Androgen Receptor Regulation of the Versican Gene through an Androgen Response Element in the Proximal Promoter*

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Versican, one of the key components of prostatic stroma, plays a central role in tumor initiation and progression. Here, we investigated promoter elements and mechanisms of androgen receptor (AR)-mediated regulation of the versican gene in prostate cancer cells. Using transient transfection assays in prostate cancer LNCaP and cervical cancer HeLa cells engineered to express the AR, we demonstrate that the synthetic androgen R1881 and dihydrotestosterone stimulate expression of a versican promoter–driven luciferase reporter vector (versican-Luc). Further, both basal and androgen-stimulated versican-Luc activities were significantly diminished in LNCaP cells, when AR gene expression was knocked down using a short hairpin RNA. Methylation-protection footprinting analysis revealed an AR-protected element between positions +75 and +102 of the proximal versican promoter, which strongly resembled a consensus steroid receptor element. Electrophoretic mobility shift and supershift assays revealed strong and specific binding of the recombinant AR DNA binding domain to oligonucleotides corresponding to this protected DNA sequence. Site-directed mutagenesis of the steroid receptor element site markedly diminished R1881-stimulated versican-Luc activity. In contrast to the response seen using LNCaP cells, R1881 did not significantly induce versican promoter activity and mRNA levels in AR-positive prostate stromal fibroblasts. Interestingly, overexpression of β-catenin in the presence of androgen augmented versican promoter activity 10- and 30-fold and enhanced versican mRNA levels 2.8-fold in fibroblasts. In conclusion, we demonstrate that AR transactivates versican expression, which may augment tumor-stromal interactions and may contribute to prostate cancer progression.

Stromal tissue mediates the induced growth and development of embryonic epithelium into differentiated prostate (1).

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†† The abbreviations used are: ECM, extracellular matrix; AI, androgen-independent; AR, androgen receptor; AREs, androgen-response elements; BPH, benign prostatic hyperplasia; CSS, charcoal-stripped serum; DHT, dihydrotestosterone; ER, estrogen receptor; DMS, dimethyl sulfate; His-ARDBD, His-tagged AR DNA-binding domain; Luc, luciferase; SRE, steroid response element; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PSA, prostate-specific antigen; PSA-Luc, prostate-specific antigen-Luciferase; shRNA, short hairpin RNA; RT, reverse transcription; Wnt, wingless; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; MeP, methylation protection.

Prostatic stroma is also necessary in the maintenance of adult prostatic secretory epithelium (2, 3). In addition, stromal-epithelial interactions play a significant role in steroid-influenced prostate carcinogenesis and in the progression to the hormone-insensitive phenotype (4).

Tumor cells must remodel the matrix to facilitate communication and escape control by the microenvironment. Remodeling can also include interactions with “alternative” extracellular matrix (ECM),3 leading to cellular proliferation, structural disruption, and circumvention of apoptosis (5). Current findings obtained through a variety of approaches increasingly point to the contribution of stromal components to oncogenic signals that mediate both phenotypic and genomic changes in epithelial cells (6–8). Versican, a chondroitin sulfate proteoglycan, is one of the main components of ECM, which provides a loose and hydrated matrix during key events in development and disease (9, 10). Versican also contributes to the development of a number of pathologic processes, including atherosclerotic vascular diseases (11, 12), cancer (13), and central nervous system injury and neurite outgrowth (14). Several reports have shown that versican plays a role in cell adhesion (15), migration (16), proliferation (17), differentiation (18), angiogenesis (19), and resistance to oxidative stress-induced apoptosis (20); all of which are important events in tumor initiation and/or progression. Versican is highly expressed in many malignancies, including prostate cancers (21). Recent studies demonstrated that prostate cells from tumor and benign prostatic hyperplasia (BPH) tissues induce host stromal cells to accumulate versican levels via a paracrine mechanism (22, 23). Further, versican has been shown to be an important modulator of tumor cell attachment to the interstitial stromal matrix of the prostate, a factor likely important in cancer cell motility and local invasion of the prostatic stroma (24). As well it has been suggested that versican may be a useful marker of disease progression in patients with early-stage prostate cancer (21).
Genes that are preferentially expressed in human prostate tissues are often regulated by androgens at the transcriptional level. The androgen testosterone, and the more potent form dihydrotestosterone (DHT), bind to and activate the androgen receptor (AR). Upon ligand activation, the AR is phosphorylated and forms a homodimer that is transported to the nucleus where it activates transcription by binding to androgen-response elements (AREs) in promoter and enhancer regions of target genes (25). Androgen withdrawal is the most effective form of systemic therapy for men with advanced prostate cancer, producing symptomatic and/or objective responses in >80% of patients (26). Unfortunately, androgen-independent (AI) progression is inevitable, and with the development of hormone-refractory disease, death occurs within 2–3 years (26). AI progression is a multifactorial process by which cells acquire the ability to both survive in the absence of androgens and proliferate using non-androgenic stimuli for mitogenesis. It probably involves variable combinations of several processes, including adaptive up-regulation of antiapoptotic genes, ligand-independent activation of the AR, and alternative signaling pathways. The elucidation of the mechanisms that mediate AI progression, including key cytoprotective molecules, is an important step toward identifying new targets for therapy.

Several lines of evidence indicate that β-catenin is important in the progression of prostate cancer. Cre-mediated excision of the β-catenin (exon3) regulatory domain leads to prostatic hyperplasia and transdifferentiation in mice at 18 weeks of age but without metastatic behavior (27). In a similar model, stabilized β-catenin appears to be important for the initiation of prostatic neoplastic lesions the precursor to invasive carcinoma (28). Also, gain-of-function, truncated forms of β-catenin occurring in metastatic prostate and breast specimens have been shown to preferentially locate to the nucleus, possibly serving as an additional “pool” of β-catenin to promote cell proliferation during the AI phenotype (29). AR and β-catenin interact by direct binding and complexing, as ascertained by yeast two-hybrid analysis (30), glutathione S-transferase pull-down assays (31), coimmunoprecipitations (32), and transcriptional reporter assays (30, 32, 33). AR/β-catenin interactions are ligand-sensitive, such that the complexes increases in the presence of androgen (33) and decreases in the presence of the pure AR antagonist, bicalutamide (30, 33).

To understand the role of the ARs in prostate cancer development and progression, it is important to determine the AR signaling cascades and the genes that are regulated by AR. Despite the importance of versican in prostate cancer, the function and regulation of expression of this versatile molecule in vitro and in vivo is unknown. Our results, for the first time, indicate that versican is transcriptionally regulated by androgens in AR-positive human prostate epithelial and stromal cancer cells. Further, we show a strong and specific binding of recombinant AR to the DNA sequences located in the +75 region of the proximal human versican promoter. Using transient transfection of synthetic reporter and endogenous gene reporter constructs in prostate stromal fibroblasts, we demonstrated that β-catenin is required for AR-mediated transcription in both ligand-dependent and ligand-independent manner in prostate stromal fibroblast cells. These data identify a novel role for β-catenin in nuclear hormone receptor-mediated transcription in prostate stromal cells.

EXPERIMENTAL PROCEDURES

**Tissue Culture**—HeLa cells, modified HeLa-FLAG-AR-overexpressing cells (a gift of M. Carey, UCLA, Los Angeles, CA), and human prostate stromal fibroblast cell line WPMY-1 (Invitrogen) were maintained in DMEM (Invitrogen), whereas the LNCaP cell line (human prostate cancer cells) was maintained in RPMI (Invitrogen). Both types of media were supplemented with 10% fetal bovine serum (Invitrogen) and 100 units/ml penicillin/streptomycin. For androgen withdrawal experiments, media was instead supplemented with 10% charcoal-stripped serum (CSS) and 100 units/ml penicillin/streptomycin for at least 24 h prior to harvesting of cells.

**Oligonucleotides**—Oligonucleotides were synthesized by Nucleic Acids Protein Services (University of British Columbia). Oligonucleotide numberings for steroid response element (SRE)-2 are relative to the transcription start site of the human versican gene. The sequences of the oligonucleotides were: SRE-2 forward, CGAGACATTTGGTGTGT; and SRE-2 reverse, ACAACACCTAATGTTCTCG.

**Electrophoretic Mobility Shift Assay**—Individual SRE-2 oligonucleotides were subjected to EMSA as previously described (34). Nuclear extracts used in EMSA were isolated from HeLa and HeLa-FLAG-AR cells using a modified Dignam method (35). For EMSA experiments, 10,000 cpn of labeled oligoduplex probes were added to 18 μg of nuclear extracts. For supershift experiments 2 μg of rabbit anti-AR polyclonal antibody (2 μg/μl, Santa Cruz Biotechnology), 2 μg of rabbit anti-AR monoclonal antibody (2 μg/μl, Santa Cruz Biotechnology), or rabbit anti-FLAG polyclonal antibody (2 μg/μl, Santa Cruz Biotechnology) were added to the binding reaction, and the mixture was incubated for 30 min on ice prior to electrophoresis.

**Probe Generation for DMS in Vitro Footprinting**—The 764-bp proximal versican promoter was excised from the pGL-3 backbone by digestion with MluI and BglII. The DNA region of interest was radiolabeled by Klenow enzyme labeling using [α-32P]deoxyadenine and deoxyctydine triphosphate. The labeled promoter fragment was then digested with PflMI to give single end-labeled fragments of 426 and 338 bp (referred to as Versican1 and Versican2, respectively). These fragments were separated from each other using a 5% polyacrylamide, 0.5 × TBE (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA) non-denaturing gel. Probes were then cut out of the gel, eluted in 500 μl of elution buffer, and ethanol precipitated to generate a single end-labeled probe for DMS footprinting.

**DMS in Vitro Footprinting: Methylation Protection Analysis**—Footprinting reactions were carried out on the Versican1 or Versican2 probes using 5–10 μg (~3 μg/μl) of His-AR-DBD as described previously (34).

**Western Blotting**—Protein samples were boiled in Laemmli sample buffer and resolved by SDS-PAGE electrophoresis. Proteins were then transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat milk in TBST (0.05% Tween 20 in phosphate-buffered saline) for 1 h and then incubated for 1–2 h at room temperature with primary AR antibodies (Santa Cruz Biotechnology, Santa Cruz,
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CA). Detection was achieved with anti-mouse horseradish peroxidase (Santa Cruz Biotechnology) and ECL Western blotting detection agents (Amersham Biosciences, RPN 2106).

Plasmid Construction—The reporter plasmid used for transfections was the versican-Luc plasmid, which contains the firefly luciferase gene under the transcriptional control of the −632 to +118 fragment of the human versican promoter (36). The pRL-TK (Promega) Renilla luciferase reporter plasmid was used as an internal transfection control. A dominant stable β-catenin construct was a kind gift of B. Gumbiner (Memorial Sloan-Kettering Cancer Center, New York, NY). The expression vector harboring the wild-type human TCF-4 gene was a gift from A. Hecht (Max-Planck-Institute of Immunobiology, Freiburg, Germany). β-Catenin/TCF luciferase reporter constructs pTOPFlash8, which has eight tandem TCF binding sites, was used for assaying TCF transcriptional activity (a gift of R. T. Moon University of Washington, Seattle, WA).

Short Hairpin RNA against AR—We recently reported the generation and cloning of short hairpin RNA (shRNA) against AR (37). In brief, the pSHAG-1-tet plasmid (gift from A. Mui, University of British Columbia, Vancouver, Canada) was used to clone the shRNA was modified from pSHAG-1 (gift from G. Hannon, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The following sequence was found to have the greatest attenuating effect on AR expression: 5′-GAAAGCAGTCGGAGATCTTTTCGCGTGCAGAAAACTTC-3′ (3539–3569). A scrambled sequence (5′-CCGTACCTACCCAGCGCCTGAC-3′) was used as a negative control. Equal molar amounts of both forward and reverse complimentary oligonucleotides were annealed, kinased, and cloned into pSHAG-1-tet plasmid.

Transfections and Luciferase Assay—HeLa and HeLa-FLAG-AR cells were plated in 6-well plates (3.0 × 10⁵ cells per well) in DMEM with 5% fetal bovine serum for 2 days or until reaching 50–60% confluency. Each well was transfected with 2.0 μg of versican-Luc reporter plasmid and 0.2 μg of pRL-TK (Promega, Madison, WI) for purposes of normalization. Plasmid DNA was mixed with of Lipofectin reagent at a ratio of 2 μg of DNA/3 μl of Lipofectin (Invitrogen) in serum-free DMEM, and the mixture was incubated for 30 min at room temperature. Cells were incubated with the transfection mix for 16 h following which transfection medium was replaced by DMEM supplemented with 5% CSS and the appropriate concentration of steroid. LNCaP cells were plated in 6-well plates (3.0 × 10⁵ cells per well) in RPMI with 5% fetal bovine serum for 2 days or until reaching 50–60% confluency. Each well was transfected with 1.0 μg of AR shRNA vector or the scramble control, 1.0 μg of versican-Luc reporter plasmid, and 0.2 μg of pRL-TK (Promega) for purposes of normalization. Plasmid DNA was mixed with of Lipofectin reagent at a ratio of 2 μg of DNA/3 μl of Lipofectin (Invitrogen) in serum-free RPMI, and the mixture was incubated for 30 min at room temperature. Cells were incubated with the transfection mix for 16 h following which transfection medium was replaced by RPMI with 5% CSS with 10 nM androsterone (R1881), and cells were collected after 24 h of incubation using passive cell lysis buffer (Promega). To inhibit AR expression, LNCaP cells were seeded at a density of 3 × 10⁵ per well in a six-well plate and cotransfected the following day using Lipo-nectin reagent (Invitrogen) with versican-Luc and either shRNA to AR or scrambled sequence in pSHAG-1-tet. After 24 h, the medium was replaced with RPMI 1640 (Invitrogen) containing 5% CSS (HyClone, West Chester, PA) supplemented with 1 nm synthetic androgen (R1881). Luciferase activities were measured using a commercial kit from Promega according to the manufacturer’s protocol. Luciferase activities were measured as luminescent units/min and normalized to Renilla control values. All transfection experiments were carried out in 3–6 replicates.

RNA Extraction, cDNA Synthesis, and RT-PCR—Total RNA was isolated from treated and untreated human prostate stromal fibroblast cell line WPMY-1 using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen). All preparations were treated with RNase-free DNase (Qiagen) to remove genomic DNA. 0.5–1 μg of RNA was reverse-transcribed in a total volume of 20 μl in the presence of 200 units of SuperScript RNase H-Reverse Transcriptase (Invitrogen), 40 units of RNaseOUT recombinant ribonuclease inhibitor (Invitrogen), and 0.5 μg of Oligo(dT) Primer (Invitrogen) according to the manufacturer’s instructions. Conventional PCR was performed using primers specific for the human versican and β-actin genes and 2 μl of the synthesized cDNA strand. Specific primers for versican were synthesized by Sigma-Genosys: sense, 5′-GCAAAAAATTTCACCCTGAC-3′; antisense, 5′-GCACCTGGATCTGTTCATTCA-3′. The samples were amplified in a thermal cycler for 35 cycles, consisting of 1 min of denaturation at 95°C, 1 min of annealing at 54°C, and 1 min of extension at 72°C.

Data Base Analyses—The versican proximal promoter was searched against the MatInspector (38) and ConSite (39) databases to detect potential SRE sites.

Statistical Analysis—Significant differences between experimental groups were determined using the Student’s t test. Results for cell culture data are expressed as means ± S.E. Calculated p values were two-sided, and those <0.05 were considered statistically significant.

RESULTS Versican Proximal Promoter Luciferase Reporter Vector Responds to Steroid Stimulation—The versican-Luc vector, in which expression of the luciferase gene is under the control of the versican proximal promoter (−634 to +118 relative to the transcriptional start site), was used in transient transfection assays to determine the effects of steroid stimulation on versican promoter activity (36). Versican-Luc transfected into LNCaP prostate cancer cells responded to the addition of a potent synthetic androgen (10 nM R1881) with an ~3-fold induction of luciferase expression while exposure of the cells to 10 nM dexamethasone, a glucocorticoid receptor agonist, provoked a ~2-fold increase in versican-Luc activity (Fig. 1A). Parallel transfections of versican-Luc into HeLa-FLAG-AR and wild-type HeLa cells were used to assess the dose response of the reporter to the natural androgen DHT. Versican-Luc responded with increasing activity across a range of DHT concentrations from 0 to 10 nM in HeLa-FLAG-AR cells while failing to respond in wild-type HeLa cells lacking AR (Fig. 1B). Taken together these results demonstrate androgen-mediated regulation of the versican promoter.
Database Searches Reveal Two Potential SRE Candidates within the Versican Proximal Promoter—To determine whether known nuclear receptor transcription factors are able to bind to the proximal versican promoter, the −634 to +118 bp of the human versican promoter sequence (Fig. 2A) was submitted to the MatInspector (38) and ConSite (39) databases to identify potential protein binding sites. Results suggested that the submitted sequence contained two potential SREs (Fig. 2B). SRE-1 (−392 to −373 bp) showed most sequence similarity to a glucocorticoid response element, whereas SRE-2 (+102 bp) most resembled a progesterone response element, although there is considerable overlap in the SRE family of recognition sequences, and both sites showed sequence similarity to the consensus ARE. Although the SRE-2 is located within exon 1 of the versican coding sequence, functional AREs within the coding regions of other genes have previously been reported (40, 41).

Methylation Protection Reveals the Interaction of the AR DNA-binding Domain to SRE-2 of the Versican Promoter—Methylation protection (MeP) analyses were conducted using single-end 32P-labeled fragments of the versican promoter to look for guanine-protein DNA contacts on incubation with bacterially produced recombinant His-tagged AR DNA binding domain (His-AR-DBD) (Fig. 4). The versican probes were incubated with His-AR-DBD or with the equivalent His extract from bacteria containing a control plasmid. In this assay, DMS was used to methylate unprotected guanines at position N7 of the DNA double helix, whereas piperidine was used to cut these methylated guanines. Following gel extraction and chemical cleavage, equal amounts of labeled DNA were then separated on a urea sequencing gel. When bound to proteins, DNA conformation may change causing certain bases to become more exposed and prone to methylation, resulting in hypersensitive sites or bases may be sterically protected from chemical modification resulting in protection. No protection or hypermethylation of the versican-promoter region corresponding to the SRE-1 was observed (data not shown). In contrast, obvious protection (guanine + 84) and hypermethylation (guanine + 92) were detected in the versican UTR exon 1 region corresponding to the previously identified SRE-2 (Fig. 3). These data positively demonstrates the binding of the AR DBD to SRE-2.

AR Binds to Versican SRE Sites in Vitro—To further explore the interaction of the AR with the SRE-2-protected region of the versican promoter, a radiolabeled, double-stranded probe corresponding to the SRE-2 region was synthesized and cloned into pBluescript (Stratagene) for EMSA studies. Fig. 4 shows the results of incubating the SRE-2 probe with nuclear extracts from HeLa and HeLa-FLAG-AR cells treated with 10 nM R1881. The SRE-2 probe yielded a number of protein-DNA complexes. Three nonspecific bands (NS, Fig. 4) of varying intensity and molecular weight were detected with both cell extracts, whereas a higher molecular weight species was present only on incubation with HeLa-FLAG-AR extract. Addition of Anti-AR (C-19 polyclonal, Sigma; N-441 monoclonal, Sigma) or Anti-FLAG (F-1804, Sigma) antibodies to the EMSA generated a further, higher molecular weight supershift in the presence of HeLa-FLAG-AR extracts. Taken together, the shift and supershift data demonstrate the binding of a full-length mammalian AR to the SRE-2 DNA sequence.

Mutation of SRE-2 Interferes with Androgen Stimulation of the Versican Promoter Activity—To further verify the direct regulation of the versican promoter by AR, site-directed mutagenesis was used to alter the SRE-2 site within the versican proximal promoter in the versican-Luc plasmid. SRE-2 site was mutated to exchange the “AA” present in the upstream half-site to “TT” (Fig. 5A). Following sequence verification, HeLa-FLAG-AR cells were transfected with either wild-type versican-Luc or the versican-Luc-SRE-2mt plasmid and the pRL-tk normalization control reporter. Cells were treated for 24 h post-transfection with 10 nM R1881 or vehicle in 5% serum DMEM. Luciferase activities were corrected for transfection efficiency and normalized to the steroid negative (Fig. 5B). The mutation of SRE-2 completely abrogated R1881 stimulation when compared with the wild-type promoter (p < 0.001), demonstrating the importance of the SRE-2 DNA element to androgen-mediated regulation of the versican promoter in an in vivo system.

**Androgen Receptor Regulation of Versican**

Add the following diagram with figure 1.
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Knock-down of AR Expression Reduces Ligand-dependent and Ligand-independent Versican Promoter Activity—in a recent study (37), we tested four shRNAs for their ability to knock down AR in LNCaP cells under in vitro and in vivo conditions. To determine which was most effective, each was screened for inhibition of activation of the androgen-responsive ARR3-tk luciferase reporter in transient transfection assays. In the present experiments, we used the most potent construct, which inhibited AR-induced luciferase expression by 97% (37), to examine the role of AR in ligand-dependent androgen-responsive promoters in prostate epithelial LNCaP cells. We treated cells with 20 mM LiCl, 1 nM R1881, or a combination of R1881 and LiCl. LiCl is an inhibitor of glycogen synthase kinase-3 (GSK-3) that induces accumulation of dephosphorylated β-catenin and increases the expression of β-catenin/TCF-dependent genes. As observed in the promoter studies, addition of R1881 or LiCl alone to prostate fibroblasts did not significantly alter versican mRNA levels (Fig. 7, A and B). We treated cells with 20 mM LiCl, 1 nM R1881, or a combination of R1881 and LiCl. LiCl is an inhibitor of glycogen synthase kinase-3β that induces accumulation of dephosphorylated β-catenin and increases the expression of β-catenin/TCF-dependent genes. As observed in the promoter studies, addition of R1881 or LiCl alone to prostate fibroblasts did not significantly alter versican mRNA levels (Fig. 7, A and B). In contrast, the combination of LiCl and R1881 resulted in 2.8-fold increase in versican-Luc activity in prostatic fibroblasts (Fig. 6A). We have recently reported that the β-catenin/TCF signaling pathway plays a critical role in versican transcription (36). Additionally, other reports suggest that AR and β-catenin interact by direct binding and that their interaction is ligand-sensitive (30–33). We showed that co-transfection of versican-Luc with β-catenin and TCF-4 expression vectors into prostate fibroblasts significantly increased versican promoter activity (~10-fold) (Fig. 6A). Interestingly, R1881 augmented overexpression of β-catenin-induced versican-Luc promoter activity in these fibroblasts. We also examined the effect of interaction between androgen and β-catenin signaling on versican mRNA levels in prostate fibroblasts (Fig. 7, A and B). We treated cells with 20 mM LiCl, 1 nM R1881, or a combination of R1881 and LiCl. LiCl is an inhibitor of glycogen synthase kinase-3β that induces accumulation of dephosphorylated β-catenin and increases the expression of β-catenin/TCF-dependent genes. As observed in the promoter studies, addition of R1881 or LiCl alone to prostate fibroblasts did not significantly alter versican mRNA levels (Fig. 7, A and B). In contrast, the combination of LiCl and R1881 resulted in 2.8-fold increase in versican mRNA levels compared with untreated cells (Fig. 7, A and B). These results suggest that β-catenin is essential for AR ligand-dependent regulation of versican gene transcription at both promoter and mRNA levels in prostate stromal fibroblasts (Figs. 6A, 7A, and 7B).

To determine whether the interaction between AR and β-catenin/TCF signaling could modulate AR- and β-catenin-dependent transcriptional activities of AR- and β-catenin/TCF-responsive promoters, prostate stromal fibroblast cells were transfected with PSA-Luc, ARR3-tk-Luc, and pTOPFlash-8-Luc with or without β-catenin and TCF-4 expression vectors in presence or absence of AR ligand, DHT. The luciferase reporter plasmid versican-Luc is both an AR- and TCF-responsive promoter; PSA-Luc and ARR3-tk-Luc reporter plasmids are AR-
responsive promoters; pTOPflashx8-Luc is responsive to activation of Wnt signaling. As mentioned above, transfection of versican-Luc, a reporter gene responsive to AR and β-catenin signaling, together with β-catenin and TCF-4 expression plasmids, mediated ~10- and 30-fold induction of luciferase activity compared with transfection of the versican-Luc reporter construct alone in the absence or presence of androgen, respectively (Fig. 6A). In contrast, co-transfection of β-catenin and TCF-4 expression plasmids with PSA-Luc (AR-responsive gene) mediated only 4- and 8-fold promoter induction (Fig. 6B).

These findings support the notion that AR and β-catenin/TCF signaling require both the TCF binding site and ARE for optimal transcriptional activation by β-catenin/TCF and AR signaling, as evidenced with the higher response of versican-Luc compared with PSA-Luc. Because β-catenin-TCF complex modulated the transcriptional activity of endogenous Wnt- and
AR-responsive promoters in the absence and presence of androgens, we investigated the ability of the β-catenin-TCF complex to modulate reporter plasmids with either synthetic ARE- or Wnt-responsive elements in prostate fibroblast cells. Co-transfected β-catenin and TCF-4 expression plasmids with ARR3-tk-Luc or pTOPflashx8-Luc augmented both AR and Wnt-responsive promoters 15- and 30-fold, in the absence or presence of 1 nM DHT, respectively (Fig. 6, C and D). These findings emphasize that in prostate stromal fibroblast cells β-catenin/TCF signaling is required for the optimal transactivation AR- and Wnt-responsive genes.

**DISCUSSION**

In the present study, we have found that androgens dose-dependently enhance human versican promoter activity in LNCaP AR-positive epithelial human prostate cancer cells (Fig. 1A). Furthermore, we have shown that inhibition of AR expression levels using shRNA diminishes both basal and androgen-stimulated versican transcriptional activities in these cells (Fig. 5C). We also demonstrated that an SRE binding site located in the first exon of the human versican gene is involved in androgen-induced versican transcription (Fig. 5, A and B) and that at least one of the members of steroid hormone family of transcription factors, AR, binds to this SRE site in the human versican promoter (Fig. 4). Finally, we showed that β-catenin is required for both ligand-dependent and ligand-independent AR-mediated transactivation in prostate stromal fibroblasts (Figs. 6 and 7).

Carcinoma cells, like normal epithelial cells, live in a complex microenvironment that includes the ECM, diffusible growth factors and cytokines, and a variety of non-epithelial cell types. Recent studies have provided evidence that stromal cells and their products can cause the transformation of adjacent cells through transient signaling that leads to the disruption of homeostatic regulation, including control of tissue architecture, adhesion, cell death, and proliferation (5, 6). Given the importance of the versican in cellular events involved in initiation and progression of tumors (15–17, 19, 20, 43), our observations may support a new concept for the role of steroid hormone signaling through the AR transactivation complex, modulating the stromal gene expression patterns and versican-rich provisional matrix formation involved in BPH (23) and aberrant prostate cancer development and progression (21, 22, 24). Interestingly, Rickard et al. (44) also identified estrogen-mediated regulation of versican gene expression. They showed that treatment of fetal osteoblast cells engineered to stably
express estrogen receptor (ER) α with 17β-estradiol increased versican mRNA expression levels. However, the same treatment of cells stably expressing ERβ failed to modulate versican mRNA abundance (44). Interestingly, there are reports suggesting that the elevated expression of versican within peritumoral stromal matrix is positively related to the relapse in women with node-negative breast cancer (45, 46). These findings might suggest that ligand-dependent activation of the ER increases versican deposition; therefore, neoplastic remodeling of ECM may facilitate local invasion and metastasis.

Growing evidence suggests the existence of intriguing similarities between the process of wound healing and that of prostate tumorigenesis. In human prostate cancers, reactive stroma is characterized by an increase in myofibroblasts, a corresponding amplification in ECM protein production, and an increase in local vascular density (47), properties similar to those seen in granulation tissue. The proteoglycan versican is one of several ECM molecules that accumulate in BPH (23) and prostate cancer (21, 22, 24). Versican is generally considered to be important in tumor initiation and/or progression because of its important roles in cell adhesion (15), migration (16), proliferation (17), differentiation (48), angiogenesis (19) and resistance to oxidative stress-induced apoptosis (20), all of which are important events in tumor formation and progression. The dynamic processes of normal prostate development and progression of prostate cancer are dependent on androgen acting through the AR (25). Androgens bind to the AR promoting dimerization and association with coactivators. AR then translocates to the nucleus and binds to AREs in the promoter regions of target genes, thereby modulating transcriptional activities. The present study suggests that versican is a positively regulated target gene of the AR. The production of versican also influences cellular functions and survival directly or indirectly. Androgens themselves have long been known to promote survival of prostatic epithelium, and they appear to increase the ability of the cell to withstand apoptotic stimuli (49). Our finding suggest that one of the molecular mechanisms by which versican influences prostate tissue behavior is via stromal-epithelial interactions, which may contribute to normal maintenance, BPH, or possibly tumor progression.

Recent data indicate that the gonadotropin hormones, luteinizing hormone and follicle-stimulating hormone, regulate versican mRNA and protein expression in the rodent ovary (50, 51). Given the role of gonadotropins in versican mRNA and protein expression (50, 51), it supports the notion that gonadotropin-mediated regulation of the versican expression occurs via either signaling transduction pathway, i.e. PI3K/PKB/Akt, or androgens. We have recently reported that the PI3K/PKB/glycogen synthase kinase-3β pathway plays a critical role in versican transcription (36). Therefore, it might be concluded that one of the mechanisms for gonadotropins to induce versican transcription is mediated by PI3K/PKB/Akt. Furthermore, it has also been reported that, in cultured granulosa cells, testosterone significantly enhanced versican mRNA and protein expression (50). In this study, for the first time, our results indicate that versican is transcriptionally regulated by androgens.

We established this regulatory sequence of events in a series of experiments. First, we showed that R1881, a potent synthetic AR agonist, significantly enhanced human versican-Luc activity in AR-positive LNCaP human prostate cancer cells (Fig. 1A). Furthermore, consistent with this result, the native androgen dose-dependently increased versican-Luc activity in HeLa-positive AR cells while failing to induce the same activation in wild-type AR-negative HeLa cells (Fig. 1B). Finally, knock-down of endogenous AR levels in AR-positive LNCaP human prostate cancer cells reduced both the basal and androgen-stimulated versican promoter-driven transcription (Fig. 5C). Overall, these results suggest that AR, at least in part, is required for androgen-mediated stimulation of versican transcription in AR-positive human prostate cancer cells.

The location, sequence, and number of AREs associated with a given androgen target gene varies, although androgen-responsive regions typically contain multiple non-consensus AREs (5′-TGTTTC-3′) (52). By analyzing the published sequence of the versican gene promoter (53), we noticed that there are two potential ARE-like motifs located in the promoter of the human versican gene (versican SRE-1, (−392)AGAAC-TagcTGCACG(−373), and versican SRE-2, (+83)AGAACAT-taGTTGTT(+102)) (Fig. 2A). We have characterized the specific binding of recombinant and native AR to the SRE-2 site by EMSA (Fig. 4), and the guanine nucleotides involved in AR-DNA interactions were localized by MeP footprinting (Fig. 3). Moreover, transient transfection analysis of wild-type and SRE-2 mutant versican-Luc constructs showed that the mutation eliminated the hormonally induced response (Fig. 5, A and B), suggesting the functional importance of this site for hormonal responsiveness and demonstrating that the versican SRE-2 functions as an independent SRE. These results suggest that AR binds to SRE-2 DNA sequence and that this binding is important in regulation of versican promoter.

β-Catenin is a potent transcriptional coactivator of AR (30, 33, 54). β-Catenin transactivates AR on minimal transcriptional reporters (30, 31) as well as endogenous targets such as PSA at a magnitude similar to CREB-binding protein (32) and steroid receptor coactivator 1 (32), thereby demonstrating the potency of β-catenin as an AR regulator. The affinity of β-catenin–AR interactions is likely attributable to the unique structural identity of the androgen receptor–ligand binding domain but is likely also accounted for by differences in the supporting network of coregulators between cell lines.

Advanced prostate cancer is often treated by total androgen ablation therapy; however, the ultimate phenotype is one of AI and death (55). Transcriptional coregulators are hypothesized to serve a critical role in promoting a more aggressive AR during AI prostate cancer. Altered ligand responsiveness of AR has been postulated as a major mechanism by which prostate cancer continues to proliferate in low androgen environments (56). Potentially, coactivator expression could result in activation of the AR and the progression of prostate cancer despite therapeutic androgen deprivation. As such, several lines of evidence indicate that β-catenin may promote the oncogenicity of AR. In light of this, it is significant that β-catenin increases AR-mediated gene activation not only in the presence of DHT, but also in the presence of the weaker adrenal androgen, androstenedione (57), which remains present in chemically castrated patients. These findings indicate that β-catenin acts as a coactivator of
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AR. Therefore, β-catenin not only alters the specificity of AR toward certain ligands but also acts as a pure coactivator. Our results demonstrate that, although the androgen agonist, R1881, induces versican promoter activity in LNCaP cells, it does not significantly induce versican-Luc promoter activity in prostate stromal fibroblasts (Fig. 6A). However, overexpression of β-catenin increased basal and ligand-dependent AR transactivation of versican-Luc activity in these cells. Furthermore, we demonstrated that, although treatment of prostatic fibroblasts with R1881 or LiCl alone did not change endogenous versican mRNA levels, the combination of these mediators significantly enhanced versican transcription (Fig. 7, A and B). We therefore hypothesize that there may be repressor proteins at play that prevent AR from binding or other positive acting factors or chromatin remodeling needed for AR to bind and initiate assembly of a transcription complex. Ongoing investigations in our laboratory seek to identify the molecular mechanisms involved in the AR-β-catenin-TCF complex-mediated regulation of androgen response genes in prostate stromal fibroblasts. Although prostate epithelial cancer cells appear to produce little or no versican, mechanisms to increase versican levels in peritumoral stroma may be an important prelude to local invasion and metastasis.

In conclusion, we have presented for the first time that versican transcription is induced by androgen-stimulated AR transactivation in human prostate cancer cells. We provide evidence of strong and specific binding of native and recombinant AR to DNA sequences located in the proximal human versican promoter. In this study, we also examined the role of β-catenin in the regulation of androgen signaling in prostate stromal fibroblast cells. Intriguingly, we demonstrated that β-catenin is required for AR-mediated transcription in prostate stromal fibroblasts. These findings provide the first line of evidence for the crucial interaction of the Wnt/β-catenin and androgen signaling pathways in prostate stromal cells, which suggests a novel mechanism for the development of androgen-independent prostate cancer. The data presented herein suggest that androgens may be cytoprotective for secretory prostatic epithelial cells in humans, in part through up-regulation of versican in stromal fibroblasts. This is not only important in the maintenance of normal prostate, but also during the progression to AI prostate cancer.

A prostate cancer cell microenvironment is crucial to its survival, progression, and metastasis. It has been shown that the growth of prostate carcinoma cells can be either inhibited or stimulated by specific stromal environments (reviewed in Ref 4). Metastasis to bone, perhaps the most clinically significant aspect of prostate cancer, is also dependent on stromal-epithelial cross-talk, because prostate carcinoma cells must induce the hospitality of bone cells to take up residence in an osseous microenvironment. Versican, one of the key components of stromal ECM, is highly expressed in many malignancies, including prostate cancers. It appears likely that prostate cancer cells with the most aggressive behavior in vivo might secrete soluble mediators leading to elevated deposition of versican by tissue fibroblasts during neoplastic remodeling of the ECM. The roles of versican in cell growth, motility, adhesion, and angiogenesis strongly suggest that this proteoglycan of the ECM might be an important downstream effector of the AR signaling pathway during normal prostate development as well as tumor initiation and/or progression. We also suggest that AR signaling, through the regulation of the expression of versican, augments tumor-stromal interactions, inducing proliferation and survival, as well as modulating adhesive, migratory, and angiogenic processes that are integral to normal prostate development and/or prostate cancer progression.

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