Opposing Effects of Cyclooxygenase-2 (COX-2) on Estrogen Receptor β (ERβ) Response to 5α-Reductase Inhibition in Prostate Epithelial Cells*

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Current pharmacotherapies for symptomatic benign prostatic hyperplasia (BPH), an androgen receptor-driven, inflammatory disorder affecting elderly men, include 5a-reductase (5AR) inhibitors (i.e. dutasteride and finasteride) to block the conversion of testosterone to the more potent androgen receptor ligand dihydrotestosterone. Because dihydrotestosterone is the precursor for estrogen receptor β (ERβ) ligands, 5AR inhibitors could potentially limit ERβ activation, which maintains prostate tissue homeostasis. We have uncovered signaling pathways in BPH-derived prostate epithelial cells (BPH-1) that are impacted by 5AR inhibition. The induction of apoptosis and repression of the cell adhesion protein E-cadherin by the 5AR inhibitor dutasteride requires both ERβ and TGFβ. Dutasteride also induces cyclooxygenase type 2 (COX-2), which functions in a negative feedback loop in TGFβ and ERβ signaling pathways as evidenced by the potentiation of apoptosis induced by dutasteride or finasteride upon pharmacological inhibition or shRNA-mediated ablation of COX-2. Concurrently, COX-2 positively impacts ERβ action through its effect on the expression of a number of steroidogenic enzymes in the ERβ ligand metabolic pathway. Therefore, effective combination pharmacotherapies, which have included non-steroidal anti-inflammatory drugs, must take into account biochemical pathways affected by 5AR inhibition and opposing effects of COX-2 on the tissue-protective action of ERβ.

Benign prostatic hyperplasia (BPH) is a common disorder affecting elderly men. Although 50% of men over the age of 50 will develop histological BPH (1, 2), less than half will go on to develop lower urinary tract symptoms (LUTS) (3, 4) such as urinary frequency, urgency, and retention (5, 6). Multiple factors including altered steroid hormone signaling and chronic inflammation contribute to the pathogenesis of BPH, ultimately leading to an increase in prostate size through tissue remodeling (4, 7–9).

The prostate epithelium is responsive to multiple sex steroids and expresses both androgen receptor (AR) and the β isoform of estrogen receptor (ERβ) (9, 10). The prostate, which is mainly an androgen-dependent organ, can convert testosterone to not only the potent AR ligand dihydrotestosterone (DHT) but also to ERβ ligands, the most abundant of which is 5α-androstan-3β,17β-diol (3β-diol) (11, 12). Although androgens regulate differentiation functions of the prostate and enhance proliferation of prostate cancer cells, these actions are opposed by estrogenic ligands, which limit proliferation and epithelial cell differentiation (12–15).

The main pharmacotherapies for treating symptomatic BPH include α-adrenergic receptor blockers and 5α-reductase (5AR) inhibitors, which limit conversion of testosterone to DHT (16, 17). α-Adrenergic blockers alleviate LUTS, whereas 5AR inhibitors reduce prostate size. Several clinical trials (Medical Therapy of Prostatic Symptoms, Reduction by Dutasteride of Prostate Cancer Events, and Prostate Cancer Prevention Trial) have examined the use of single and combination therapies in prostate cancer patients with the concurrent examination of BPH progression. The Prostate Cancer Prevention Trial showed that finasteride, a 5AR type 2 inhibitor, decreased the number of BPH events over a 7-year period as compared with placebo (18). In the Reduction by Dutasteride of Prostate Cancer Events trial, dutasteride, a 5AR type 1 and 2 inhibitor, reduced the number of BPH events compared with placebo over a 2-year period (19). The Medical Therapy of Prostatic Symptoms trial examined the combination of α-adrenergic receptor blockers with 5AR inhibitors and the effectiveness of combination therapy over monotherapy (20). Treatment with either drug significantly reduced BPH symptoms compared with placebo, but the combination therapy performed signifi-

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3 The abbreviations used are: BPH, benign prostatic hyperplasia; 5AR, 5α-reductase; AR, androgen receptor; DHT, dihydrotestosterone; EMT, epithelial-mesenchyme transition; LUTS, lower urinary tract symptoms; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; ROS, reactive oxygen species; qPCR, quantitative PCR; BPH-1 scr, BPH-1 cells stably expressing a scrambled shRNA; ER, estrogen receptor; 3β-diol, 5α-androstane-3β,17β-diol; COX, cyclooxygenase; pSmad3, phosphorylated Smad3; ANOVA, analysis of variance; RNA-seq, high-throughput RNA sequencing.
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Experimental Procedures

**Cell Culture and Treatment**—Human prostate epithelial cells derived from BPH patients, BPH-1 and BPHPrE1, were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (Corning, Manassas, VA) and 50:50 Dulbecco’s modified Eagle’s medium (DMEM)/F-12 supplemented with 5% FBS, 1% insulin-transferrin-selenium-ethanolamine (Gibco), 0.4% bovine pituitary extract (Life Technologies), 1:1000 10 ng/ml epidermal growth factor (EGF; Life Technologies), and 1% penicillin/streptomycin (Corning), respectively (28, 29). RWPE-1 cells, derived from normal human prostatic epithelial cells, were grown in keratinocyte medium supplemented with bovine pituitary extract and EGF (Life Technologies). Cells were seeded at 2.5 × 10^5 cells/well and grown overnight. The following day, cells were treated with dutasteride (Sigma-Aldrich), finasteride (Sigma-Aldrich), 1 μM 3β-diol (Steraloids, Newport, RI), 5 nM WAY20070 (Tocris Bioscience, Ellisville, MO), 1 μM NS398 (Cayman Chemical, Ann Arbor, MI), 9 nM SC560 (Cayman Chemical), 200 μM aspirin (Sigma-Aldrich), testosterone (Sigma-Aldrich), or dihydrotestosterone (Sigma-Aldrich).

**RNA Isolation and qPCR**—RNA was isolated from cells using TRIzol (Life Technologies) and chloroform (Sigma-Aldrich) with procedures described previously (30). cDNA synthesis was performed using the iScript CDNA synthesis kit containing a mixture of oligo(dT) and random hexamer primers (Bio-Rad) as described by the supplier. Gene-specific primers were used to validate gene expression levels using the comparative CT method (Table 1).

**Western Blotting**—Cell-free protein lysates were obtained from cells using radiolabeled precipitate assay buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS), and total protein levels were quantified using the Lowry method (Table 1).

**Indirect Immunofluorescence**—Cells were plated on glass coverslips, fixed in 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100. Cells were blocked with Super-Block Blocking Buffer (Pierce) and then incubated with E-cadherin (Cell Signaling Technology) antibody overnight. Cells were washed with PBS and incubated with Alexa Fluor 488 secondary antibody (ThermoFisher Scientific, Grand Island, NY) that COX-2 inhibition on the biological functions of ERβ with monotherapy with finasteride (27). Combination therapy showed no increased efficacy compared with non-steroidal anti-inflammatory drugs (NSAIDs) and 5AR inhibitors may improve LUTS. A small clinical study designed to investigate the short term effects of NSAIDs plus finasteride showed that patients treated with both drugs improved in both international prostate symptom score and urinary flow within the first 4 weeks; however, within 24 weeks, combination therapy showed no increased efficacy compared with monotherapy with finasteride (27).

In this report, we examine the impact of combined 5AR and COX-2 inhibition on the biological functions of ERβ in a human BPH-derived cell line (i.e. BPH-1). Our results show that COX-2 promotes ERβ activity through the regulation of steroidogenic enzyme gene expression, ultimately leading to the enhanced production of ERβ ligands. Our results suggest that the anti-inflammatory benefits of NSAIDs in prostatic disease may be counterbalanced by a reduction in tissue-protective effects of ERβ. Therefore, any combination pharmacotherapies that attempt to limit the inflammatory component of benign or malignant prostate disease may need to include compounds that maintain ERβ signaling.

**Table 1**

| Gene name | Forward (5’–3’) | Reverse (5’–3’) |
|-----------|-----------------|----------------|
| ACTB/actin | TCCGCACTTTGATGACTGCCCTG | CGACCGGCAGATTGCTGGACCTT |
| AKR1A1 | TATTAAGCTGAGGGAATCTTGA | CTGGCGTCAACTGAAAT |
| AKR1C1 | CTTGAAGCTGAGGGAATCTTGA | CTGGCGTCAACTGAAAT |
| AKR1C3 | TGAGATTAGCTGAGGGAATCTTGA | CTGGCGTCAACTGAAAT |
| CDH-1/E-cadherin | TGAGATTAGCTGAGGGAATCTTGA | CTGGCGTCAACTGAAAT |
| COX-2 | ATCCAGCTCTTCCCATCCGGAC | CGGAGGAGGCTGAGGGAATCTTGA |
| CVIP/B1 | GATGCAAGATGAGCCGTTGTA | GCCTTCCTTTTTCCTGAGT |
| ESRI | CTATTGCTGTTTCTGTTGAGT | CACCTTTGCTTTTACTGTC |
| HSD17B4 | AGCTGAGCATTGGTGGTT | GCTTTAACACAAACAAACCTA |
| SMAD4 | AGCTGAGCATTGGTGGTT | GCTTTAACACAAACAAACCTA |
| SRD5A1 | CTTGAGCATCTGAGGTTGTC | GCTTTAACACAAACAAACCTA |
| SRD5A2 | AGCTTATGCTGTTGTTGTC | GCTTTAACACAAACAAACCTA |
NY). For F-actin labeling, cells were incubated for 30 min with Alexa Fluor 488-conjugated phalloidin (ThermoFisher Scientific). Nuclei were stained with DAPI, and slides were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL). Images were captured using an Olympus Fluoview 1000 confocal microscope.

**Lentivirus Infection**—Lentivirus sets containing five unique shRNA sequences were purchased from the University of Pittsburgh Cancer Institute Lentiviral Core Facility (Pittsburgh, PA). Cells were plated in 6-well plates and allowed to grow until 50% confluence at which point culture medium was replaced with 1 ml of complete medium containing 16 μg/ml Polybrene and 1 ml of lentivirus expressing shRNAs of interest (i.e. COX-2, ESR2, and SMAD4) or scrambled control containing a puromycin resistance gene marker. Following an overnight infection, virus-containing medium was replaced with complete medium containing 1 μg/ml puromycin to select for resistant cells. Cells were maintained in selection medium until experiments.

**Chromatin Immunoprecipitation and qPCR**—BPH-1 cells were treated at 80% confluence with either control (EtOH) or dutasteride (0.1 μM) for 24 h. The medium was replaced with fresh growth medium containing 1% formaldehyde for cross-linking DNA-protein complexes and incubated for 10 min at 37°C. The cross-linking reaction was halted with the addition of glycine to a final concentration of 125 mM, and the cells were incubated for 10 min at room temperature. Cells were sonicated at 3 × 15 s at 30 A and then at 3 × 15 s at 40 A on ice. Fragment size was verified by DNA gel electrophoresis. Chromatin was then incubated with antibodies against phospho-Smad3 (Cell Signaling Technology), as well as enrichment of DNA-protein complexes was observed as an input, and the remainder was then linked to Protein A-Sepharose beads (GE Healthcare). Beads were then washed, and cross-linking was reversed. DNA was isolated using phenol-chloroform-isooamyl alcohol and resuspended in Tris-EDTA buffer containing RNase A. DNA was analyzed using RT-qPCR using primers listed in Table 2. RT-qPCR results were calculated as relative enrichment over input control.

**Flow Cytometry**—Apoptosis was quantified by flow cytometry using annexin V labeling to detect the phosphatidylserine found on the outer membrane of apoptotic cells. 2.5 × 10⁵ cells were plated in 6-well plates and allowed to grow overnight prior to being subjected to treatments. Cells were then harvested, washed with ice-cold PBS, and then resuspended in 100 μl of binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂) containing 5 μl of FITC-conjugated annexin V (BD Biosciences) and 5 μl of 1× propidium iodide (Sigma-Aldrich). Cells were gently mixed and incubated for 15 min in the dark, and cell volumes were brought up to 500 μl with binding buffer prior to flow cytometry. Cells were counted using an LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo (Flowjo, Ashland, OR). Only cells labeled singly with annexin V were considered apoptotic; cells labeled with propidium iodide were considered dead.

**RNA Sequencing and Analysis**—Libraries were prepared using the Illumina HiSeq 2000 (Tufts core facility, Medford, MA). All sequence mapping and filtering were performed in house. FASTQ files were aligned to the human genome (hg19) using TopHat (32). Cufflinks was then used to assemble RNA reads into transcripts and calculate fragments per kilobase of transcript per million mapped to determine differential gene expression (33).

**Steroid Metabolism**—BPH-1 cells stably expressing a scrambled shRNA (BPH-1 scr) or BPH-1 shCOX-2 cells were seeded in 12-well plates at 50% confluence 1 day before steroid metabolism analysis. On the day of analysis, the cells in each well were incubated at 37°C with 1 ml of medium containing 0.1 μM testosterone or dihydrotestosterone with 1,000,000 cpm [³H]-labeled steroid (PerkinElmer Life Sciences). Aliquots (0.3 ml) of medium were removed at 2, 4, and 12 h, and steroids were extracted with 1 ml of dichloromethane and dried under a nitrogen stream. Steroids were analyzed using an Agilent 1260 Infinity high performance liquid chromatography (HPLC) system with UV detector and β-RAM4 in-line scintillation counter (LabLogic, Brandon, FL). Excreted steroid products were dissolved in 20 μl of methanol, and 5-μl injections were resolved with a 50 × 2.1-mm, 2.6-μm, C₅ Kinetex column (Phenomenex, Torrance, CA) at a flow rate of 0.4 ml/min and a methanol/water linear gradient: 27% methanol from 0 to 0.5 min, 39% to 16 min, 44% to 20 min, 60% to 22 min, 71% to 30 min, 75% to 30.5 min, and 27% to 33 min. Products were identified by retention times of external standards chromatographed at the beginning and end of the experiments. The flow rate of the scintillation mixture (Bio-SafeII, Research Products International, Mount Prospect, IL) was 1.2 ml/min, and the data were processed with Lahore software (LabLogic (34)).

**Prostaglandin E₂ (PGE₂) Accumulation**—Cells were plated in 10-cm plates at 7.5 × 10⁵ cells and incubated overnight. Cells were then subjected to appropriate treatments for 24 h in complete medium. Cells were scraped and stored as a lystate in 2 ml of PBS at −80°C for prostaglandin analysis. The internal standard, PGE₂-d₆ (100 ng), was added to the cell lystate and allowed to equilibrate for 5 min before adding 5 ml of CHCl₃/MeOH (2:1). Samples were then vortexed and set to shake on low for 10 min before centrifugation at 2800 × g for 10 min. The organic

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**TABLE 2**

| ChIP Primers | Forward (5’→3’) | Reverse (5’→3’) |
|--------------|-----------------|-----------------|
| COX-2 site 1 (−9723 to −9569) | TTTTCTCCCTCCTGTTGAGCT | TTTGGGATAGCAATGATT |
| COX-2 site 2 (−1787 to −1993) | AACATGCGTCTCATTGCCAAGA | GTTCCACCATTAGGGACTCC |
| COX-2 site 3 (−348 to −150) | TGGATCCCGCAACTGAGTTC | TTACCCACGGAATGAGAAA |
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ERβ agonist efficiently induced expression of E-cadherin, an established ERβ target gene (Fig. 2A). These data corroborate previous studies from other laboratories that revealed functional ERβ in BPH-1 cells (37, 38). Because ERβ activation by a potent synthetic ligand enhances apoptosis in normal human prostate epithelial and stromal cells in mouse xenograft studies (38), we examined the contribution of ERβ ligands to apoptosis in cultured BPH cell lines. As shown in Fig. 2B, treatment with either ERβ ligand did not trigger a significant increase in apoptosis. To eliminate the possible contribution of endogenous ERβ ligands to basal apoptosis, we treated BPH-1 and BHPtE1 cells with the 5AR type 1 and 2 inhibitor dutasteride, which would block the production of potent DHT-derived ERβ ligands and potentially reduce basal apoptosis in these cells. Surprisingly, dutasteride treatment (0.5 μM) on its own increased apoptosis (Fig. 2C). This induction of apoptosis is not observed upon treatment of BPH-1 cells with the 5AR type 2-specific inhibitor finasteride (Fig. 2D). The expression of both 5AR enzymes was detected in BPH-1 cells by quantitative RT-PCR (see Fig. 8E). 5AR inhibitors increase apoptosis in prostate cancer cells (39, 40) through limiting the action of DHT on the prosurvival effects of AR. The limited expression of AR in BPH-1 cells makes it unlikely that dutasteride-induced apoptosis in these cells is mediated by reduced AR action (28). Furthermore, dutasteride-induced apoptosis in BPH-1 cells requires ERβ as shown by the lack of this response upon stable shRNA-mediated ablation of ERβ (Fig. 3, A and B). The results of poly(ADP-ribose) polymerase 1 cleavage assays (Fig. 3C) corroborate our conclusion that dutasteride induces apoptosis as revealed by annexin V staining. Furthermore, dutasteride does not exert a significant effect on the generation of the cleaved form of LC-3, arguing against an effect on autophagy (Fig. 3D). The contribution of ERβ to dutasteride-induced apoptosis is further supported by enhanced apoptosis in BPH-1 cells treated with a suboptimal concentration of dutasteride and the potent synthetic ERβ ligand WAY20070 (Fig. 3E), either of which alone does not promote apoptosis. Stable ablation of ERβ eliminated the proapoptotic effect of combined suboptimal concentrations of dutasteride and WAY20070 (Fig. 3F). In summary, our results confirm previous results establishing the role for ERβ in triggering apoptosis in BPH-derived cells and tissues (38) but uniquely reveal an ERβ-dependent effect on apoptosis in these cells in response to inhibition of 5AR types 1 and 2. The inability of finasteride alone to trigger apoptosis in BPH-1 cells suggests that dutasteride may be having an off-target effect to drive apoptosis, which prompted further investigation into the mechanism of dutasteride effects on ERβ-dependent apoptosis.

Dutasteride-mediated Repression of E-cadherin mRNA Expression in BPH-1 Cells Requires ERβ—To further examine the impact of dutasteride on ERβ action, we measured the expression of the ERβ target gene E-cadherin in response to dutasteride treatment. Estrogenic ligands such as 3β-diol activate ERβ in prostate epithelial cells and maintain epithelial cell differentiation in part by limiting epithelial-mesenchyme transition (EMT) (41, 42) through the induction of cell adhesion proteins such as E-cadherin (43). As shown in Fig. 4A, dutasteride does not promote or enhance the induction of E-cadherin but rather represses its expression in BPH-1 cells. In the

Results

Dutasteride-mediated Apoptosis in BPH-1 Cells Requires ERβ—Within the normal prostate epithelium, testosterone can be metabolized to a potent AR ligand (DHT) and ERβ (e.g. 3β-diol) ligands through different steroid-converting enzymes (Fig. 1) (35, 36). Therefore, normal prostate size and differentiated function are maintained in the presence of testosterone through the opposing actions of AR and ERβ. Perturbations of this balance, either by up-regulation of AR-dependent pathways (i.e. proliferative) or down-regulation of ERβ-dependent pathways (i.e. proapoptotic), may contribute to BPH progression.

To examine the impact of 5AR inhibition on ERβ action, we used human BPH-derived cell lines BPH-1 and BHPtE1 (28, 29). BPH-1 and BHPtE1 cells express functional ERβ as treatment with either a natural (3β-diol) or synthetic (WAY20070)
absence of ERβ, there is no significant change in E-cadherin expression in any treatment group assessed (Fig. 4B), indicating that dutasteride repression of E-cadherin mRNA expression requires ERβ. Because a combined treatment with testosterone does not alter dutasteride repression of E-cadherin mRNA expression (Fig. 4A), inhibition of 5ARs appears to be required for the effect of dutasteride on E-cadherin expression. This conclusion is further supported by the elimination of the repressive effect of dutasteride on E-cadherin expression by a combined treatment with DHT, the product of 5AR. Treatment of BPH-1 scr cells with dutasteride results in a decrease in total E-cadherin protein, an effect not observed in ERβ-ablated BPH-1 cells (Fig. 4C). However, indirect immunofluorescence analysis did not reveal any dramatic effects on plasma membrane localization of E-cadherin or organization of F-actin into stress fibers (Fig. 4D) following a 24-h treatment with dutasteride. Thus, dutasteride treatment alone is not sufficient to drive EMT in BPH-1 cells.Treatment with the natural (3β-diol) or synthetic (WAY20070) ERβ ligand significantly increases E-cadherin mRNA levels in BPH-1 cells and overcomes the repressive effect of dutasteride (Fig. 4E). The induction of E-cadherin mRNA expression by 3β-diol and WAY20070 and repression by dutasteride are not observed in ERβ-ablated BPH-1 cells (Fig. 4F). Thus, dutasteride generates opposing effects on ERβ responses in BPH-1 cells, acting to promote ERβ-dependent apoptosis (Fig. 2) but limiting one component of the EMT-inhibitory action of the receptor as reflected in reduced E-cadherin expression.

Canonical TGFβ Signaling Is Required for Dutasteride Repression of E-cadherin mRNA Expression—EMT is a key component in the pathogenesis of BPH and occurs through a TGFβ-dependent mechanism (41, 44). In the prostate, TGFβ1 regulates cell growth, EMT, apoptosis, and carcinogenesis (45, 46). Tissue specimens from human BPH patients show increased pSmad3 staining in areas of the prostate undergoing the phenotypic changes associated with EMT (44). Treatment of BPH-1 cells with dutasteride increases the phosphorylation of Smad3 significantly within 30 min with no changes in total Smad3 protein (Fig. 5A). Pretreatment of BPH-1 cells with TGFβ-neutralizing antibody inhibited the dutasteride-mediated repression of E-cadherin (Fig. 5B). In the canonical TGF-β signaling pathway, Smad2 and Smad3 can either form homo- or heterodimers upon activation. However, phosphorylated Smad2/3 associates with Smad4, translocates to the nucleus, and then binds to Smad-binding elements of TGFβ-regulated genes. Given that the inhibition of E-cadherin induction by dutasteride is blocked by a TGFβ-neutralizing antibody, we chose to ablate Smad4 in BPH-1 cells to inhibit this pathway (Fig. 5C). As shown in Fig. 5D, the repression of E-cadherin mRNA expression by dutasteride is not observed in the Smad4-ablated cells (Fig. 5D) with expression levels similar to that of cells pretreated with TGFβ-neutralizing antibody (Fig. 5B). Therefore, dutasteride treatment of BPH-1 cells triggers a rapid activation of the canonical TGFβ signaling pathway, which then mediates the ERβ-dependent repression of E-cadherin mRNA expression.

FIGURE 2. Apoptosis in prostate epithelial cell lines derived from BPH patients is mediated by dutasteride. A, treatment with 3β-diol (natural ERβ ligand) and WAY20070 (synthetic ERβ ligand) induces E-cadherin mRNA expression in BPH-1 and BHPrE1 cells. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. **, p value <0.001; n = 3. B, treatment with 3β-diol and WAY20070 does not induce apoptosis in BPH-derived cells as measured by annexin V staining and flow cytometry. n = 3. C, there was an increase in apoptosis in both BPH-1 and BHPrE1 cells treated with 0.5 μM dutasteride. *, p value <0.05. Representative histograms of flow cytometry data for both BPH-1 (left) and BHPrE1 (right) are shown. n = 3. D, finasteride treatment of BPH-1 scr did not increase apoptosis. A representative histogram of flow cytometry data for finasteride treatment in BPH-1 scr cells is shown. n = 3. Error bars represent S.E. ns, not significant.
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**FIGURE 3. Dutasteride-mediated apoptosis in prostate epithelial cell lines requires ERβ.** A. BPH-1 cells were stably ablated of ERβ (BPH-1 shESR2) with an shESR2-expressing lentivirus. A Student’s t test was performed. *, p value < 0.05; n = 3. B, dutasteride triggers apoptosis in BPH-1 scr cells but not BPH-1 shESR2 as measured by flow cytometry. A two-way ANOVA followed by Bonferroni post hoc test was performed. *, p value < 0.05; n = 3. C, dutasteride triggers apoptosis in BPH-1 scr cells but not BPH-1 shESR2 as measured by poly(ADP-ribose) polymerase (PARP) cleavage. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. *, p value < 0.05; n = 3. D, apoptosis is not increased when BPH-1 cells are treated with 3β-diol, WAY20070, or a suboptimal dose of dutasteride (1 μM). However, a combination treatment with WAY20070 and dutasteride significantly increases apoptosis. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. *, p value < 0.05; n = 3. E, treatment with 3β-diol, WAY20070, and dutasteride either singly or in combination does not induce apoptosis in ERβ-ablated BPH-1 cells. n = 3. Error bars represent S.E. ns, not significant.

**TGFβ-mediated Induction of COX-2 in Response to Dutasteride Limits ERβ Activation**—BPH is commonly characterized as a disease of chronic inflammation and is associated with overexpression of COX-2 in luminal epithelial cells (25). Consistent with the contribution of COX-2 to BPH pathology, we found that basal COX-2 protein expression is much higher in BPH-derived cell lines than in the normal prostate epithelial cell line RWPE-1 (Fig. 6A). As shown in Fig. 6B, dutasteride treatment of BPH-1 cells induces COX-2 expression. This induction is lost with the addition of DHT but not testosterone, supporting the dependence on 5αR inhibition for this response. Furthermore, dutasteride induction of COX-2 in BPH-1 cells requires TGFβ as it is not observed in Smad4-ablated BPH-1 cells (Fig. 6C). Pretreatment with TGFβ-neutralizing antibody also inhibits the dutasteride-mediated induction of COX-2 (Fig. 6D). Several putative Smad-binding elements have been identified in the promoter region of COX-2 (47, 48). Examination of these Smad-binding elements by chromatin immunoprecipitation (ChIP) qPCR revealed that dutasteride treatment increases the binding of pSmad3 complexes to the COX-2 promoter (Fig. 6E). In contrast to these direct effects of the canonical TGFβ pathway on COX-2 gene induction by dutasteride, repression of E-cadherin gene expression by TGFβ is likely to be indirect, involving Smad3 regulation of E-cadherin targeting microRNAs (49).

Previous studies from our laboratory revealed an inhibitory effect of COX-2-generated ROS on the transcriptional response of ERβ in the DU145 prostate cancer cell line (50). To determine whether ERβ action in BPH-1 cells is likewise affected by ROS, we treated cells with a physiologic concentration of H$_2$O$_2$ (i.e. 50 μM) along with the ERβ ligand WAY20070. As shown in Fig. 7A, ERβ induction of E-cadherin is inhibited by H$_2$O$_2$. ROS may further promote EMT in BPH as evidenced by the induction of EMT markers Snail and Slug in BPH-1 cells (Fig. 7, B and C). These EMT-promoting effects of ROS cannot be overcome by treatment with WAY20070.

**Disruption of COX-2 Alters BPH-1 Response to Dutasteride**—To more definitively examine whether COX-2 contributes to ERβ action in BPH-1 cells, we used both pharmacological and molecular approaches to reduce its activity and expression. As shown in Fig. 8A, stable shRNA-mediated ablation of COX-2 expression (Fig. 8C) or specific inhibition of COX-2 activity by NS398 increased apoptosis in BPH-1 cells in response to dutasteride. Given the ERβ dependence of dutasteride-induced apoptosis (Fig. 3D), these results suggest that COX-2 may indeed limit the proapoptotic action of ERβ in prostate epithelial cells. Furthermore, the ERβ-dependent repression of E-cadherin by dutasteride is lost upon COX-2 ablation (Fig. 8D). The sensitization of BPH-1 cells to dutasteride-mediated apoptosis is also observed for finasteride (Fig. 8B), which is ineffective on its own
FIGURE 4. Dutasteride repression of E-cadherin mRNA is mediated by canonical TGFβ signaling. A, dutasteride treatment of BPH-1 cells represses E-cadherin mRNA expression. Dutasteride-induced repression of E-cadherin mRNA expression is overcome by a co-treatment with DHT but not testosterone. A one-way ANOVA followed by Tukey’s multiple comparison test was performed. *, p value < 0.05; n = 3. B, in the absence of ERβ, neither dutasteride treatment nor dutasteride and testosterone co-treatment represses E-cadherin mRNA expression. n = 3. C, E-cadherin protein decreases upon dutasteride treatment only in the BPH-1 scr cell line. A representative Western blot is shown below. A Student’s t test was performed. *, p value < 0.05; n = 3. D, indirect immunofluorescence of E-cadherin in BPH-1 scr cells treated with dutasteride showed no significant change in cell surface localization. Dutasteride treatment also did not result in the reorganization of F-actin (phalloloid staining) into stress fibers in BPH-1 scr cells. E, dutasteride repression of E-cadherin mRNA expression is relieved upon co-treatment with an ERβ ligand (3β-diol or WAY20070). A one-way ANOVA followed by Tukey’s multiple comparison test was performed. *, p value < 0.05; **, p value < 0.01; n = 3. F, E-cadherin mRNA expression is not affected by dutasteride or ERβ ligands upon ablation of ERβ in BPH-1 cells. n = 3. Error bars represent S.E. ns, not significant.

(Fig. 2B) to promote apoptosis. Examination of 5α-reductase reductase type 1 (SRD5A1) and type 2 (SRD5A2) genes showed that they are both expressed in BPH-1 (Fig. 8E). However, expression of SRD5A1 is significantly increased upon COX-2 knockdown. This suggests that the increased efficacy of dutasteride and finasteride could be due in part to an increase in expression of the target enzyme. Additionally, the increase in COX-2 expression as observed in BPH tissue may negatively influence the ability of ERβ to drive apoptosis and limit EMT in prostate epithelial cells.

COX-2 Regulates Steroid Hormone Metabolism—The impact of COX-2 on various ERβ responses in dutasteride-treated BPH-1 cells could be due to multiple mechanisms of action. For example, although 5αR inhibition by dutasteride will limit the production of ERβ ligands from DHT, other ligands could be generated via backdoor pathways utilizing alternative steroid precursors and steroidogenic enzymes (36). We therefore initially confirmed that BPH-1 cells contained the enzymatic machinery to generate DHT from testosterone and ERβ ligands from DHT as initially described by Hayward et al. (28) (Fig. 9, A and B). Surprisingly, although COX-2 ablation did not affect the conversion of testosterone to DHT in BPH-1 cells, the production of ERβ ligands from DHT is severely compromised in COX-2-ablated cells (Fig. 9, C and D). Therefore, although COX-2 may limit ERβ activity through the production of ROS, it is required for efficient production of endogenous ERβ ligands in BPH-1 cells.

To identify potential genetic alterations caused by COX-2 ablation in BPH-1 cells, RNA-seq was performed on both BPH-1 scr and BPH-1 shCOX-2 cells. Sequencing was performed on three biological replicates of each cell line, and only genes that were greater than one fragment per kilobase of transcript per million mapped were considered to be sequenced above baseline. To be considered differentially expressed between the two conditions, expression differences had to be greater than 2-fold in at least two replicates with a p value < 0.05. Of the 16,007 genes sequenced that were greater than one fold change between the two conditions, expression differences had to be greater than 2-fold in at least two replicates with a p value < 0.05. Of the 16,007 genes sequenced that were greater than one fragment per kilobase of transcript per million mapped, 6,952 genes were differentially expressed. Among these, expression of 10 steroidogenic enzyme genes was altered when COX-2 was ablated in BPH-1 cells (Table 3). Specifically, the expression of two enzymes responsible for the conversion of DHT to 3β-diol and 3α-diol was significantly reduced. Although both AKR1C1 and AKR1C2 can metabolize DHT to diols, AKR1C1 is predominantly responsible for the metabolism of DHT to 3β-diol, and AKR1C2 is responsible for the
metabolism of DHT to 3α-diol (51). Additionally, the expression of the enzyme responsible for the metabolism of 3β-diol to its inactive metabolite 5α-androstanetriol, CYP7B1, was elevated in shCOX-2 cells (Fig. 9E). The alterations of these steroidogenic enzyme genes agree with the reduction of ERβ ligands as determined by mass spectrometry (Fig. 9, C and D). Therefore, the absence of COX-2 limits ERβ activation through a dual mechanism: reduction of the metabolism of DHT to diols as well as increased metabolism (inactivation) of any 3β-diol that is produced.

Inhibition of COX-2 Influences ERβ Activity through Mediation of Prostaglandin Synthesis—In addition to the alteration in steroid metabolism enzymes, the RNA-seq data also revealed differential expression of two prostaglandin synthase genes, PTGES and PTGES2; ablation of COX-2 leads to decreased expression of these enzymes. Previous studies have shown that several steroid metabolism enzymes are dependent on PGE2 activation of the PKA pathway (52, 53). Examination of PGE2 accumulation in control and COX-2-ablated BPH-1 cells (both genetically and pharmacologically) by mass spectrometry showed that, in the absence of COX-2, intracellular PGE2 levels are reduced (Fig. 9F). Treatment with a COX-2-specific inhibitor in the COX-2-ablated cell line showed no additional decrease in PGE2 accumulation, indicating that the genetic ablation and the pharmacological inhibition of COX-2 is at a maximal level. NS398 treatment of BPH-1 cells results in the same levels of PGE2 accumulation as is seen in shCOX-2 cells. Interestingly, treatment with a COX-1 inhibitor or a nonspecific COX inhibitor, aspirin, showed an even greater decrease in PGE2 accumulation, suggesting that there is some COX-1 compensation when COX-2 is ablated in BPH-1 cells.

Discussion

Current monotherapies for symptomatic BPH have limited efficacy in decreasing prostate size and reducing LUTS due to the complex etiology of the disease. BPH progression is linked to both chronic inflammation and a down-regulation of ERβ-dependent pathways. In this report, we examined the influence of one inflammatory mediator, COX-2, on ERβ function in BPH-derived epithelial cells. We initially focused on a current standard pharmacotherapy approach for BPH, which seeks to limit the action of AR through inhibition of the enzymes that convert testosterone to the more potent AR ligand DHT. Although both dutasteride (inhibitor of 5AR types 1 and 2) and finasteride (inhibitor of 5AR type 2 only) prevent the conversion of testosterone to DHT, they also decrease the production of ERβ ligands generated from DHT and therefore could limit potential tissue-protective effects of this receptor manifested
partly through an ERβ-dependent promotion of apoptosis (Fig. 10). However, we found that dutasteride, but not finasteride, induces apoptosis in BPH-derived cells in an ERβ-dependent manner. In nearly all experiments presented in this report, the response of BPH-1 cells to Dutasteride was overcome by the addition of DHT but not testosterone, confirming that the inhibition of 5AR activity and production of DHT was responsible for the effects observed. Therefore, ERβ may promote apoptosis due to activation by ligands generated by backdoor pathways that do not require 5AR activity or through ligand-independent action that is enhanced in the absence of potent DHT-derived ERβ ligands.

The fact that finasteride on its own does not trigger apoptosis in BPH-1 cells suggests either that inhibition of testosterone to DHT must be complete for ERβ to promote apoptosis or that dutasteride is having off-target effects to promote ERβ-depen-
dent apoptosis and gene expression responses. Dutasteride treatment of BPH-1 cells leads to rapid activation of the canonical TGFβ pathway, which Smad4 ablation experiments revealed contributes to some of the biological effects of dutasteride (e.g. repression of E-cadherin). Prostate samples collected from BPH patients that failed α-blocker and dutasteride combination therapy showed a decrease in E-cadherin expression compared with patients that failed α-blocker monotherapy along with an increase in pSmad3 (54). Therefore, dutasteride activation of the TGFβ pathway, as observed in our studies with BPH-1 cells, can occur in patients subjected to 5αR inhibition, although it is unlikely on its own to drive EMT as supported by our in vitro findings. Nonetheless, these clinical results as well as data reported herein suggest that the clinical utility, or lack thereof, of 5αR inhibitors to treat symptomatic BPH is not only related to its reduction in AR signaling but perhaps influenced by the activation of TGFβ within individual BPH patients.

One of the TGFβ responses to dutasteride in BPH-1 cells that may be of particular relevance to BPH, given the importance of inflammation in this disease, is the induction of COX-2 expression. Inflammation associated with BPH may trigger an increase in COX-2 expression. Because COX-2-derived ROS can limit ERβ action in a prostate cancer cell line (50), we predicted that reducing COX-2 expression in BPH-1 cells would potentiate ERβ responses in these cells. In fact, the ERβ-dependent induction of apoptosis by dutasteride in BPH-1 cells was promoted by either molecular or pharmacological inhibition of COX-2. Furthermore, finasteride, which is ineffective on its own to promote apoptosis in BPH-1 cells, enhanced ERβ-dependent apoptosis upon COX-2 ablation. Therefore, the inhibition of COX-2 may be required to optimize any pharmacotherapies that seek to limit BPH symptoms through reduced production of DHT or activation of ERβ. A recent clinical trial found that ERβ agonist treatment alone was insufficient to reduce LUTS in BPH patients (55), which was unexpected given the presumed benefit of ERβ derived from numerous preclinical studies in rodents (9). These disappointing clinical trial data highlight the need to understand more fully the various signaling pathways that impact ERβ action in prostate cells to develop effective combination pharmacotherapies for individual BPH patients.
Although COX-2 plays an important role in BPH disease, the clinical benefits of NSAIDs are indeterminate. A small clinical trial showed that combination therapy with NSAIDs and a 5α-AR inhibitor alleviated LUTS more quickly than monotherapy (27). However, because this effect does not persist with long term treatment, ablation of COX-2 may generate potential compensatory mechanisms that alter treatment efficacy. We uncovered one outcome of COX-2 inhibition that would oppose its beneficial effects in prostate and thereby potentially limit its therapeutic benefit. Specifically, we found that ablation of COX-2 in BPH-1 cells leads to a dramatic alteration in endogenous ERβ ligand metabolism. Specifically, although COX-2 does not impact 5α-AR activity (i.e. conversion of testosterone to DHT), it is required for the efficient generation of potent ERβ ligands in BPH-1 cells.

RNA-seq analysis identified several steroid metabolism enzyme genes whose expression was altered upon COX-2 ablation such as AKR1C1 and AKR1C2, which are responsible for the conversion of DHT predominantly to 3β-diol and 3α-diol, respectively. These genes are down-regulated upon COX-2 ablation in BPH-1 cells, which would lead to decreased conversion of DHT to ERβ ligands. Furthermore, ablation of COX-2 leads to an increase in CYP7B1 gene expression, an enzyme that converts 3β-diol to an inactive and excreted metabolite (5α-androstanetriol). Hence, inhibition of COX-2 leads to decreased activation of ERβ, which may explain the lack of long

**FIGURE 9.** COX-2 ablation alters steroid metabolism in BPH-1 cells. A, tritiated testosterone (7) is metabolized into 4-androstene-3,17-dione (AD), 5α-androstanedione (1), and 5α-diol (2) in BPH-1 scr cells. B, tritiated DHT is metabolized into 5α-androstanedione and 5α-diol in BPH-1 scr cells. C, in COX-2-ablated BPH-1 cells, metabolism of tritiated testosterone to DHT is maintained, but its ultimate conversion to 4-androstene-3,17-dione, 5α-androstanedione, and 5α-diol is reduced. D, direct conversion of tritiated DHT to these metabolites is also reduced in COX-2-ablated BPH-1 cells. E, quantitative RT-PCR validation of effects of COX-2 ablation in BPH-1 cells (i.e. shCOX-2) on steroidogenic enzyme mRNA expression revealed in RNA-seq analysis. *, p value < 0.05; n = 4. F, PGE2 accumulation measured by mass spectrometry was reduced upon pharmacological inhibition of COX enzymes in BPH-1 scr or shRNA-mediated ablation of COX-2 (i.e. shCOX-2). The following COX inhibitors were used: aspirin (ASA; COX-1 and COX-2 inhibitor; 200 μM), SC560 (COX-1 selective inhibitor; 9 nM), and NS398 (COX-2 selective inhibitor; 1 μM). A one-way ANOVA was performed following a Dunnett’s post hoc test with BPH-1 scr as control. *, p value < 0.05; **, p value < 0.01; ***, p value < 0.001; n = 4. Error bars represent S.E.

**TABLE 3**

Expression of steroidogenic enzyme genes upon COX-2 ablation in BPH-1 cells

| Gene name | -Fold difference (shCOX-2/scr) |
|-----------|-------------------------------|
| AKR1C1    | 0.31                          |
| CYP36A1   | 0.22                          |
| CYP7B1    | 6.22                          |
| HSD17B1   | 0.37                          |
| HSD17B8   | 0.38                          |
| HSD17B10  | 0.45                          |
| HSD17B11  | 2.75                          |
| HSD3B7    | 0.20                          |
| PTGES     | 0.22                          |
| PTGES2    | 0.38                          |

**FIGURE 10.** Dutasteride-mediated ERβ activation pathway in BPH-derived epithelial cells. See text for details.
term efficacy of COX-2 inhibitors in clinical management of BPH.

Other genes that are differentially regulated by COX-2 in BPH-1 cells include two prostaglandin synthase genes, **PTGES** and **PTGES2**. The expression of some steroid metabolism enzymes is PGE$_2$-dependent through the activation of the PKA pathway (52, 56, 57). In the absence of COX-2, there was a significant decrease in PGE$_2$ accumulation. Therefore, reduced PGE$_2$ accumulation would limit activation of steroid metabolism enzymes that are PGE$_2$-dependent, providing yet another mechanism through which chronic COX-2 inhibition serves to alter the intraprostatic hormonal milieu. In addition to selective COX-2 inhibitors, nonspecific COX inhibitors have been evaluated for their effectiveness in limiting BPH symptoms (58–60). Treatment of BPH-1 cells with a COX-1 inhibitor and a nonspecific COX inhibitor showed an even greater decrease in PGE$_2$ accumulation than inhibition of COX-2 alone. Even though COX-2 is the inducible isoform and COX-1 is constitutively expressed, these data and our results in BPH-1 cells suggest that within the prostate epithelial cells COX-1 is participating in the production and accumulation of PGE$_2$. Additionally, it has been shown that COX inhibition by NSAIDs can inhibit the AKR1C enzymes, further reducing the metabolism and accumulation of ERβ ligands (51).

Combination therapy with NSAIDs and 5AR inhibitors was considered a promising treatment for men with symptomatic BPH, but their clinical effectiveness has been disappointing. Although 5AR inhibitors do increase apoptosis in BPH cells and could potentially decrease prostate size, these compounds also alter the steroid hormone milieu through decreased production and increased metabolism of ERβ ligands that normally function to protect and maintain prostate size and differentiation. Additionally, in an effort to treat the inflammation inherent in BPH, treatment with NSAIDs also further decreases the protection provided by ERβ activation through a dual mechanism: in the absence of COX-2, there is a significant decrease in steroid metabolism enzymes necessary for the production of ERβ ligands as well as a decrease in PGE$_2$ accumulation, which serves to limit the activation of PGE$_2$-dependent steroid-metabolizing enzymes. Future therapies designed to limit proliferation and chronic inflammation but maintain ERβ activation may provide more rational treatment strategies and thus serve to provide better clinical outcomes for patients with symptomatic BPH.

**Author Contributions**—T. T. L. and M. J. G. conducted most of the experiments, analyzed the results, and wrote most of the paper. K. A. F. conducted the ChIP and indirect immunofluorescence experiments. S. G. W. conducted the experiments measuring the PGE$_2$ accumulation. J. L. and R. J. A. conducted the experiments examining steroid metabolism. DBD. conceived the idea for the project. D. B. D., R. J. A., and W. A. R. interpreted the results and critically revised the paper. All authors approved the final version of the manuscript.

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**References**

1. Kirby, R. S. (2000) The natural history of benign prostatic hyperplasia: what have we learned in the last decade? Urology 56, 3–6
2. Berry, S. J., Coffey, D. S., Walsh, P. C., and Ewing, L. L. (1984) The development of human benign prostatic hyperplasia with age. J. Urol. 132, 474–479
3. Chute, C. G., Panser, L. A., Girman, C. J., Oesterling, J. E., Guess, H. A., Jacobsen, S. J., and Lieber, M. M. (1993) The prevalence of prostatism: a population-based survey of urinary symptoms. J. Urol. 150, 85–89
4. Isaacs, T. J., and Coffey, D. S. (1989) Etiology and disease process of benign prostatic hyperplasia. Prostate Suppl. 2, 33–50
5. Barry, M. J., Fowler, F. J., Jr., O’Leary, M. P., Bruskewitz, R. C., Holmgren, H. L., Mebus, W. K., and Cockett, A. T. (1992) The American Urological Association symptom index for benign prostatic hyperplasia. The Measurement Committee of the American Urological Association. J. Urol. 148, 1549–1557; discussion 1564
6. McVary, K. T., Roehrborn, C. G., Avins, A. L., Barry, M. J., Bruskewitz, R. C., Donnell, R. F., Foster, H. E., Jr., Gonzalez, C. M., Kaplan, S. A., Penson, D. F., Ulchaker, J. C., and Wei, J. T. (2011) Update on AUAGuideline on the management of benign prostatic hyperplasia. J. Urol. 185, 1793–1803
7. Nickel, J. C., Roehrborn, C. G., O’Leary, M. P., Bostwick, D. G., Somerville, M. C., and Rittmaster, R. S. (2008) The relationship between prostate inflammation and lower urinary tract symptoms: examination of baseline data from the REDUCE trial. Eur. Urol. 54, 1379–1384
8. Kramer, G., and Marberger, M. (2006) Could inflammation be a key component in the progression of benign prostatic hyperplasia? Curr. Opin. Urol. 16, 25–29
9. McPherson, S. J., Elem, S. J., Patchev, V., Fritzemeier, K. H., and Risbridger, G. P. (2006) The role of ERα and ERβ in the prostate: insights from genetic models and isoform-selective ligands. Ernst Schering Found. Symp. Proc. 131–147
10. Pelletier, G. (2008) Expression of steroidogenic enzymes and sex-steroid receptors in human prostate. Best Pract. Res. Clin. Endocrinol. Metab 22, 223–228
11. Oliveira, A. G., Coelho, P. H., Guedes, F. D., Mahecha, G. A., Hess, R. A., and Oliveira, C. A. (2007) 5α-Androstan-3β,17β-diol (3β-diol), an estrogenic metabolite of 5α-dihydrotestosterone, is a potent modulator of estrogen receptor ERβ expression in the ventral prostate of adult rats. Steroids 72, 914–922
12. Weihua, Z., Rathe, L., Warner, M., and Gustafsson, J. A. (2002) An endocrine pathway in the prostate, ERβ, AR, 5α-androstan-3β,17β-diol, and CYP17B1, regulates prostate growth. Proc. Natl. Acad. Sci. U.S.A. 99, 13589–13594
13. Cunha, G. R. (1996) Growth factors as mediators of androgen action during male urogenital development. Prostate Suppl. 6, 22–25
14. Wu, C. T., Altuwaijri, S., Ricke, W. A., Huang, S. P., Yeh, S., Zhang, C., Niu, Y., Tsai, M. Y., and Chang, C. (2007) Increased prostate cell proliferation and loss of cell differentiation in mice lacking prostate epithelial androgen receptor. Proc. Natl. Acad. Sci. U.S.A. 104, 12679–12684
15. Simanainen, U., Allan, C. M., Lim, P., McPherson, S., Jimenez, M., Zajac, J. D., Davey, R. A., and Handelsman, D. J. (2007) Disruption of prostate epithelial androgen receptor impedes prostate lobe-specific growth and function. Endocrinology 148, 2264–2272
16. Lepor, H. (1990) Role of α-adrenergic blockers in the treatment of benign prostatic hyperplasia. Prostate Suppl. 3, 75–84
17. Roehrborn, C. G., Boyle, P., Nickel, J. C., Hoefner, K., and Andriole, G.; ARIA3001, ARIA3002, and ARIA3003 Study Investigators (2002) Efficacy and safety of a dual inhibitor of 5α-reductase types 1 and 2 (duasteride) in men with benign prostatic hyperplasia. Urology 60, 434–441
18. Parsons, J. K., Schenk, J. M., Arnold, K. B., Messer, K., Till, C., Thompson, I. M., and Kristal, A. R.; Prostate Cancer Prevention Trial; Urologic Diseases in America Project (2012) Finasteride reduces the risk of incident clinical benign prostatic hyperplasia. Eur. Urol. 62, 234–241
19. Roehrborn, C. G., Nickel, J. C., Andriole, G. L., Gagnier, R. P., Black, L., Wilson, T. H., and Rittmaster, R. S. (2011) Duasteride improves outcomes of benign prostatic hyperplasia when evaluated for prostate cancer risk
reduction: secondary analysis of the REDuction by DuTasteride of prostate Cancer Events (REDUCE) trial. *Urology* 78, 641–646
20. McConnell, I. D., Roehrborn, C. G., Bautista, O. M., Andriole, G. L., Jr., Dixon, C. M., Kusek, J. W., Lepor, H., McVary, K. T., Nyberg, L. M., Jr., Clarke, H. S., Crawford, E. D., Dinolo, A., Foley, J. P., Foster, H. E., Jacobs, S. C., et al. (2003) The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia. *N. Engl. J. Med.* 349, 2387–2398
21. Barkin, J., Guimarães, M., Jacobi, G., Pushkar, D., Taylor, S., and van Viersen Trip, O. B. (2003) a-Blocker therapy can be withdrawn in the majority of men following initial combination therapy with the dual 5a-reductase inhibitor dutasteride. *Eur. Urol.* 44, 461–466
22. Nickel, J. C., Downey, J., Young, I., and Boag, S. (1999) Asymptomatic inflammation and/or infection in benign prostatic hyperplasia. *BJU Int.* 84, 976–981
23. Kramer, G., Steiner, G. E., Hanidisuya, A., Stix, U., Halet, A., Knerer, B., Gesel, A., Lee, C., and Marberger, M. (2002) Increased expression of lymphocyte-derived cytokines in benign hyperplastic prostate tissue, identification of the producing cell types, and effect of differentially expressed cytokines on stromal cell proliferation. *Prostate* 52, 43–58
24. König, J. E., Senge, T., Allhoff, E. P., and König, W. (2004) Analysis of the inflammatory network in benign prostate hyperplasia and prostate cancer. *Prostate* 58, 121–129
25. Wang, W., Bergh, A., and Damber, J. E. (2004) Chronic inflammation in benign prostate hyperplasia is associated with focal upregulation of cyclooxygenase-2, Bcl-2, and cell proliferation in the glandular epithelium. *Prostate* 61, 60–72
26. Baltaci, S., Orhan, D., Gögüs, C., Türkölmez, K., Tulunay, O., and Gögüs, O. (2001) Inducible nitric oxide synthase expression in benign prostatic hyperplasia, low- and high-grade prostatic intraepithelial neoplasia and prostatic carcinoma. *BJU Int.* 88, 100–103
27. Di Silverio, F., Bosman, C., Salvadori, M., Albanesi, L., Piroietti Pannunzi, L., Ciacciariello, M., Cardi, A., Salvadori, G., and Sciara, A. (2005) Combination therapy with rofecoxib and finasteride in the treatment of men with lower urinary tract symptoms (LUTS) and benign prostatic hyperplasia (BPH). *Eur. Urol.* 47, 72–78; discussion 78–79
28. Hayward, S. W., Dahiya, R., Cunha, G. R., Bartek, J., Deshpande, N., and Narayan, P. (1995) Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1. *In Vitro Cell Dev. Biol.* 31, 14–24
29. Jiang, M., Strand, D. W., Fernandez, S., He, Y., Yi, Y., Birbach, A., Qiu, Q., Grant, M., Chandrasiri, U. P., Toivanen, R., Wang, Y., Taylor, R. A., and Risch-Bediger, G. P. (2010) Estrogen receptor-beta activated apoptosis in benign hyperplasia and cancer of the prostate is androgen independent and TNFα mediated. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3123–3128
30. Lazier, C. B., Thomas, L. N., Douglas, R. C., Vessey, J. P., and Rittmaster, R. S. (2004) Dutasteride, the dual 5a-reductase inhibitor, inhibits androgen action and promotes cell death in the LNCaP prostate cancer cell line. *Prostate* 58, 130–144
31. Schmidt, L. J., Murillo, H., and Tindall, D. J. (2004) Gene expression in prostate cancer cells treated with the dual 5α-reductase inhibitor dutasteride. *J. Androl.* 25, 944–953
32. Mak, P., Leav, I., Pursell, B., Bae, D., Yang, X., Taglianti, C. A., Gouvin, L. M., Sharma, V. M., and Mercurio, A. M. (2010) ERβ impedes prostate cancer EMT by destabilizing HIF-1α and inhibiting VEGF-mediated snail nuclear localization: implications for Gleason grading. *Cancer Cell* 17, 319–332
33. Thomas, C., and Gustafsson, J. Å. (2011) The different roles of ER subtypes in cancer biology and therapy. *Nat. Rev. Cancer* 11, 597–608
34. Guerini, V., Sau, D., Scaccianoce, E., Rusmini, P., Ciana, P., Maggi, A., Martini, P. G., Katzenellenbogen, B. S., Martini, L., Motta, M., and Poletti, A. (2005) The androgen derivative 5α-androstan-3β,17β-diol inhibits prostate cancer cell migration through activation of the estrogen receptor β subtype. *Caner Res.* 65, 5445–5453
35. Alonso-Magdalena, P., Brössner, C., Reiner, A., Cheng, G., Sugiyama, N., Warner, M., and Gustafsson, J. A. (2009) A role for epithelial–mesenchymal transition in the etiology of benign prostatic hyperplasia. *Proc. Natl. Acad. Sci. U.S.A.* 106, 2859–2863
36. Wikström, P., Stattin, P., Franck-Lissbrant, I., Damber, J. E., and Bergh, A. (1998) Transforming growth factor β is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. *Prostate* 37, 19–29
37. Xie, L., Law, B. K., Chytil, A. M., Brown, K. A., Akre, M. E., and Moses, H. L. (2004) Activation of the Erk pathway is required for TGF-β1-induced EMT in vitro. *Neoplasia* 6, 603–610
38. Supperregui, A. R., Gamell, C., Rodríguez-Carballo, E., Ortuño, M. J., Bartrons, R., Rosa, J. L., and Ventura, F. (2011) Noncanonical BMP signaling regulates cyclooxygenase-2 transcription. *Mol. Endocrinol.* 25, 1006–1017
39. Ren, R., Charles, P. C., Zhang, C., Wu, Y., Wang, H., and Patterson, C. (2007) Gene expression profiles identify a role for cyclooxygenase 2-dependent prostate generation in BMP6-induced angiogenic responses. *Blood* 109, 2847–2853
40. Ahn, S. M., Cha, J. Y., Kim, J., Kim, D., Trang, H. T., Kim, Y. M., Cho, Y. H., Park, D., and Hong, S. (2012) Smad3 regulates E-cadherin via miRNA-200 pathway. *Oncogene* 31, 3051–3059
41. Grubisha, M. J., Cifuentes, M. E., Hammes, S. R., and Defranco, D. B. (2010) A local paracrine and endocrine network involving TGF-β, Cox-2, ROS, and estrogen receptor β influences reactive stromal cell regulation of prostate cancer cell motility. *Mol. Endocrinol.* 26, 940–954
42. Steckelbroeck, S., Jin, Y., Gopishetty, S., Oyesanmi, B., and Penning, T. M. (2004) Human cytosolic 3α-hydroxysteroid dehydrogenases of the aldo-keto reductase superfamily display significant 3β-hydroxysteroid dehydrogenase activity: implications for steroid hormone metabolism and action. *J. Biol. Chem.* 279, 10784–10795
43. Tremblay, Y., and Beaudoin, C. (1993) Regulation of 3 β-hydroxysteroid dehydrogenase and 17 β-hydroxysteroid dehydrogenase messenger ribonucleic acid levels by cyclic adenosine 3',5'-monophosphate and phorbol myristate acetate in human choriocarcinoma cells. *Mol. Endocrinol.* 7, 355–364
53. Beaudoin, C., Bonenfant, M., and Tremblay, Y. (1997) Regulation of cytochrome P450 cholesterol side-chain cleavage, 3\(\beta\)-hydroxysteroid dehydrogenase/\(\Delta\Delta5\) to \(\Delta\Delta4\) isomerase type 1 and estradiol-17\(\beta\)-hydroxysteroid dehydrogenase mRNA levels by calcium in human choriocarcinoma JEG-3 cells. *Mol. Cell. Endocrinol.* **133**, 63–71

54. Kim, H. K., Zhao, C., Choi, B. R., Chae, H. J., Kim do, S., and Park, J. K. (2013) Is transforming growth factor-\(\beta\) signaling activated in human hypertrophied prostate treated by 5-\(\alpha\) reductase inhibitor? *Dis. Markers* **35**, 679–685

55. Roehrborn, C. G., Spann, M. E., Myers, S. L., Serviss, C. R., Hu, L., and Jin, Y. (2015) Estrogen receptor \(\beta\) agonist LY500307 fails to improve symptoms in men with enlarged prostate secondary to benign prostatic hyperplasia. *Prostate Cancer Prostatic Dis.* **18**, 43–48

56. Payne, A. H., and Sha, L. L. (1991) Multiple mechanisms for regulation of 3\(\beta\)-hydroxysteroid dehydrogenase/\(\Delta\Delta5\) to \(\Delta\Delta4\) isomerase, 17\(\alpha\)-hydroxylase/C17–20 lyase cytochrome P450, and cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid levels in primary cultures of mouse Leydig cells. *Endocrinology* **129**, 1429–1435

57. Sewer, M. B., and Waterman, M. R. (2002) cAMP-dependent transcription of steroidogenic genes in the human adrenal cortex requires a dual-specificity phosphatase in addition to protein kinase A. *J. Mol. Endocrinol.* **29**, 163–174

58. St Sauver, J. L., Jacobson, D. J., McGree, M. E., Lieber, M. M., and Jacobsen, S. J. (2006) Protective association between nonsteroidal antiinflammatory drug use and measures of benign prostatic hyperplasia. *Am. J. Epidemiol.* **164**, 760–768

59. Schenk, J. M., Calip, G. S., Tangen, C. M., Goodman, P., Parsons, J. K., Thompson, I. M., and Kristal, A. R. (2012) Indications for and use of nonsteroidal antiinflammatory drugs and the risk of incident, symptomatic benign prostatic hyperplasia: results from the prostate cancer prevention trial. *Am. J. Epidemiol.* **176**, 156–163

60. Sutcliffe, S., Grubb Iii, R. L., Platz, E. A., Ragard, L. R., Riley, T. L., Kazin, S. S., Hayes, R. B., Hsing, A. W., and Andriole, G. L. (2012) Non-steroidal anti-inflammatory drug use and the risk of benign prostatic hyperplasia-related outcomes and nocturia in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. *BJU Int.* **110**, 1050–1059