Signal Transduction

Significance of Divergent Expression of Prostaglandin EP4 and EP3 Receptors in Human Prostate Cancer

Hosea F. S. Huang, Ping Shu, Thomas F. Murphy, Seena Aisner, Valerie A. Fitzhugh, and Mark L. Jordan

Abstract

PGE\textsubscript{2} has been implicated in prostate cancer tumorigenesis. We hypothesized that abnormal prostaglandin receptor (EPR) expression may contribute to prostate cancer growth. Twenty-six archived radical prostatectomy specimens were evaluated by immunohistochemistry (IHC) and Western blotting for the expression of EP1, EP2, EP3, and EP4. As a corollary, EPR expression in one normal (PZ-HPV7) and four prostate cancer cell lines (CA-HPV10, LNCaP, PC3, and Du145) were assessed by Western blotting. Prostate cancer and normal cell growth were compared in vitro after EPR blockade, siRNA EPR knockdown, or overexpression. EP1, EP2, EP3, and EP4 receptors were detected by IHC in all areas of benign tissue within the clinical prostate cancer specimens. In areas of prostate cancer, EP4 and EP2 were overexpressed in 85\% (22 of 26) and 75\% (18 of 24) and EP3 expression was reduced in all (26 of 26, 100\%) specimens (P < 0.05 vs. benign tissue). EP1 showed no specific differential expression pattern. Increased EP4 and reduced EP3 was confirmed by Western blotting in fresh clinical specimens and in prostate cancer cell lines (CA-HPV10, LNCaP, PC3, and Du145) compared with the normal prostate cell line (PZ-HPV7). EP2 and EP4 siRNA knockdown resulted in reduced in vitro growth and metastasis-related gene expression (MMP9 and Runx2) of prostate cancer lines, and in vitro migration was inhibited by EP4 antagonists. As a corollary, EP3-overexpressing PC3 cells displayed impaired growth in vitro. Human prostate cancer is associated with EP4 and EP2 overexpression and reduced EP3 expression. These data suggest that targeting specific EPR may represent a novel therapeutic approach for prostate cancer. Mol Cancer Res; 11(4); 427–39. ©2013 AACR.

Introduction

Prostate cancer accounts for 30\% of all new male cancer diagnoses, and next to lung cancer, is the second leading cause of cancer death in men (1). More than 32,000 U.S. men died of prostate cancer in 2010, and the incidence is predicted to increase as the male population continues to age (1). Epidemiologic studies have shown a direct link between the incidence of prostate cancer and dietary lipid and its metabolism (2). For example, there is a higher incidence of prostate cancer in U.S. Caucasian and African American males than in Asian males, and the incidence of prostate cancer increases in Asian males who adopt a North American diet (3). Arachidonic acid (AA) has been shown to promote prostate cancer cell growth mediated, in part, by the formation of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). PGE\textsubscript{2} is known to have potent effects on immune responses and in oncogenesis of a variety of tumors (4, 5). In prostate cancer, for example, PGE\textsubscript{2} acts as a tumor promoter by stimulating cell proliferation and inhibiting apoptosis (6, 7). Furthermore, COX-2, the rate-limiting enzyme for PGE\textsubscript{2} synthesis from arachidonic acid, is overexpressed in prostate cancer (8, 9). These observations, taken together with the known suppression of prostate cancer cell growth by COX-2 inhibitors and non-steroidal anti-inflammatory agents, have suggested that COX inhibition may present a logical therapeutic approach for prostate cancer (10). However, translation of these findings into clinical use has been hampered by incomplete knowledge of the mechanisms involved in the effects of PGE\textsubscript{2} on prostate cancer, and COX-2 inhibitors have as yet not shown clinical efficacy (11).

PGE\textsubscript{2} exhibits multiple regulatory effects on cell proliferation, apoptosis, motility (4, 6), immune surveillance (12, 13) and induces angiogenesis (14). PGE\textsubscript{2} exerts both autocrine and paracrine functions by binding to and activation of the specific G-protein–coupled receptors EP1, EP2, EP3, and EP4 (15, 16) which are frequently co-expressed in the same cells but use different and/or opposing signaling pathways. Altered EPR expression has been reported in various malignancies. For example, EP2 overexpression has been reported in skin and esophageal cancer (17, 18). EP4 is upregulated in colon and breast cancer (19, 20) and glioblastoma (21). The functional relevance of EP2 and EP4 overexpression in these cancers has been corroborated by
enhanced cell proliferation, migration, invasion in vitro and tumorigenesis and metastasis in vivo induced by EP2 and EP4 agonists (18–21). As a corollary, EP2 and EP4 antagonists suppress cancer cell growth, migration/invasion, and colony formation in vitro and attenuate tumorigenesis and metastasis in vivo (22, 23). These data suggest that targeting specific EP receptors may offer an attractive and more specific therapeutic approach for the treatment of prostate cancer than has been previously accomplished with broad-based COX inhibition. However, the characteristics of EP receptor expression in human prostate cancer have not been well-established, thus potential rationale for targeting of specific receptors is unclear. The current study was undertaken to characterize EPR expression in normal and prostate cancer cells in prostate tissue from patients undergoing radical prostatectomy for clinically localized prostate cancer and to determine whether tissue expression of EP receptors in human prostate cancer correlates with Gleason grade, stage, and clinical outcome. The patterns of EPR expression we detected in resected prostate cancer were recapitulated by the results observed in human prostate cancer cell lines. We also showed that siRNA knockdown of the EP2 and EP4 genes and stable overexpression of EP3 attenuated the in vitro growth of human prostate cancer cells. EP2 and EP4 knockdown was also associated with reduced MMP9 and Runx2 expression. These observations provide a scientific rationale for targeting EPR signaling as a therapeutic approach in the management of prostate cancer.

Materials and Methods

Patients and tissues

All human studies described were approved by the Institutional Review Board (IRB) of the New Jersey Medical School (NJMS). Archived, formalin-fixed, paraffin-embedded human prostate tissue from 26 patients who underwent radical prostatectomy for clinically localized prostate cancer in the Division of Urology, University of Medicine and Dentistry of New Jersey (UMDNJ)–NJMS were used. None of these patients had undergone prior hormonal treatment or radiation. Immunohistochemistry (IHC) using affinity-purified polyclonal antibodies and Alexa Fluor–conjugated secondary antibody assessed expression of EPR in both prostate cancer and normal prostate epithelia within the same patient sample. Tissue sections (8 μm) were first stained with hematoxylin/eosin (HE) for pathologic evaluation by 1 of 2 board-certified pathologists (S. Aisner and V. Fitzhugh). Adjacent sections were subsequently used for immunostaining of EPR in our laboratory. Fresh specimens were examined immediately after radical prostatectomy in an additional 3 cases. The tissue blocks (2 × 2 × 2 mm³) from areas representing a significant cluster (>80%) of prostate cancer or benign glands were dissected and stored at −80°C for future assay.

Immunohistochemistry of EPR

Tissue sections for immunostaining were processed by deparaffinization in xylene, rehydration in graded ethanol, antigen retrieval in boiling 0.01 mol/L sodium citrate (pH 6), and blocking with 10% normal goat serum (NGS). After washing in PBS (5 minutes × 2), sections were incubated with affinity purified anti-EPR antibodies (1:100, Cayman biological) diluted in 4% NGS in PBS overnight at 4°C. As negative controls, sections were incubated with normal rabbit serum (1:100) or antigen-absorbed primary antibody, washed in PBS (5 minutes × 2) and incubated in darkness with anti-rabbit IgG conjugated with Alexa Fluor 488 fluorescent dye (1: 100 in PBS) for 60 minutes. Sections were examined with a Nikon Eclipse 400 microscope and photographed. Intensities of immunostaining of EPR in prostate cancer and adjacent normal epithelia were compared with a color chart representing increasing degrees of green fluorescence and scored 1 to 4 (1, negative or background; 2, weak; 3, moderate; 4, strong). In each area, a total of 6 to 12 clusters of cancer glands/cells or normal epithelia were scored.

Cell lines and culture

Human normal prostate epithelial cells line PZ-HPV7 and prostate cancer cell lines from primary tumor (CA-HPV10) or metastasis (LNCaP, PC3, and Du145) were purchased from American Type Culture Collection. All cells were maintained in the recommended medium until the monolayers reached 85% to 90% confluence.

RNA isolation and RT-PCR

Cells were seeded onto 6-well plates in their respective medium, cultured until 80% confluence, then rinsed with PBS twice and total RNA was isolated using RNeasy Mini kit (Qiagen). Gene expression was examined by reverse transcription polymerase chain reaction (RT-PCR) using specific primer pairs (Supplementary Data). To identify EP3 isoforms, total RNA from normal and prostate cancer cells were subjected to RT-PCR using combinations of primer pairs (Supplementary Data) specific for different EP3 isoforms (24).

Real-time PCR

Quantitative real-time PCR was carried out using Power SYBR Green PCR Master Mix (Applied Biosystems) and monitored using 7500 Real time PCR system (Applied Biosystems). Negative controls without template were included, and all reactions were conducted in triplicate. The amplification conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

Western immunoblotting

Cultured prostate cancer cells or homogenized freshly obtained human prostate tissue were lysed in ice-cold lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail. Protein concentrations were determined by using BCA protein assay kit (Pierce). Protein extracts were mixed with 2× Laemmli sample buffer (Bio-Rad Laboratories) and denatured at 95°C for 5 minutes and then separated by SDS-PAGE (Bio-Rad Laboratories) and
transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). The membrane was subjected to immunoblot analysis with each primary antibody (EP1 1:500, EP2 1:500, EP3 1:500, EP4 1:600, Cayman Chemical; actin 1:800, Santa Cruz Biotechnology) followed by horseradish peroxidase–conjugated secondary antibody (1:5,000 dilution, Santa Cruz Biotechnology). The protein bands were visualized using Amersham ECL Western blotting analysis system (GE Healthcare).

**Transient EPR knockdown**

Cells (PZ-HPV7, CA-HPV10, and Du145) were seeded in 6-well plates in 2 mL of appropriate medium to achieve 30% to 40% confluence after 24 hours. 2.5 μL of SMARTpool siRNA duplex that specifically targets EP2 or EP4 or nontargeting pool (100 μmol/L, Dharmacon) and 5 μL of Lipofectamine RNAiMAX (Invitrogen) were first diluted in 250 μL Opti-MEM I reduced serum medium separately and then combined and added to each well, mixed gently, and incubated at 37°C in 5% CO₂ for 48 to 72 hours. Individual EPR expression after knockdown was assessed by RT-PCR. To determine the effects of EP2/EP4 knockdown on cell growth, PZ-HPV7, CA-HPV10, or Du145 cells (2 × 10⁵ per well) were seeded in 96-well plates and allowed to settle overnight. On day 2, EP2/EP4 siRNA knockdown was conducted using the siRNA-Lipofectamine RNAiMAX mixture described above. The transfection mixture was removed 6 hours later and cell growth determined 48 to 120 hours later by MTT assay.

**Preparation of conditioned media**

To prepare conditioned medium, 48 hours after siRNA transfection, cells were rinsed with serum-free medium and incubated with serum-free medium for 24 hours. Medium was collected and centrifuged at 1,000 rpm for 5 min at 4°C to eliminate cellular debris. Cell number was determined for each sample. The medium was concentrated using Amicon Ultra-4 centrifugal filter units (Millipore) with a molecular weight cutoff of 30 KDa.

**Gelatin zymography**

Equal amounts of protein from conditioned medium were mixed with 2 × Laemmli sample buffer (Bio-Rad) and electrophoresed on 8% PAGE containing 1 mg/mL gelatin. The gel was washed with 2.5% Triton X-100 and incubated for 24 hours at 37°C in 1 × Developing buffer (Bio-Rad). After incubation, the gel was stained with 0.5% Coomassie Blue R-250 in 50% methanol and 10% acetic acid and destained in methanol:acetic acid:water (50:10:40). The intensity of the digested bands on zymograms was quantified densitometrically with AlphaImager 3400 (Alpha Innotech).

**Construction of EP3II expression plasmid**

EP3II cDNA was amplified from the pcDNA3.1zeo (−)EP3II plasmid (a kind gift from Dr. Barrie Ashby, Temple University, Philadelphia, PA) with the following primers: the sense primer included nucleotides 1–23 (bold) and contained a *HindIII* site (italics); 5 ′-ATTATTAGCT-TATGAAGGAGACCCGGGG-CTACGG-3 ′. Antisense primer represented nucleotides 1,144 to 1,167 (bold) and contained an XhoI site (italics); 5 ′-CCGCCGCTCGAGT-CATGCTTCTGTCTGTA-TTATTTC-3 ′. PCR products were electrophoresed on 1% agarose gel, excised, and purified using QIAquick gel purification kit (Qiagen). To construct the EP3II expression vector, the EP3II cDNA and the pcDNA3.1(+) expression vector were digested using the restriction enzymes *HindIII* and XhoI. The EP3II fragment was then ligated into the pcDNA3.1(+) vector using T4 DNA ligase. The plasmid clones were sequenced using T7 and BGH rev primers at NJMS Molecular Resource Facility. The plasmid generated (pcDNA3.1/EP3II) was subsequently expanded and used for transfection experiments.

**Stable overexpression of EP3 receptor genes**

PC3 cells seeded onto 6-well plates were grown to 50% to 60% confluence in complete medium and then transfected with the EP3 expression plasmid using Lipofectamine 2000 for 6 hours. After 48 hours, 500 μg/mL G418 (Invitrogen) was added, and colonies derived from single cells were selected for cells carrying the plasmid. After 2 weeks, G418-resistant colonies were expanded in the presence of culture medium with G418 at 500 μg/mL. EP3 overexpression was verified by RT-PCR and immunostaining with specific antibody.

**In vitro migration assay**

PC3 cells (2.5 × 10⁵) suspended in RPMI-1640 medium supplemented with 10% BCS were seeded into the upper compartment of a modified Boyden chamber (membrane pore size, 8 μm) in 12-well plates in triplicates and cultured overnight. The cells were exposed to reagents in the presence or absence of 0.1% BCS added to the lower chamber for an additional 24 hours. The cells that migrated through the membrane into the lower chamber were enumerated after 24 hours.

**Extracellular signal–regulated kinase phosphorylation**

PZ-HPV7, CA-HPV10, and Du145 cells were seeded in 6-well plates for 24 hours and then serum-starved (Du145) for an additional 24 hours or protein and growth factor–starved (PZ-HPV7 and CA-HPV10) for 48 hours and then were treated with PGE₂ (0.5 μmol/L) for 5 to 60 minutes. Cellular proteins were subsequently isolated and subjected to SDS-PAGE. After transfer to PDVF membranes, phosphorylated ERK (p-ERK) was detected using anti-phosphorylated ERK antibody. The membranes were then stripped and reprobed with antibody against total ERK. To determine effects of EP2 or EP4 blockade, serum or protein/growth factor–starved cells were preincubated with the EP2 antagonist AH6809 or EP4 antagonist AH23848 (10 or 50 μmol/L, respectively) or vehicle [0.1% or 0.5% dimethyl sulfoxide (DMSO), respectively] for 60 minutes before PGE₂ (0.5 μmol/L) treatment. The cells were collected at various time points after addition of PGE₂, and total and p-ERK were determined and quantified by densitometry. The p-ERK level of each sample was normalized against the
corresponding total ERK level. Untreated samples were set as unity, and the fold change in p-ERK level of each sample was calculated as a ratio against the corresponding reference sample.

Statistics
All experiments were carried out a minimum of 3 times and results analyzed by ANOVA and Student’s t test as appropriate. When significant \( (P < 0.05) \), Dunnett’s multiple range test was used to compare means among groups.

Results
Expression of EPR in human prostate cancer tissue
The demographics, Gleason scores, pathologic stage, pre- and postoperative prostate-specific antigen (PSA), and EPR expression scores as determined by IHC from the 26 archived clinical samples are presented in Table 1. In all 26 patients, EP1, EP2, EP3, and EP4 receptors were detected to varying degrees both in areas of normal prostate (NP) epithelia and prostate cancer (representative patient sample Fig. 1A). However, in prostate cancer, the overall intensity of EP4 immunostaining was consistently stronger \( (P < 0.001) \) and EP3 was significantly weaker \( (P < 0.001) \) than in the adjacent NP (Fig. 1B). Increased EP4 and decreased EP3 in prostate cancer cells were observed in 22 of 26 \( (85\%) \) and 25 of 25 \( (100\%) \) specimens examined. Increased EP2 expression in prostate cancer was also observed in 18 of 24 \( (75\%) \) patients \( (P < 0.005 \) vs. NP). In contrast, the intensity of EP1 immunostaining in areas of prostate cancer compared with the adjacent NP exhibited no specific pattern. (Table 1 and summarized in Fig. 1B). Within each patient, the intensities of EP2, EP3, and EP4 immunostaining in prostate cancer cells did not correlate with Gleason grade \( (P > 0.1) \).

Western blotting of EPR in fresh clinical samples
To further validate the IHC results obtained from archived specimens, Western blot analysis of freshly obtained prostate cancer specimens using the same antisera that had been used for IHC was conducted. Figure 1C shows representative Western blots of EP1–4 in 3 normal and 3 prostate cancer patient samples assayed in the same blot, showing greater EP4 and reduced EP3 protein levels in prostate cancer when compared with NP. These observations were confirmed by quantitative comparison of the intensities of protein bands after normalization against that of \( \beta \)-actin intensity of each sample (Fig. 1D). EP1 and EP2 protein levels were not statistically different (not shown).

EPR in human cell lines
To determine whether the differential EPR expression we observed in the clinical specimens (Table 1) would be recapitulated in human prostate cancer lines, the expression of EPR was determined in normal (PZ-HPV7, normal prostate) and prostate cancer cell lines (CA-HPV10, primary tumor; LNCaP, PC3, and Du145, prostate cancer lines metastatic to lymph node, bone, and brain, respectively).

Consistent with our observations in resected human prostate cancer tissue (Table 1 and Fig. 1B), the prostate cancer lines exhibited lower EP3 and higher EP4 expression (compared with the benign prostate line PZ-HPV7) after normalization against \( \beta \)-actin in each sample (Fig. 1E). There was no consistent pattern of EP1 and EP2 in prostate cancer cells compared with those of normal lines (not shown).

Specificity of siRNA knockdown of EP2 and EP4 receptor genes
The increased EP4 and EP2 expression in our clinical prostate cancer specimens suggested that increased expression might contribute to prostate cancer tumorigenesis. We conducted transient EP2 and EP4 siRNA knockdown in PZ-HPV7 (NP line) and CA-HPV10 (primary tumor line). These lines were selected as surrogates for normal and nonmetastatic prostate cancer, respectively. The Du145 (brain metastatic cell line) was also used as a surrogate for metastatic prostate cancer for comparison with the primary tumor line CA-HPV10. Figure 2A confirms that the receptor gene knockdown was specific to the siRNA used and there was no interference with the expression of the nonrelevant EPR.

Physiologic relevance of EP2/EP4 signaling in prostate cancer cells
We next assessed transient siRNA knockdown of EP2 and EP4 on the growth of CA-HPV10, PZ-HPV7, and Du145 cells. Growth of normal (PZ-HPV7) cells was not affected by EP2 KD \( (P > 0.05 \) vs. nontargeting siRNA) but was moderately suppressed by EP4 KD \( (P < 0.05 \) at 96 hours (Fig. 2B). In contrast, the growth of CA-HPV10 cells was suppressed by more than 50% \( (P < 0.001, P < 0.01) \) by both EP2 and EP4 KD at 72 and 96 hours. EP2 KD also suppressed the growth of Du145 cells \( (P < 0.001 \) at 72 and 96 hours; however, EP4 KD did not affect Du145 cell growth significantly until 96 hours \( (P < 0.05 \). This was confirmed in 3 subsequent replicate experiments.

EP2 and EP4 KD reduce MMPs and Runx2
As MMP2 and MMP9 have been shown to be involved in prostate cancer metastasis and invasion (25) and PGE2 promotes tumor cell migration, we hypothesized that MMP2 and 9 may be downstream targets responsive to EPR signaling. Figure 2C shows real-time PCR results of MMP2, MMP9, and Runx2 expression 72 hours after transient EP2 and EP4 KD in PZ-HPV7, CA-HPV10, and Du145 cell lines. EP2 and EP4 KD resulted in reduced expression of MMP9 mRNA in both PZ-HPV7 and CA-HPV10 cells. MMP2 expression was more variably affected: MMP2 mRNA was reduced in PZ-HPV7 after both EP2 and EP4 KD but only by EP2 KD in CA-HPV10. In Du145 cells, EP2 KD resulted in reduced MMP9 but had no effect on MMP2 expression, whereas EP4 KD appears to have paradoxically increased MMP2 and MMP9. As Runx2 is abnormally expressed in metastatic prostate cancer cells but not in benign cells, and Runx2 knockdown has recently been shown to inhibit prostate cancer cell migration in vitro and
**Table 1. Patient demographic data and EPR expression**

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**MEAN**

N = 26

P Value (ANOVA) = 0.0000

95% CI

-0.169 to 0.398

-0.670 to -0.202

0.596 to 0.897

-0.713 to -0.292
growth in vivo (26), the effects of EP2/4 knockdown on Runx2 expression were also examined. EP2 and EP4 KD resulted in decreased Runx2 expression in all 3 cell lines. The potential reasons for differential signaling via EP2 and EP4 in Du145 compared with the 2 other cell lines are explored in more detail in the ERK experiment below.

MMP2 and MMP9 enzymatic activity after EP2 and EP4 knockdown

To determine whether the altered mRNA expression observed by real-time PCR in Fig. 2C correlated with functional enzymatic activity of MMP2 and MMP9 after EP2 and EP4 KD, conditioned medium of each siRNA-transfected cell line was collected, and equal amounts of protein were subjected to gelatin zymography. The intensity of the digested bands corresponding to MMP9 and MMP2 (Fig. 3A) was measured by densitometry and expressed as fold level of the nontargeting siRNA-transfected control which was expressed as unity (Fig. 3B). These results recapitulated the PCR data, with the exception of MMP2 enzymatic activity after EP4 KD of Du145 which was not elevated.

EP4 and prostate cancer migration

In vitro migration as a surrogate for invasive potential was assessed using a Boyden chamber assay. PC3 was used as a representative metastatic cell line in this assay. Control migration in Dulbecco’s Modified Eagle’s Media (DMEM) was assessed in the presence or absence of 1% BCS as a positive and negative control for chemotaxis, respectively. PGE2 (10–1,000 nmol/L) did not induce chemotaxis in the absence of BCS. However, PGE2 at physiologic concentrations (1,000 nmol/L) significantly (P < 0.05) increased BCS-induced chemotaxis compared with BCS alone (Fig. 4A). PC3 cells were then pretreated with the EP4 antagonists AH23848 and L161,982 (10 and 50 μmol/L) or vehicle (0.1%–0.5% DMSO) 1 hour before induction of migration.
by BCS (1%) + PGE₂ (1,000 nmol/L). As shown in Fig. 4B, EP4 antagonist pretreatment of the cells suppressed migration toward PGE₂ + BCS by 50% (P < 0.01) compared with respective vehicle (0.1% or 0.5% DMSO). These results indicate that PGE₂-induced PC3 migration is mediated, in part, by EP4.

**EP2/4 blockade inhibits PGE₂-induced ERK phosphorylation**

Phosphorylation of ERK is necessary for EP4-mediated cell migration and cell growth (27, 28) and PGE₂-mediated prostate cancer angiogenesis (14). We therefore examined whether the effects of EP2-4 blockade were mediated, in part, via ERK phosphorylation. Figure 5A shows that maximal induced ERK1/2 phosphorylation occurred within 5 to 10 minutes after exposure to 0.5 μmol/L of PGE₂ in the 3 cell lines (PZ-HPV7, CA-HPV10, and Du145) evaluated. Cells were then pretreated with the EP2 antagonist AH6809 or EP4 antagonist AH23848 (10 or 50 μmol/L, respectively), or vehicle (0.1% or 0.5% DMSO) for 1 hour, followed by addition of 0.5 μmol/L PGE₂. Resultant p-ERK and total ERK were determined by Western blotting at the corresponding time point of peak PGE₂-induced ERK phosphorylation for each cell line (Fig. 5A: 5 minutes for PZ-HPV7 or 10 minutes for CA-HPV10 and Du145). Figure 5B shows that PGE₂-elicited ERK1/2 phosphorylation was not affected by vehicle (DMSO, 0.1% or 0.5%) but was markedly suppressed by the EP2 antagonist (AH6809) at both 10 and 50 μmol/L in PZ-HPV7, CA-HPV10, and Du145 cells. The effects of the EP4 antagonist AH23848 were more variable. AH23848 suppressed PGE₂-induced ERK phosphorylation in CA-HPV10 at 10 and 50 μmol/L, but in PZ-HPV7 cells at only 50 μmol/L, AH23848. AH23848 had minimal effects on PGE₂-induced ERK phosphorylation in Du145 cells. These results are consistent with the effects of EP2 and EP4 blockade on PZ-HPV7 and CA-HPV10 and Du145 growth shown in Fig. 2B.

**EP3II is the major isoform in human prostate cells**

The consistently lower EP3 protein expression observed in both the prostate cancer specimens and cell lines (Table 1 and Fig. 1) suggests that altered EP3 expression may have biologic significance in prostate cancer. Because of the complexity of EP3 isoforms and their diverse cellular functions (29, 30), we sought to identify EP3 isoforms that are expressed in NP and prostate cancer cells. For this purpose, different primers specific for EP3 isoforms (ref. 24; Supplementary Data) were used in RT-PCR. EP3II (primer pairs P1 + P3) was the only EP3 isoform detected in all prostate cell lines (PZ-HPV7, CA-HPV10, LNCaP, PC3, and Du145) used in our studies (Supplementary Data). This isoform was also detected in 2 additional human prostate lines (PREC and PWR-1E). Primer pairs P1 + P4 (Supplementary Data) and P1 + P2 (not shown) did not result in specific EP3 product.
These results provide rationale for using the EP3II isoform in subsequent overexpression studies.

**Stable EP3 overexpression inhibits prostate cancer growth in vitro**

We cloned the human EP3II isoform and sequence analysis of the insert revealed 100% match with published sequences of human EP3II (24). We then constructed an expression plasmid of human EP3II (Materials and Methods). Stable transfection of PC3 cells with the EP3II plasmid resulted in a PC3 subline overexpressing EP3II (EP3II OE) as shown by RT-PCR, Western blotting, and IHC (Fig. 6A–C). Figure 6D shows that EP3 II OE reduces PC3 growth in vitro compared with WT PC3. PC3 WT and EP3II OE cells were cultured in the presence of indomethacin (10 μmol/L) to prevent endogenous PGE2 production with either vehicle (0.01% DMSO) or the EP3 agonist, sulprostone (1 μmol/L), for 5 days. The reduced growth of EP3II OE cells compared with WT PC3 cells (P < 0.01, Fig. 6D) was further exacerbated by sulprostone (P < 0.01). WT PC3 growth was not affected by sulprostone (Fig. 6E, P > 0.1). These preliminary results strongly suggest that the overexpressed EP3 receptor is functionally responsive to exogenous EP3 stimulation by the EP3 agonist sulprostone.

**Discussion**

It is generally reported that COX-2 is weakly expressed in normal prostate tissue but overexpressed in CaP (8, 9). Overexpression of COX-2 in prostate cancer tissues (8, 9) is thought to result in higher local endogenous PGE2 synthesis which may contribute to disease progression. The concurrence of increased COX-2 expression, local inflammation, neovascularization, and prostatic intraepithelial neoplasia (PIN) in human prostate tissues (31) is consistent with COX2/PGE2-mediated carcinogenesis. Multiple in vitro studies have also suggested that COX-2/PGE2 signaling plays a role in deregulated prostate cancer cell growth. For example, arachidonic acid–induced PC3 cell proliferation is preceded by increased PGE2 production, and these effects were associated with an increase in c-fos that in turn stimulates cell proliferation (32). When treated with COX-2 inhibitors (NS398 or celecoxib), both LNCaP and PC3 cells exhibit increased apoptosis (33), suggesting that endogenous PGE2 might promote prostate cancer cell growth by suppression of apoptosis.
PGE₂ exerts its cellular effects by binding to and activation of the membrane-bound E-prostanoid receptors EP1, EP2, EP3, and EP4 (15, 16). Among these, EP2 and EP4 have been shown to be overexpressed in various cancers (17–20) and to promote cell growth and tumorigenesis, as well as metastasis-related functions including angiogenesis, cell motility, and invasion (14, 19–21). More recently, the expression of EPR subtypes has been described in human prostate cancer (14, 34, 35). Most studies of EP4 in prostate cancer growth had generally shown indirect receptor effects by the use of pharmacologic antagonists or through intermediate effects on angiogenesis factors (14). More recent specific data from Terada and colleagues (35) reported upregulation of EP4 in prostate cancer xenografts grown in castrated nude mice compared with xenografts in intact mice, suggesting involvement of EP4 in the development of the castration-resistant prostate cancer (CRPC) phenotype. These authors also reported that overexpression of EP4 facilitated androgen-independent growth in LNCaP cells in vitro, which was attenuated by specific EP4 antagonist administration (35). However, no studies to date have shown direct involvement of EP2 and EP4 in the growth of human prostate cancer cells or established a direct causal relationship between EP2/EP4 expression status and the development of primary tumor.

Our studies in 26 resected prostate cancer specimens from our clinical urology program revealed consistent overexpression of EP4 and reduced EP3 expression compared with benign prostate tissue. In vitro experiments using human normal prostate and prostate cancer cell lines verified these clinical findings by showing higher EP4 protein levels when compared with immortalized benign prostate cells. We next evaluated the effects of siRNA knockdown of EP2 and EP4 genes on the growth of PZ-HPV7 (normal) and CA-HPV10 (primary tumor). Reduced in vitro growth of CA-HPV10 cells after EP4 knockdown strongly suggests a role for EP4 in...
primary prostate cancer tumorigenesis. The inhibitory effects of EP4 knockdown on human prostate cancer cell growth reported herein have been corroborated by our recent findings showing that stable siRNA knockdown of EP4 in murine prostate cancer RM1 cells inhibited their growth in vitro and tumorigenesis in vivo (36).

The effects of EP2 and EP4 knockdown were next examined in Du145 cells as a surrogate for metastatic prostate cancer. EP4 signaling has been reported to contribute to the development of the androgen-independent phenotype of prostate cancer cells (35). As Du145 cells are highly metastatic and exhibit androgen-independent growth, the lack of a significant effect of EP4 knockdown on the growth of Du145 cells may suggest less sensitivity of these cells to EP4 receptor–mediated cell growth regulation compared with that of the primary Ca-HPV10 line. Although the growth of PZ-HPV7 cells was not affected by EP2 knockdown, both CA-HPV10 and Du145 cell growth was significantly reduced after EP2 knockdown. Although EP2 and EP4 act through similar signaling pathways via adenylate cyclase (AC) activation, the amino acid identity between these EP2 and EP4 is only 31%. EP2 actually shares more homology with the prostacyclin receptor (IP) than with EP4 (40% vs. 31%; ref. 15). These differences may therefore have functional implications: for example, EP4 regulates murine dendritic cell migration, although both EP2 and EP4 are expressed by these cells (37). Our current study showed that PGE2–induced PC3 migration is inhibited by 2 different EP4 receptor antagonists, suggesting that human prostate cancer migration may also be mediated by EP4. Therefore, it is plausible that although EP2 and EP4 may share similar signaling pathways, these receptors may exert different downstream effects in prostate cancer as we observed.

The downstream effects of EP2 and EP4 signaling can be mediated by MMP2 and MMP9 and the Runx family transcription factors (38). Previous studies have shown that MMP2 and MMP9 are overexpressed in prostate cancer (25, 38), and the effects of PGE2 on MMP9 expression are mediated through the activation of Runx 2 transcription factors that are abnormally expressed in metastatic prostate cancer cells (38). Recent studies also reported knockdown of
the Runx2 gene results in inhibition of prostate cancer cell migration and growth in vivo (26). A previous study also suggested that Runx 3 transcription factor might be silenced by hypermethylation in prostate cancer (39). We did not observe consistent changes in the expression of MMP2 and Runx 2 after EP2 or EP4 knockdown. On the other hand, we did observe a significant reduction of MMP9 mRNA in PZ-HPV and CA-HPV10 cells after either EP2 or EP4 knockdown. The effects of EP2 and EP4 KD on Du145 MMP and Runx2 differed from those in the primary prostate cancer HPV10 line in that both EP2 and EP4 KD resulted in reduced expression of Runx2, EP2 KD in reduced MMP9 expression, and EP4 KD increased MMP2 and MMP9 expression. Whether the differences observed in the Du145 line may be attributable to diminished EP4 regulatory capacity after acquisition of androgen independence (35) remains to be determined.

Distinct effects of EP4 siRNA knockdown on the growth of primary CA-HPV10 cells and metastatic Du145 suggest variability of EP4-mediated effects occur during disease progression or metastasis. ERK phosphorylation has been shown to mediate effects of EP4 signaling on various cellular functions including cell growth (14, 27, 28). The EP2 antagonist AH6809 reduced PGE2-induced ERK phosphorylation of PZ-HPV7, CA-HPV10, and Du145 cells; however, the effects of the EP4 antagonist AH23848, although inhibitory, were less marked than that of the EP2 antagonist, especially in the normal prostate cell line PZ-HPV7 and the androgen-independent line Du145. This is consistent with the idea that primary prostate cancer cells (represented by the Ca-HPV10 line) overexpressing EP4 may be more susceptible to EP4-mediated inhibition of deregulated growth than metastatic or androgen-independent prostate cancer (Du145). The marginal effects of the EP4 antagonist AH23848 on the PGE2-induced ERK phosphorylation in Du145 cells also provide a possible explanation for the lack of significant inhibition of the growth of this line after EP4 KD.

Of the 4 EPR subtypes, EP3 is least well-understood and its role in tumorigenesis unclear with divergent reports of both tumor promoting and suppressing properties (29, 30). Several studies have suggested a tumor promoter role for EP3: It was reported that PGE2-induced HCA-7 human colon cancer cell migration may be mediated by EP3-induced VEGFR-1 expression (40). Others reported that tumor-associated angiogenesis is reduced in EP3 knockout (KO) mice (41). EP3 KO mice exhibit reduced growth of carcinogen-induced skin (42) of sarcoma 180 and metastasis of murine Lewis lung cancer cells compared with wild-type mice (43). EP3 agonists have been shown to stimulate the proliferation of hepatocytes (44) and endometrial stromal cells (45). In contrast, other studies have found EP3 to be antineoplastic role; EP3 expression is reduced in colon cancer in mice and humans compared with normal mucosa, and EP3 agonists caused decreased colon cancer cell viability, and AOK-induced colon cancer was enhanced in EP3 KO mice (46), and overexpression of murine EP3 variants impairs human colon cancer (HCT116) and human embryonic kidney cell (HEK293) growth (47). EP3 also promotes S-phase arrest of 3T6 fibroblasts (48). EP3 agonists also induced atypical cell death in neutrophils (49). The apparent discrepant activities of EP3 signaling may be due, in part, to the existence of a variety of EP3 isoforms and signaling pathways. For example, it is known that multiple EP3 receptor isoforms are generated by alternative splicing of the C-terminal tail (50). Functional differences among these splice variants have been reported. Macias-Perez and colleagues (49) showed that overexpression of murine EP3 α, β, and γ receptor variants in HCT116 cells resulted in decreased in vitro growth and in vivo tumorigenesis in nude mice and was associated with EP3 coupling to Gi protein. These emerging data suggest a tumor-suppressive role for EP3, at least, in colon cancer. However, to our knowledge, no studies have examined the role of EP3 in prostate cancer. There are at least 8 human EP3II isoforms which have been identified and cloned (24, 30). These EP3 isoforms exert their function through membrane Gs (EP3I, EP3III, and EPIV) or Gi (EP3II) proteins, or Ca++/phospholipase signaling cascades (15, 24, 29, 30). We identified EP3II as the major (if not the only) EP3 isoform expressed in the normal and prostate cancer cell lines studied herein. We hypothesized that reduced EP3 expression may be consistent with tumor-suppressive properties of EP3 signaling in prostate cancer. Impaired cell growth after EP3 overexpression in human PC3 cells and exacerbation of this effect by an EP3 agonist supports this idea. We have also observed that murine EP3II OE results in reduced RM1 prostate cancer cell growth in vitro and WT RM1 cell colony formation was promoted by an EP3 antagonist (unpublished data). We are undertaking further experiments to further characterize EP3II overexpression in human prostate cancer in our laboratory.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.F.S. Huang, M.L. Jordan
Development of methodology: H.F.S. Huang, P. Shu, T.F. Murphy, M.L. Jordan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.F.S. Huang, S. Aisner, V. Fitzhugh, M.L. Jordan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.F.S. Huang, P. Shu, T.F. Murphy, M.L. Jordan
Writing, review, and/or revision of the manuscript: H.F.S. Huang, P. Shu, T.F. Murphy, M.L. Jordan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.F.S. Huang, P. Shu, T.F. Murphy, V. Fitzhugh, M.L. Jordan
Study supervision: H.F.S. Huang, M.L. Jordan

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# Significance of Divergent Expression of Prostaglandin EP4 and EP3 Receptors in Human Prostate Cancer

Hosea F. S. Huang, Ping Shu, Thomas F. Murphy, et al.


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