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Inactivation of AR/TMPRSS2-ERG/Wnt signaling networks attenuate the aggressive behavior of prostate cancer cells

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Abstract

The development of prostate cancer (PCa) and its progression to castrate-resistant prostate cancer (CRPC) after anti-androgen ablation therapy are driven by persistent biological activity of androgen receptor (AR) signaling. Moreover, studies have shown that more than 50% of human PCa over-express ERG 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 PCa cells, and further assessed the effects of BR-DIM and CDF (natural agents-derived synthetic formulation and analogue of 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 over-expression and nuclear translocation activated the activity of Wnt signaling. Furthermore, forced over-expression of ERG promoted invasive capacity of PCa cells. More importantly, 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 PCa cell invasion. In addition, BR-DIM and CDF inhibited proliferation of PCa cells and induced apoptotic cell death. Based on our findings, we conclude that because BR-DIM and CDF down-regulate 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 PCa.

Keywords

TMPRSS2-ERG; Wnt; prostate cancer; DIM; CDF

Introduction

Despite significant effort made in the fight against cancers, prostate cancer (PCa) 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 PCa (CRPC) contribute to high mortality of patients diagnosed with PCa. Emerging evidence suggests that the mechanisms involved in the progression of PCa include

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the deregulation of androgen receptor (AR), Akt, Wnt, and Hedgehog signaling (2–5). Among them, AR signaling is more critical for the development of PCa and the progression of PCa to CRPC (5). It is believed that because of AR over-expression and androgen hypersensitivity found in CRPC, AR is easily activated by low concentration of androgen present in the prostates of men treated with anti-androgen therapy (6, 7).

Moreover, studies have shown that a significant fraction (more than 50%) of human PCa expresses a fusion gene product, especially the AR-regulated over-expression 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 demonstrated that over-expression of ERG transcript is sufficient for the initiation of PCa (10). Importantly, the patients with TMPRSS2-ERG mediated over-expression of ERG have shown a significantly higher risk of recurrence (58.4% at 5 years) compared to the patients lacking TMPRSS2-ERG fusion gene (8.1%, $P < 0.0001$) (9). The frequency in the expression of AR-regulated ERG due to TMPRSS2-ERG fusion was found to be increased in moderate to poorly differentiated PCa (11). Importantly, ERG over-expression could cooperate with the loss of PTEN, promoting tumor progression of PCa (12, 13). Moreover, TMPRSS2-ERG fusion has been associated with activated AR and Wnt signaling in CRPC (14, 15). Furthermore, the crosstalk between ERG and Wnt or EZH2 in Epithelial-to-Mesenchymal Transition (EMT) phenotypic cells or prostate lineage-specific differentiation has been documented (16, 17). Although controversies exist regarding the role of TMPRSS2-ERG fusion in PCa (9, 18), it is generally believed that AR-regulated over-expression 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 PCa. 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 up-regulation 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 PCa, and thus β -catenin is believed to play important roles in the progression of PCa (20, 21). Moreover, experimental studies have shown that β -catenin also modulates AR signaling at multiple levels including trans-activation of AR (22), suggesting the novelty and importance of a signaling network between AR-regulated over-expression of ERG due to TMPRSS2-ERG fusion and the activation of Wnt signaling in PCa. Therefore, it is important to investigate the regulation of AR/TMPRSS2-ERG/Wnt signaling in PCa progression and design novel strategies by which one could attenuate these signaling networks for the prevention or treatment of PCa 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 down-regulate the expression of AR (23); therefore, BR-DIM could in turn inhibit the expression of ERG in PCa cells that are positive for TMPRSS2-ERG fusion because ERG expression could be down-regulated due to 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 has shown rapid metabolism and lacks systemic and target tissue bioavailability (26, 27). To overcome such a problem of curcumin, we have recently reported the development of a synthetic analogue of curcumin (CDF) which showed much

superior bioavailability (28), suggesting that CDF could be useful for the prevention of PCa progression. In this study, we investigated the mechanistic role of AR/TMPRSS2-ERG/Wnt signaling network in the aggressive behavior of PCa cells, and further investigated the effects of BR-DIM and CDF on the regulation of AR/TMPRSS2-ERG/Wnt signaling in PCa cells.

Materials and Methods

Cell lines, reagents, and antibodies

VCaP (ATCC, Manassas, VA), LNCaP (ATCC), C 4-2B and ARCaP (Novicure, Birmingham, AL) prostate cancer cells were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a 5% CO₂ 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 (STR) profiling using the PowerPlex® 16 System from Promega (Madison, WI). BR-DIM [BioResponse, Boulder, CO; formulated-DIM with higher bioavailability *in vivo* (29)] was generously provided by Dr. Michael Zeligs and was dissolved in DMSO to make a 50 mM stock solution. CDF [3,4-difluoro-benzo-curcumin or simply difluorinated curcumin (28)] was dissolved in DMSO to make a 5 mM stock solution. Anti-AR (Santa Cruz, Santa Cruz, CA), anti-ERG (Santa Cruz), anti-ERG (Epitomics, Burlingame, CA), anti-PSA (Santa Cruz), anti-CBP (Santa Cruz), anti-Wnt-16 (Santa Cruz), anti-β-catenin (Cell Signaling, Danvers, MA), anti-Wnt-3a (Cell Signaling), anti-LRP6 (Cell Signaling), anti-Naked2 (Cell Signaling), anti-Axin1 (Cell Signaling), anti-GAK-3β (Cell Signaling), anti-β-actin (Sigma, St. Louis, MO) 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 PCa cells were treated with 25 µM BR-DIM or 2.5 to 5 µM CDF for 24 and 48 hours. Some samples were followed by 1 nM DHT or 10 nM testosterone treatment for 24 hours. After treatment and harvesting, the cells were resuspended in lysis buffer (0.08 M KCl/ 35 mM HEPES pH 7.4/ 5 mM K-phosphate pH 7.4/ 5 mM MgCl₂/ 25 mM CaCl₂/ 0.15M sucrose/ 2mM PMSF/ 8mM DTT) and frozen at -80°C overnight. The cell suspension was thawed and passed through a 28 gauge needle three times. A small aliquot of the cells were checked for cell membrane breakage 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 by using Coomassie Plus Protein Assay kit (Pierce, Rockford, IL). For total protein extraction, BR-DIM or CDF-treated VCaP, LNCaP and C4-2B PCa cells were lysed in RIPA buffer. After centrifugation, the concentration of total protein was measured using BCA protein assay (PIERCE, Rockford, IL).

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) and incubation for 1 hour, the samples were centrifuged. The agarose pellet was then washed three times, resuspended in Laemmli buffer, and boiled for 5 minutes. The boiled samples were centrifuged and 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, Rockford, IL) and quantified by using AlphaEaseFC (Alpha Innotech, Santa Clara, CA). 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 CMV-driven N-terminally truncated ERG cDNA expression construct (10) was transiently transfected into LNCaP and C4-2B cells using ExGen 500 (Fermentas, Hanover, MD). After 5 hours, the transfected cells were washed and incubated with complete RPMI 1640 medium overnight followed by treatment with 25 μ M BR-DIM or 5 μ M 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 using specific antibodies as shown under 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) or control RNA duplex (Santa Cruz) by DharmaFECT (Thermo, Rockford, IL) for 48 hours followed by 1 nM DHT treatment for 24 hours or followed by 25 μ M BR-DIM and 5 μ M 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 by using BD BioCoat Tumor Invasion Assay System (BD Biosciences, Bedford, MA) 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 μ M BR-DIM or 2.5 μ M CDF were seeded into the upper chamber of the system. Bottom wells in the system were filled with complete medium and same reagent treatment as upper chamber. After 24 hours of incubation, the cells in the upper chamber were removed, and the cells, which invaded through matrigel matrix membrane, were stained with 4 μ g/ml Calcein AM in Hanks buffered saline at 37°C for one hour. Then, fluorescence of the invaded cells was read in a ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 530/590nm. These fluorescently labeled invasive cells were also photographed under a fluorescent microscope.

TOPflash assay for measuring activity of Wnt signaling

TOPflash assay was performed according to the manufacture's instruction (Millipore, Temecula, CA). TOPflash contains six 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 co-transfected with ERG cDNA construct into LNCaP and C4-2B cells using ExGen 500 (Fermentas) with or without 25 μ M BR-DIM or 2.5 μ M CDF treatment. In addition, the TOPflash or FOPflash reporter plasmid was also transiently co-transfected with ERG siRNA into VCaP cells using 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 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and WST-1 assays

VCaP, LNCaP and C4-2B PCa cells were seeded in 96-well plates. After 24 hours, the cells were treated with 25 μ M BR-DIM or 5 μ M 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, Palo Alto, CA) 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, San Diego, CA).

Histone/DNA ELISA for detection of apoptosis

VCaP, LNCaP and C4-2B PCa cells were seeded in 6-well plates. After 24 hours, the cells were treated with 25 μ M BR-DIM or 5 μ M 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 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 PCa cells was statistically evaluated by Student's t-Test using GraphPad StatMate software (GraphPad Software Inc).

Results

Androgen receptor (AR) induced ERG expression through TMPRSS2-ERG fusion

Since VCaP cells harbor TMPRSS2-ERG fusion gene while C4-2B and LNCaP cells have no such fusion gene, we first tested the specificity of five ERG antibodies from different companies. We observed strong signal of ERG in VCaP cells even under very short exposure time and no signal detected in LNCaP cells under short exposure except with the antibody from abcam (Supplementary Figure 1). However, after long exposure we observed ERG signal in LNCaP cells by all five antibody staining (Supplementary Figure 1), suggesting that LNCaP cells have 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 and Epitomics for our subsequent studies because clear-cut ERG signal was observed by using these antibodies.

In order 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 PSA and caused AR nuclear translocation in VCaP, C4-2B and LNCaP cells (Fig. 1A, 1B, and Supplementary Figure 2); however, ERG induction and nuclear translocation after DHT treatment was only observed in VCaP cells (Fig. 1A and Supplementary Figure 2), 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 TMPRSS2-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 1C). Importantly, we also found that Wnt signaling was activated after DHT treatment. Several Wnt ligands and co-regulators including β -catenin (Figure 1C), Wnt-3a, Wnt-16, and LRP6 (Fig. 1D) were up-regulated while Naked2 and Axin1, which are known to inhibit Wnt signaling, were down-regulated (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 crosstalk between AR-regulated TMPRSS2-ERG fusion and Wnt signaling.

It has been known that transcriptional co-activator 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 genes. It has been found that CBP regulates the activation of Wnt signaling by interacting with β -catenin (32). Therefore, we conducted immunoprecipitation experiments 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-3 β , or β -catenin in the nucleus of VCaP cells (Fig. 2A). Importantly, we observed that the activity of CBP binding to ERG, AR, GSK-3 β , 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.

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 up-regulation 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 which have very low expression of ERG and found that ERG cDNA transfection up-regulated the level of Wnt signaling molecules such as Wnt-16, LRP6, p-GSK-3 β and β -catenin (Fig. 2C), further confirming the crosstalk 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 which commonly reflects the activity of Wnt signaling. We found that ERG cDNA transfection increased the activity of Wnt signaling while ERG siRNA transfection down-regulated the activity of Wnt-signaling (Fig. 2D). Western blot analysis showed that the forced over-expression of ERG by cDNA transfection up-regulated the expression of c-Myc and cyclin D1 (Fig. 2D), two important target genes of Wnt signaling, and that ERG siRNA transfection decreased 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

Since 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 3B), suggesting that high expression of ERG could promote invasion of PCa cells through activation of Wnt signaling. In order to further confirm this observation, we transfected ERG siRNA into VCaP cells to inactivate ERG expression, and performed invasion assay. We found that the inhibition of ERG decreased the invasive capacity of VCaP cells (Fig. 3C and 3D), 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 decreased the expression of AR, leading to the inhibition of PCa cell growth (33). In this study, we tested the effect of BR-DIM and CDF on AR expression and found that both BR-DIM and CDF could down-regulate the expression of AR (Fig. 4A and Supplementary Figure 3). To investigate whether the inhibition of AR could lead to the down-regulation 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 which harbor TMPRSS2-ERG fusion gene. We found that testosterone or DHT treatment significantly up-regulated the expression of ERG and PSA as expected and increased nuclear translocation of AR and ERG (Fig. 4B, 4C, and Supplementary Figure 3), suggesting that the expression of ERG in VCaP cells is regulated by AR. Importantly, we found that BR-DIM or CDF pre-treatment abrogated the up-regulation of ERG and PSA, and inhibited the nuclear translocation of AR and ERG stimulated by testosterone or DHT. These results demonstrate that BR-DIM and CDF could inhibit AR-regulated expression of ERG in cells containing TMPRSS2-ERG fusion gene.

More importantly, 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 down-regulation of Wnt-3a, Wnt-16, and LRP6 by BR-DIM or CDF, and up-regulation of Naked2 and Axin1 by BR-DIM (Fig. 4D). These results suggest that the anti-tumor 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 up-regulation in the expression of Wnt signaling molecules such as Wnt-16, LRP6, β -catenin and p-GSK-3 β (Fig. 5A). Moreover, BR-DIM and CDF treatment resulted in the inhibition in 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 up-regulation of CBP binding to ERG, AR, GSK-3 β , 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 up-regulation 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 (Fig. 6B) cells, suggesting its inhibitory effects on AR and Wnt signaling.

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

By invasion assay, we found that forced over-expression 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 PCa cells stimulated by ERG as shown earlier (Fig. 3A and 3B). Moreover, BR-DIM and CDF also showed their inhibitory effect on cell invasion of VCaP cells (Fig. 3C and 3D). Similar inhibitory effect on cell invasion was observed in ERG siRNA transfected VCaP cells (Fig. 3C and 3D). 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 PCa 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 PCa cell growth and invasive capacity.

Discussion

It is well known that fusion genes play important roles in carcinogenesis. More than 50% of human PCa harbor TMPRSS2-ERG fusion gene, suggesting the importance of this fusion gene in the tumorigenesis of PCa. 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 II β (TOP2B) (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 PCa 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 PCa 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 PCa 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). Over-expression of ERG combined with PTEN loss could promote progression of prostate 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 PCa progression (42, 43). Our results are consistent with a recent study showing that ERG could activate Wnt signaling in PCa cells (16). Moreover, our study showed that forced over-expression of ERG could promote invasion of PCa cells. All of these results clearly suggest that TMPRSS2-ERG fusion gene, occurred prior to or after malignant transformation, could result in the progression of PCa; therefore, inhibition of TMPRSS2-ERG expression could be a newer strategy for the prevention of prostatic intraepithelial neoplasia progression to invasive PCa.

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 in order to answer how ERG activates Wnt signaling.

It is known that c-Myc is one of the Wnt target genes. We found that ERG over-expression 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 over-expression 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 over-expression. The over-expression of c-Myc has been correlated with high invasive activity of cancer cells (47, 48). We also found that ERG over-expression, which activated Wnt signaling and increased c-Myc expression, promoted invasion of PCa cells. These results suggest that AR regulated expression of TMPRSS2-ERG fusion could lead to PCa 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 down-regulation of Akt, AR and NF- κ B signaling (33). Curcumin also down-regulates AR and NF- κ B 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 PCa 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 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 up-regulated by BR-DIM while CDF only up-regulated 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 PCa progression either alone or in combination with other therapeutics. The biological activity of BR-DIM and CDF is mediated through the down-regulation 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 PCa and its progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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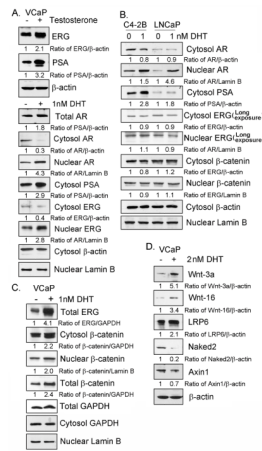
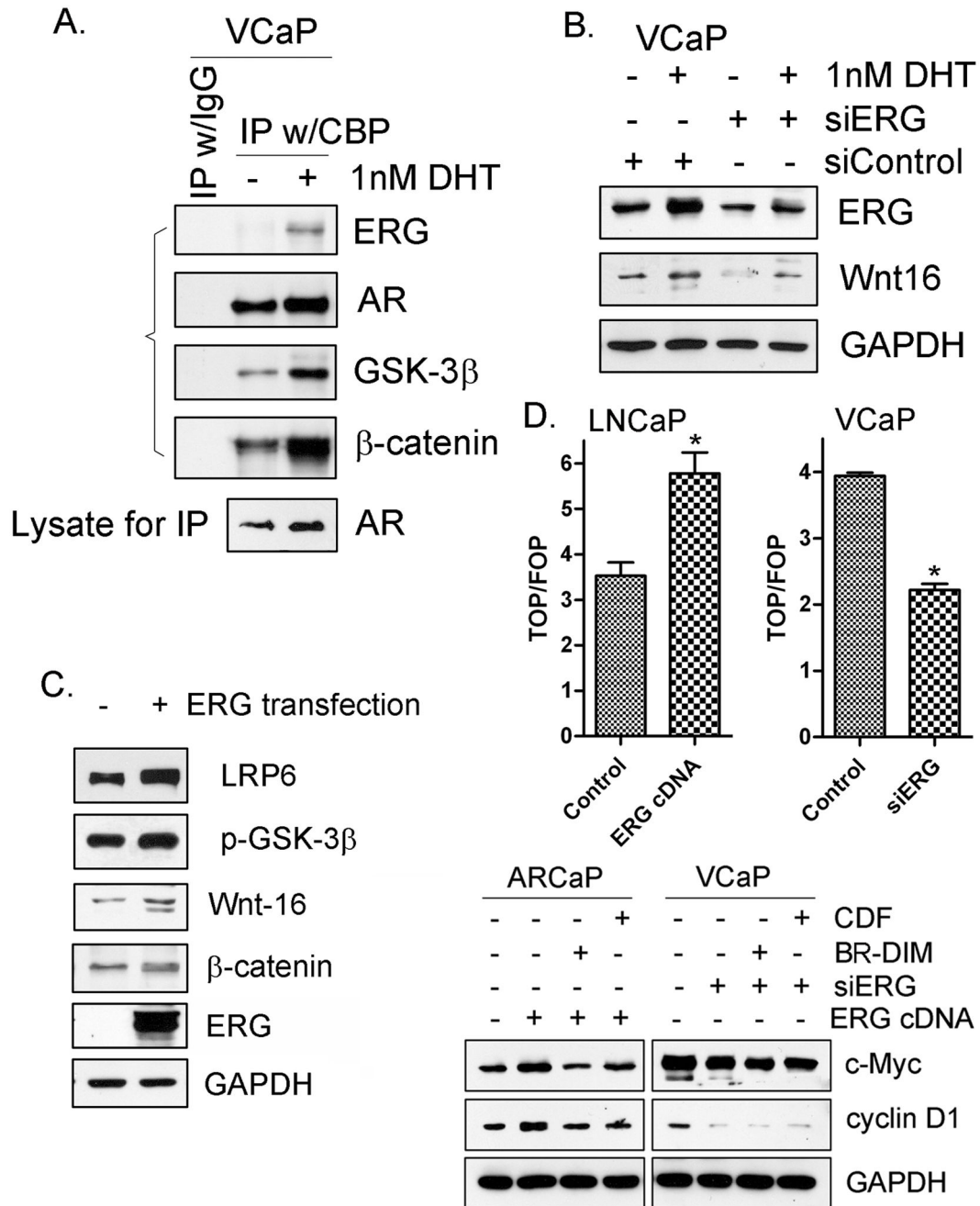


Figure 1. AR/TMPRSS2-ERG/Wnt signaling was up-regulated by testosterone or DHT treatment. (A) VCaP cells were treated with 10 nM testosterone or 1 nM DHT for 24 hours. (B) C4-2B and LNCaP cells were treated with 1 nM DHT for 24 hours. (C) VCaP cells were treated with 1 nM DHT for 24 hours. (D) VCaP cells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

**Figure 2.**

Over-expression of ERG activated Wnt signaling. (A) VCaP cells were treated with 1 nM DHT for 24 hours. Immunoprecipitation 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 nM DHT treatment for 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 up-regulated by ERG cDNA transfection and down-regulated by ERG siRNA (siERG) transfection. Western Blot analysis showed that the

expression of Wnt targets, c-Myc and cyclin D1, was up-regulated by ERG cDNA transfection and down-regulated by ERG siRNA, BR-DIM and CDF. (*: $p < 0.05$ compared to control; n=3;)

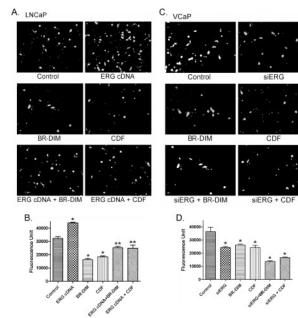


Figure 3.

ERG over-expression promoted the invasive activity of prostate cancer cells while ERG siRNA, BR-DIM, and CDF inhibited the invasion of PCa cells. (A) Invasion assay was conducted to test the invasive activity of LNCaP cells transfected ERG cDNA and/or treated with 25 μ M BR-DIM or 2.5 μ M CDF. (B) The graph showed the value of 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 μ M BR-DIM or 2.5 μ M CDF. (D) The graph showed the value of fluorescence from the invaded VCaP cells. The value indicated the comparative amount of invaded VCaP cells. (*: $p < 0.05$ compared to control; **: $p < 0.05$ compared to ERG cDNA; $n = 3$;)

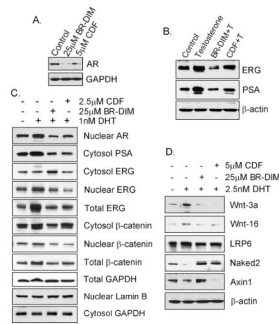
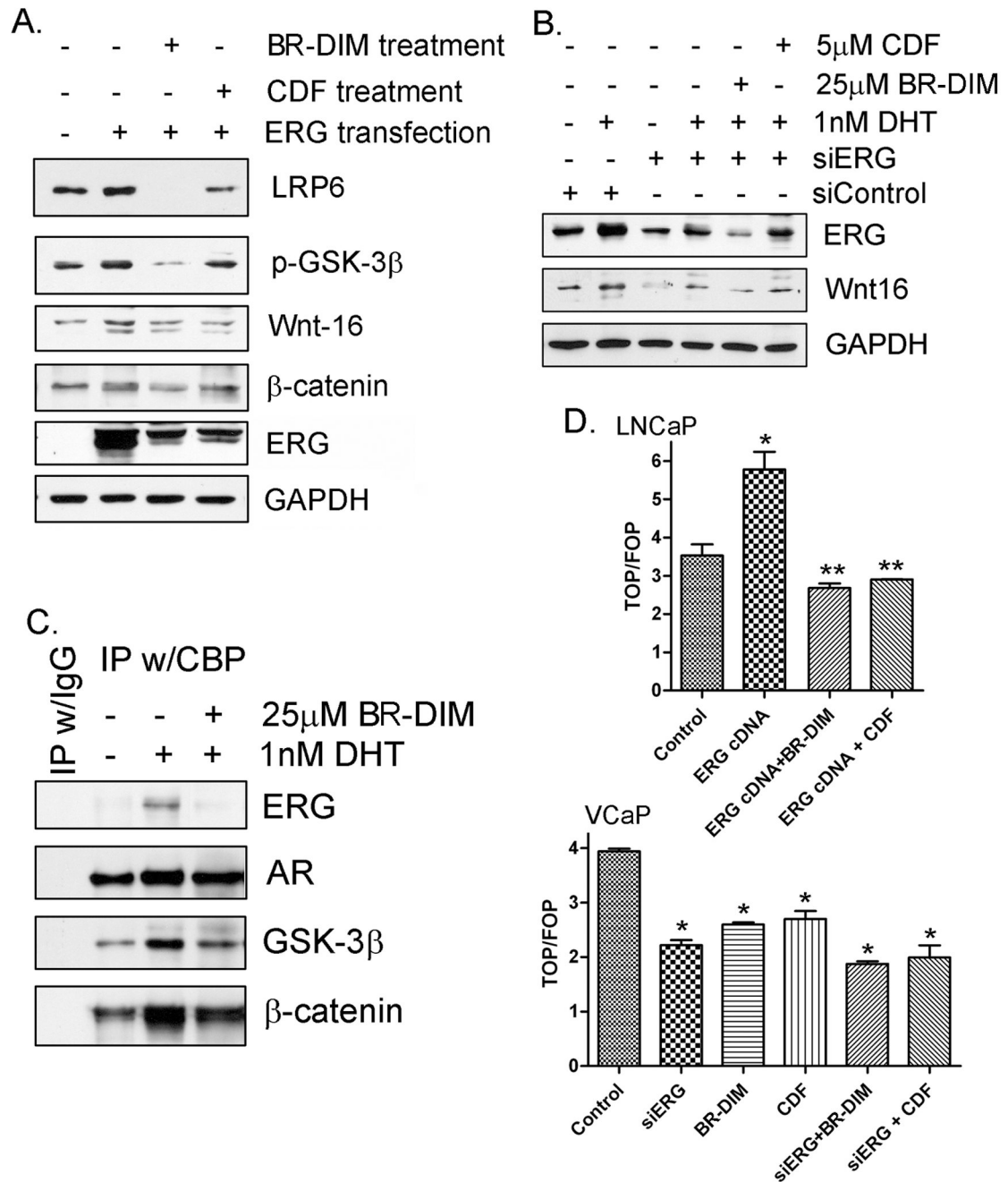


Figure 4.

AR/TMPRSS2-ERG/Wnt signaling was down-regulated by BR-DIM and CDF. (A) VCaP cells were treated with 25 μ M BR-DIM or 5 μ M CDF for 48 hours. (B) VCaP cells were treated with 25 μ M BR-DIM or 5 μ M CDF for 48 hours followed by 10 nM testosterone for 24 hours. (C) VCaP cells were treated with 25 μ M BR-DIM or 2.5 μ M CDF for 48 hours followed by 1 nM DHT treatment for 24 hours. (D) VCaP cells were treated with 25 μ M BR-DIM or 5 μ M CDF for 48 hours followed by 2.5 nM DHT treatment for 24 hours. Western Blot analysis was conducted to measure the expression of molecules in AR, ERG, and Wnt signaling.

**Figure 5.**

BR-DIM and CDF abrogated activation of Wnt signaling stimulated by the over-expression of ERG. (A) ARCaP cells were transfected with ERG cDNA for 24 hours followed by 25 μM BR-DIM or 2.5 μM 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 nM DHT treatment for 24 hours or first treated with 25 μM BR-DIM and 5 μM CDF for 48 hours and then with 1 nM DHT treatment for 24 hours. Western Blot analysis was conducted. (C) VCaP cells were treated with 25 μM BR-DIM for 48 hours followed by 1 nM DHT treatment for 24 hours. Immunoprecipitation was conducted using anti-CBP and anti-IgG (control) antibodies. Western Blot analysis was conducted to

measure the binding activity of ERG, AR, GSK-3 β , and β -catenin to CBP. (D) TOPflash assay showed that Wnt signaling activity was up-regulated by ERG cDNA transfection and down-regulated by ERG siRNA (siERG) transfection or 25 μ M BR-DIM and 2.5 μ M CDF treatment for 48 hours. (*: p<0.05 compared to control; **: p<0.05 compared to ERG cDNA; n=3;)

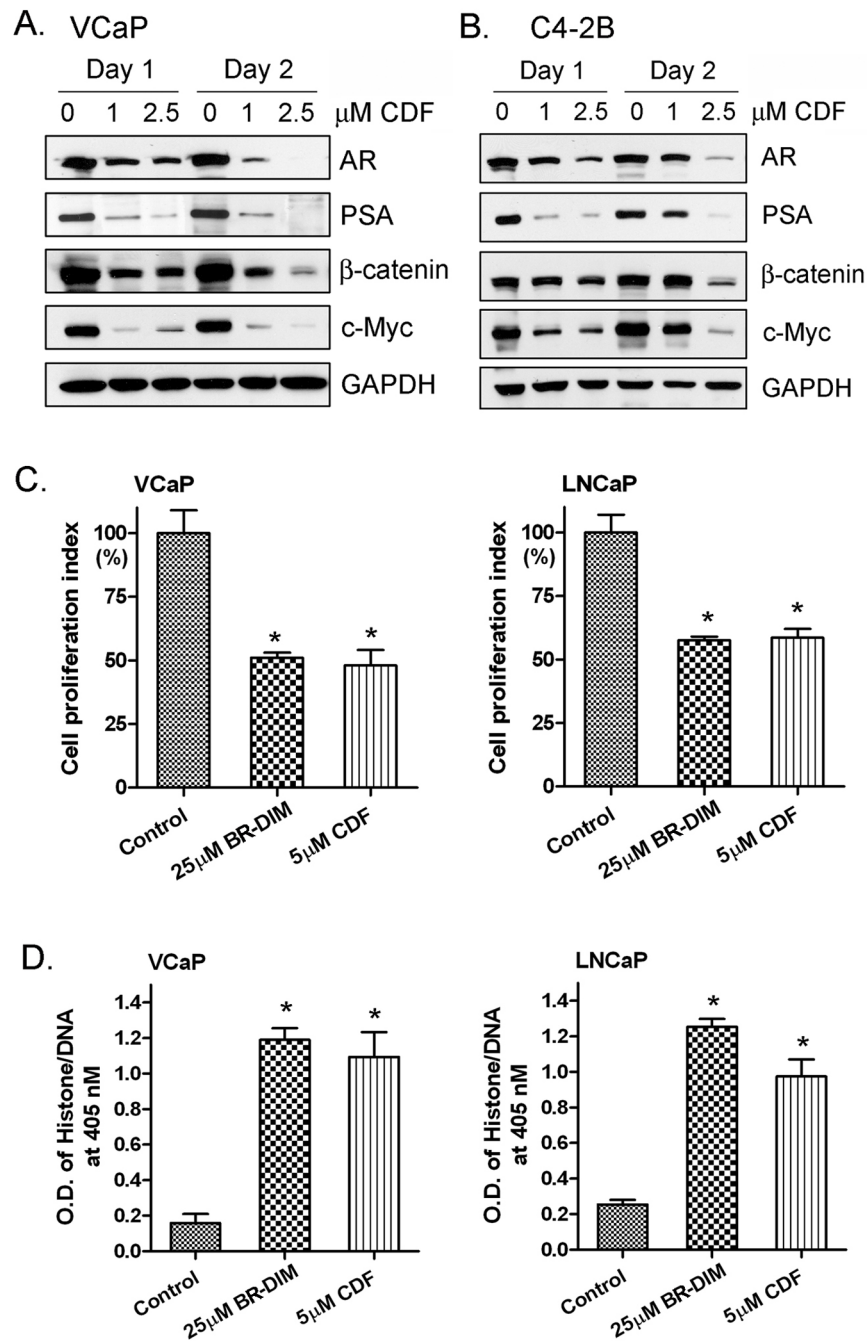


Figure 6. Western Blot analysis showed that CDF inhibited the expression of molecules in AR (AR & PSA) and Wnt (β -catenin & c-Myc) signaling in both VCaP (A) and C4-2B (B) cells. BR-DIM and CDF treatment for 48 hours significantly inhibited cell proliferation (C) and induced apoptosis (D) in both VCaP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