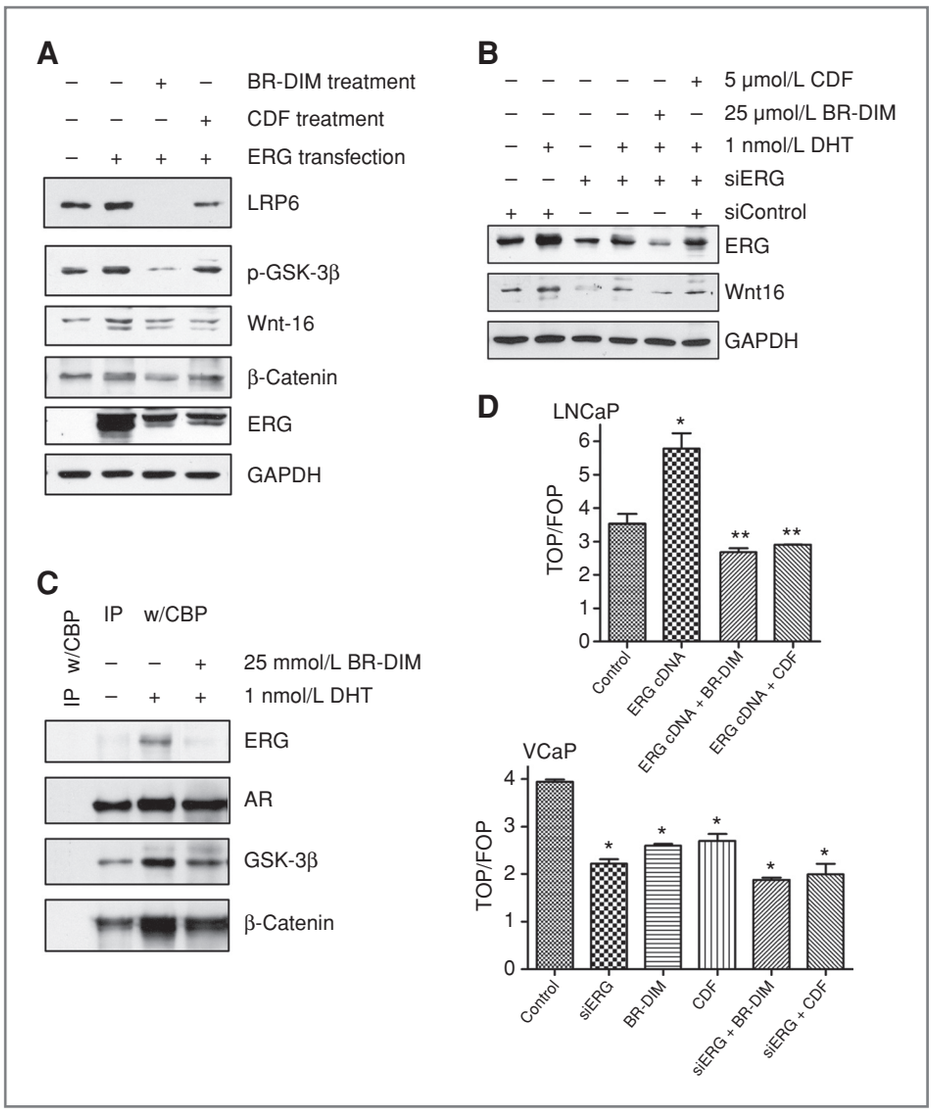

Figure 5. BR-DIM and CDF abrogated activation of Wnt signaling stimulated by the overexpression of ERG. A, ARCaP cells were transfected with ERG cDNA for 24 hours followed by 25 μmol/L BR-DIM or 2.5 μmol/L CDF treatment for 48 hours. Western blot analysis was conducted. B, VCaP cells were transfected with ERG siRNA (siERG) or control siRNA (siControl) for 48 hours followed by 1 nmol/L DHT treatment for 24 hours or first treated with 25 μmol/L BR-DIM and 5 μmol/L CDF for 48 hours and then with 1 nmol/L DHT treatment for 24 hours. Western blot analysis was conducted. C, VCaP cells were treated with 25 μmol/L BR-DIM for 48 hours followed by 1 nmol/L DHT treatment for 24 hours. Immunoprecipitation (IP) was conducted using anti-CBP and anti-IgG (control) antibodies. Western blot analysis was conducted to measure the binding activity of ERG, AR, GSK-3β, and β-catenin to CBP. D, TOPflash assay showed that Wnt signaling activity was upregulated by ERG cDNA transfection and downregulated by ERG siRNA (siERG) transfection or 25 μmol/L BR-DIM and 2.5 μmol/L CDF treatment for 48 hours. *, $P < 0.05$ compared with control; **, $P < 0.05$ compared with ERG cDNA; $n = 3$.

(Fig. 6B) cells, suggesting its inhibitory effects on AR and Wnt signaling.

BR-DIM and CDF inhibited invasion of prostate cancer cells through the regulation of AR/TMPRSS2-ERG/Wnt signaling network

By invasion assay, we found that forced overexpression of ERG by ERG cDNA transfection promoted cell invasion; however, BR-DIM and CDF inhibited cell invasion and abrogated the induction of invasive capacity of prostate cancer cells stimulated by ERG as shown earlier (Fig. 3A and B). Moreover, BR-DIM and CDF also showed their inhibitory effect on cell invasion of VCaP cells (Fig. 3C and D). Similar inhibitory effect on cell invasion was observed in ERG siRNA–transfected VCaP cells (Fig. 3C and D). These results suggest that BR-DIM and CDF inhibits cell invasion, which, is in part mediated through the down-regulation of TMPRSS2-ERG/Wnt signaling.

BR-DIM and CDF inhibited cell proliferation and induced apoptotic cell death

We have previously reported that BR-DIM inhibits the growth of LNCaP and C4-2B cells (33). In the current study, we found that both BR-DIM and CDF inhibited the growth of VCaP and LNCaP prostate cancer cells (Fig. 6C). Furthermore, we found that BR-DIM and CDF significantly induced apoptotic cell death in VCaP and LNCaP cells (Fig. 6D). These results suggest that BR-DIM and CDF could be promising agents for the inhibition of prostate cancer cell growth and invasive capacity.

Discussion

It is well known that fusion genes play important roles in carcinogenesis. More than 50% of human prostate cancers harbor *TMPRSS2-ERG* fusion gene, suggesting

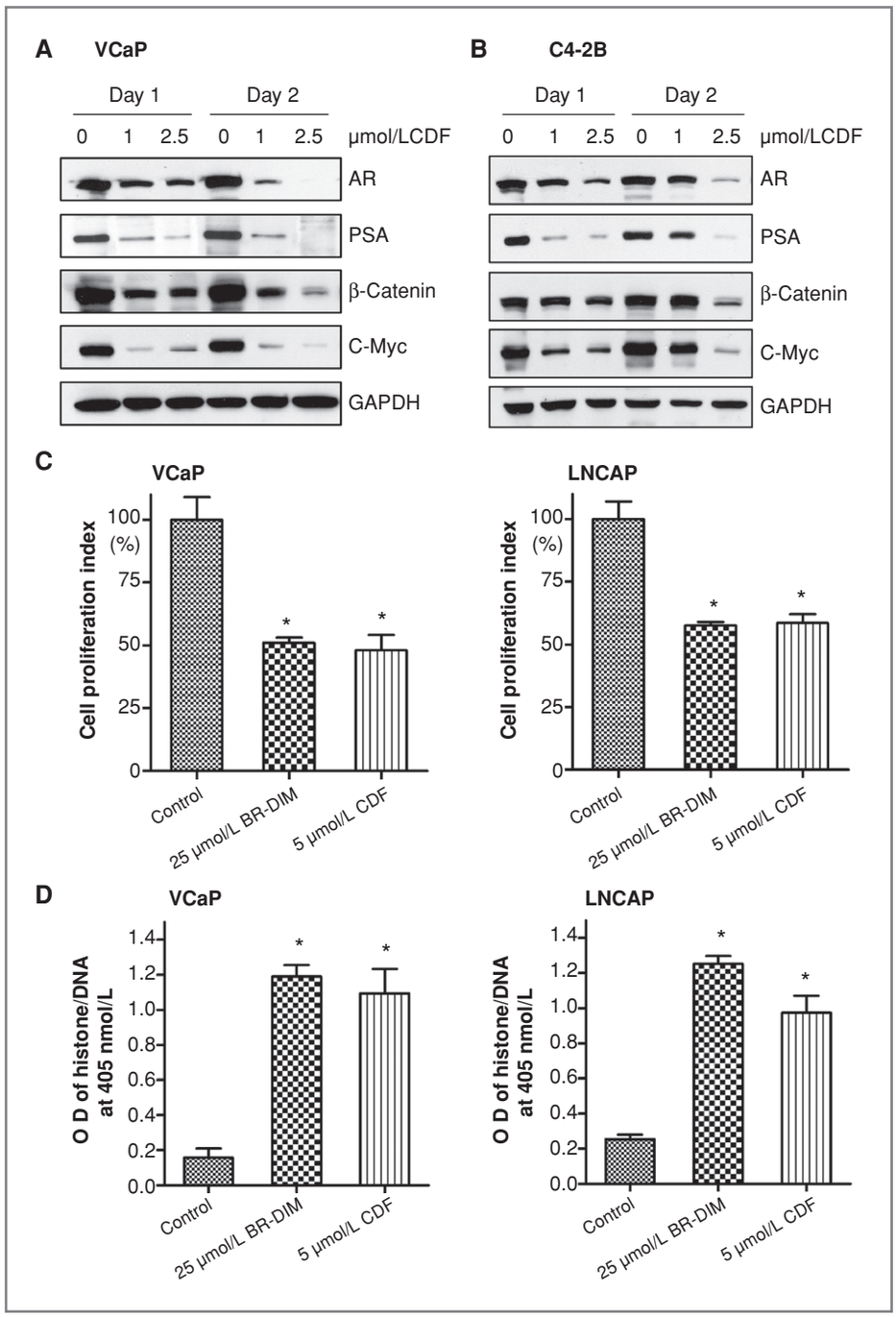

Figure 6. Western blot analysis showed that CDF inhibited the expression of molecules in AR (AR and PSA) and Wnt (β-catenin and c-Myc) signaling in both VCaP (A) and C4-2B (B) cells. BR-DIM and CDF treatment for 48 hours significantly inhibited cell proliferation (C) and induced apoptosis (D) in both VCaP and LNCaP cells. *, $P < 0.05$ compared with control; $n = 3$.

the importance of this fusion gene in the tumorigenesis of prostate cancer. The mechanisms leading to these fusions are unclear and somewhat controversial. Studies have shown that the treatment of cells with androgen can induce the *TMPRSS2-ERG* fusion through induction of chromosomal proximity (34) and DNA double-strand breaks mediated by androgen-induced topoisomerase IIβ (TOP2B; ref. 35) or ligand and genotoxic stress-induced cytidine deaminase and LINE-1 repeat-encoded ORF2 endonuclease (36). The induction of *TMPRSS2-ERG* fusion by androgen could be observed in both malignant and nonmalignant prostate epithelial cells, although prolonged exposure to androgen was required to detect the fusion transcript in nonmalignant cells (37), suggesting that the fusion can be induced by AR after or before malignant transformation. The roles of *TMPRSS2-ERG* fusion in prostate cancer progression are also unclear and somewhat controversial. Several studies on clinical

samples and *in vitro* experiments have shown that the expression of *TMPRSS2-ERG* fusion gene is correlated with prostate cancer progression (38, 39). It was found that ERG expression was fully restored by AR reactivation in VCaP xenografts that relapsed after castration, suggesting that expression of *TMPRSS2-ERG* fusion, similarly to other AR-regulated genes, could be restored in CRPC and may contribute to prostate cancer progression (15). Moreover, clinical observations indicated that certain *TMPRSS2-ERG* fusion isoforms were significantly correlated with more aggressive disease (40). Furthermore, *TMPRSS2-ERG* fusion has been found to be an early molecular event associated with prostate cancer invasion (41). Overexpression of ERG combined with *PTEN* loss could promote progression of prostate prostatic intraepithelial neoplasia (PIN) to invasive adenocarcinoma (12).

Our study showed that AR could regulate the expression of *TMPRSS2-ERG* fusion gene and, in turn, led to the activation of Wnt signaling, which has been recognized as inducer of prostate cancer progression (42, 43). Our results are consistent with a recent study showing that ERG could activate Wnt signaling in prostate cancer cells (16). Moreover, our study showed that forced overexpression of ERG could promote invasion of prostate cancer cells. All of these results clearly suggest that *TMPRSS2-ERG* fusion gene, which occurred before or after malignant transformation, could result in the progression of prostate cancer; therefore, inhibition of *TMPRSS2-ERG* expression could be a newer strategy for the prevention of PIN progression to invasive prostate cancer.

Recent studies have shown that Wnt signaling, another critical signaling pathway in cancer cells, also plays important role in the development and progression of CRPC (43, 44). However, the regulation between AR and Wnt signaling remains controversial. It has been reported that Wnt molecules could be negatively regulated by androgen (45). In our study, we did not find activation of Wnt signaling after DHT treatment in LNCaP and C4-2B cells. However, we found the activation of Wnt signaling in VCaP cells treated with DHT or in LNCaP cells transfected with ERG cDNA. These results suggest that the activation of Wnt signaling by DHT that we have observed is mediated through AR/TMPRSS2-ERG signaling. Therefore, the effect of androgen on the regulation of Wnt signaling could be dependent on the existence of fusion gene or other signaling perturbation. Thus, the complexities in the regulation of AR and Wnt signaling need further in-depth investigation to answer how ERG activates Wnt signaling.

It is known that *c-Myc* is one of the Wnt target genes. We found that ERG overexpression induced the activation of Wnt signaling, leading to the higher expression of *c-Myc*. Other investigators also found the regulation of *c-Myc* expression by ERG and a significant correlation between *c-Myc* overexpression and *TMPRSS2-ERG* fusion was reported (46), suggesting that *TMPRSS2-ERG* fusion could lead to the activation of Wnt signaling and the subsequent *c-Myc* overexpression. The overexpression of *c-Myc* has been correlated with high invasive activity of cancer cells (47, 48). We also found that ERG overexpression, which activated Wnt signaling and increased c-Myc expression, promoted invasion of prostate cancer cells. These results suggest that AR-regulated expression of *TMPRSS2-ERG* fusion could lead to prostate cancer progression including invasion through activation of AR/TMPRSS2-ERG/Wnt signaling axis.

We have previously reported that DIM could inhibit cancer cell growth, which was in part mediated through the downregulation of Akt, AR, and NF-κB signaling (33). Curcumin also downregulates AR and NF-κB signaling, resulting in the inhibition of cell proliferation. Importantly, in this study we found that BR-DIM and CDF could also inhibit AR/TMPRSS2-ERG/Wnt signal transduction, leading to the inhibition of prostate cancer cell invasion. Although both BR-DIM and CDF showed inhibitory effects on Wnt signaling activity, the Wnt molecules and the expression levels of Wnt molecules altered by BR-DIM or CDF were not the same, suggesting that other regulatory molecules are responsible for differential alterations in the Wnt signaling by these agents. Both Axin1 and Naked2 are negative regulators of Wnt signaling; however, they also form complex with molecules in other signaling pathway. We found that both Axin1 and Naked2 were upregulated by BR-DIM whereas CDF only upregulated Naked2. We also found that CDF significantly inhibited the expression of molecules including AR, PSA, β-catenin, and c-Myc that are important in the AR and Wnt signaling axis in both VCaP cells harboring *TMPRSS2-ERG* fusion and C4-2B cells without such fusion. These results suggest that CDF could inhibit Wnt activation by regulation of other signal transduction pathways in addition to the AR/TMPRSS2-ERG/Wnt signaling.

In conclusion, BR-DIM and CDF could be useful for the prevention of prostate cancer progression either alone or in combination with other therapeutics. The biological activity of BR-DIM and CDF is mediated through the downregulation of multiple signaling pathways including AR/TMPRSS2-ERG/Wnt signaling, which makes them very promising newer agents that could become useful for designing novel therapeutic strategies for the prevention and/or treatment of prostate cancer and its progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This study was supported by National Cancer Institute, NIH (5R01CA108535 to F.H. Sarkar). The Guido and Puschelberg Foundation provided generous contribution for the completion of this study.

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Received February 7, 2011; revised May 4, 2011; accepted May 26, 2011; published OnlineFirst June 16, 2011.

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