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Tumorigenesis and Neoplastic Progression

PAGE4 Positivity Is Associated with Attenuated AR Signaling and Predicts Patient Survival in Hormone-Naive Prostate Cancer

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Aberrant activation of the androgen receptor (AR) plays a key role during prostate cancer (PCa) development and progression to castration-resistant prostate cancer (CR-PCa) after androgen deprivation therapy, the mainstay systemic treatment for PCa. New strategies to abrogate AR activity and biomarkers that predict aggressive tumor behavior are essential for improved therapeutic intervention. PCa tissue microarrays herein reveal that prostate-associated gene 4 (PAGE4), an X-linked cancer/testis antigen, is highly up-regulated in the epithelium of preneoplastic lesions compared with benign epithelium, but subsequently decreases with tumor progression. We show that AR signaling is attenuated in PAGE4-expressing cells both *in vitro* and *in vivo*, most likely via impaired androgen-induced AR nuclear translocation and subsequently reduced AR protein stabilization and phosphorylation at serines 81 and 213. Consistently, epithelial PAGE4 protein levels inversely correlated with AR activation status in hormone-naive and CR-PCa clinical specimens. Moreover, PAGE4 impaired the development of CR-PCa xenografts, and strong PAGE4 immunoreactivity independently predicted favorable patient survival in hormone-naive PCa. Collectively, these data suggest that dysregulation of epithelial PAGE4 modulates AR signaling, thereby promoting progression to advanced lethal PCa and highlight the potential value of PAGE4 as a prognostic and therapeutic target. ***(Am J Pathol 2012, 181:1443–1454; http://dx.doi.org/10.1016/j.ajpath.2012.06.040)***

Androgens play a key role in regulating prostate cellular homeostasis[1] via the androgen receptor (AR), a ligand-inducible transcription factor whose dysregulation is associated with the development/progression of prostate cancer (PCa), the second leading cause of male cancer death in Western societies.[2] On initial diagnosis, 80% to 90% of PCa are androgen-dependent.[3] Approximately 90% of patients respond to current first-line androgen deprivation therapy, however, many patients experience disease progression and succumb to castration-resistant PCa (CR-PCa) within 3 years.[4,5] Despite low circulating androgen levels, AR signaling frequently is reactivated in CR-PCa and plays a key role in disease progression.[6,7] AR reactivation may be attributed to AR hypersensitivity, promiscuous AR activation, local tumoral androgen production, and altered recruitment/expression of AR coregulators.[8] Such *cis*-acting AR regulators confer promoter specificity of androgen signaling by modulating AR maturation and posttranslational modification, AR-protein interactions, histone modification, or local chromatin remodeling.[9–16] Thus, the AR and regulators thereof remain therapeutic targets for CR-PCa.

We previously reported up-regulation of prostate-associated gene 4 (*PAGE4*) on exposure of primary prostatic epithelial and stromal cells to transforming growth factor β.[17,18] *PAGE4* is a related member of the germ cell-associated gene cancer/testis antigens (CTAs),[19] an X-linked gene family with restricted expression to germ cells in normal tissues yet present in tumor cells of diverse histologic origin.[20] In addition to testis, the six-membered *PAGE* subfamily is expressed highly in placenta and at lower levels in normal prostate and uterus.[19]

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Because of their high immunogenicity and restricted spatial expression, CTAs are considered potential tumor-specific diagnostic and therapeutic targets.[21] Although the biological function of most CTAs including PAGE4 is unknown, recent studies have shown that PAGE4 and PAGE5 are highly intrinsically disordered proteins, a characteristic that favors low affinity but highly specific interactions.[22–24] Consequently, intrinsic disorder is prevalent among regulatory and signaling proteins, including nuclear hormone receptors such as AR.[25] This study aimed to investigate the function of PAGE4 and evaluate its potential as a diagnostic and/or therapeutic target in PCa.

Materials and Methods

Reagents and Cell Culture

Reagents were from Sigma Aldrich (St. Louis, MO) unless otherwise specified. α-Tubulin was from Santa Cruz Biotechnology (Santa Cruz, CA), AR (PG-21) and pSer81-AR were from Millipore (Billerica, MA), pSer213-AR was from Imgenex Corp. (San Diego, CA), E-tag was from Bethyl Laboratories (Montgomery, TX), horseradish-peroxidase–conjugated secondary antibodies were from Promega (Madison, WI), and Alexa Fluor–labeled secondary antibodies were from Invitrogen (Carlsbad, CA). The proteasome inhibitor MG132 was from Calbiochem (La Jolla, CA), and bicalutamide was from Molekula (Munich, Germany). Cell lines were from ATCC (LGC Standards, Wesel, Germany). For steroid hormone stimulation, cells were incubated for 16 hours in media supplemented with charcoal-treated bovine calf serum (Hyclone Laboratories, Logan, UT) before addition of dihydrotestosterone (DHT), progesterone, dexamethasone, or vehicle equivalent at the indicated concentration for the duration stated. For analysis of pSer213-AR, cells were incubated for 30 hours in media supplemented with charcoal-treated bovine calf serum before addition of DHT or insulin-like growth factor-1 (IGF-1), either alone or in combination for 4 hours.

Plasmids and Generation of PAGE4-Overexpressing LNCaP Sublines

PAGE4:Etag was described previously,[17] other plasmids were generous gifts: wild-type AR expression vector (pARO) and androgen-responsive luciferase reporter (pARE2tataLuc; Guido Jenster, Rotterdam, The Netherlands). Glucocorticoid receptor (GR) alpha expression vector α expression vector (pGRα), glucocorticoid-responsive luciferase reporter (pGREtkLuc), and pMMTV-Luc (Stoney Simons Jr, Bethesda, MD). LNCaP and PC3 cells were stably transfected with Sleeping Beauty transposase (SB11) and shuttle transposon (pT2) containing the PAGE4 open reading frame downstream of the cytomegalovirus promoter. Polyclonal lines resistant to 1 μg/mL neomycin were selected.

Table 1. Primer Sequences

Gene symbol	Gene ID	Primer sequence*
AR	367	F: 5′-CCTGGCTTCCGCAACTTACAC-3′
		R: 5′-GGACCTGTGCATGCGGTACTCA-3′
HMBS	3145	F: 5′-CCAGGACATCTTGGATCTGG-3′
		R: 5′-ATGGTAGCCTGCATGGTCTC-3′
IGFBP3	3486	F: 5′-CAAGCGGGAGACAGAATATG-3′
		R: 5′-TTATCCACACACCAGCAGAA-3′
KLK3 (PSA)	354	F: 5′-TTGACCCCAAAGAAACTTCA-3′
		R: 5′-TGACGTGATACCTTGAAGCA-3′
NKX3-1	4824	F: 5′-GAGACGCTGGCAGAGACC-3′
		R: 5′-ATCACCTGAGTGTGGGAGAA-3′
PAGE4	9506	F: 5′-AATGGATCTGGAAAAGACTCG-3′
		R: 5′-GTGACATCAGCCATGTGTGTA-3′
RGS2	5997	F: 5′-CCCAAAAGCTGTCCTCAAAA-3′
		R: 5′-TTCTGGGCAGTTGTAAAGCA-3′
TAT	6898	F: 5′-CAGGGAGCTCTGAAAAGCAT-3′
		R: 5′-CAACGCCCCATAACAGAGAT-3′
TSC22D3 (GILZ)	1831	F: 5′-TGGTGGCCATAGACAACAAG-3′
		R: 5′-CAGGGTCTTCAACAGGGTGT-3′
TMPRSS2	7113	F: 5′-GGCTTTGAACTCAGGGTCAC-3′
		R: 5′-GGTAGTACTGAGCCGGATGC-3′

*Primer sequences are given 5′ to 3′, annealing temperature for all primers in quantitative PCR is 56°C, and all primers span at least one intron.
F, forward; R, reverse.

PAGE4 Antibody Generation

Synthetic peptides corresponding to residues 45 to 60 of the PAGE4 amino acid sequence (NP_008934) were coupled to a lysine core generating multiple antigenic peptides (AltaBioscience, Birmingham, UK). BALB/C mice were immunized with 100 μg peptide diluted with an equal volume of Freund's adjuvant. Once optimal titers were obtained, mice were surrendered for monoclonal antibody isolation.[26]

Real-Time Quantitative PCR

RNA isolation, cDNA synthesis, and quantitative PCR were performed as described.[27] Primer sequences are shown in Table 1. For quantitative PCR, cDNA concentrations were normalized to the internal standard hydroxymethylbilane synthase, a moderate copy number housekeeping gene not regulated under the experimental conditions used. Fold change in gene expression was determined using the mathematical model ratio $2^{-\Delta\Delta CT}$.[28]

Luciferase Reporter Assays, Transfection, and siRNA-Mediated Knockdown

Cells seeded in triplicate in 24-well plates in media supplemented with charcoal-treated bovine calf serum were transfected with firefly luciferase reporter vector (225 ng/well) and where indicated with 75 ng/well pARO/GR α expression vector and 400 ng/well PAGE4:Etag using Lipofectamine 2000 (Invitrogen). pGL4.73, a SV40-driven renilla luciferase reporter vector (Promega), served as transfection control. Equal DNA concentrations were maintained using appropriate empty vectors. Six hours after transfection, media were exchanged supplemented with the indicated steroid hormone or vehicle equivalent

for 24 hours. Luciferase activity was measured using the Dual-Glo luciferase assay system (Promega) on a Chameleon luminometer (HVD Life Sciences, Vienna, Austria) and values were normalized to renilla luciferase activity and total protein content determined by Bradford assay (Bio-Rad). For knockdown experiments, JEG3 cells were transfected with scrambled or PAGE4 small-interfering RNA (siRNA) duplexes (Invitrogen) for 96 hours before processing for quantitative PCR or transfection with pGREtkLuc (for luciferase assays) as described earlier.

Subcellular Fractionation, Western Blotting, and PSA Determination

Nuclear and cytosolic fractions were prepared using the NE-PER extraction kit according to the manufacturer's instructions (Thermo Fisher Scientific, Vienna, Austria). Total cell lysates were prepared as described[27] and normalized against total protein content via Bradford assay (Bio-Rad Laboratories, Hercules, CA) before SDS-PAGE, Western blotting, and densitometric quantification as described.[27] As specificity control for pSer81- and pSer213-AR immunoblotting, lysates were incubated with or without 400 U lambda phosphatase (Cell Signaling Technology, Danvers, MA) as indicated at 30°C for 30 minutes before SDS-PAGE. Densitometric data were normalized against β-actin or total AR (for pSer81-AR and pSer213-AR) as indicated. Prostate-specific antigen (PSA) concentration in conditioned media was determined using the ADVIA Centaur clinical diagnostic PSA test (Siemens Health Care Diagnostics, Inc., Tarrytown, NY) and normalized to total protein concentration of the corresponding cell lysates.

Cell Proliferation

Cell proliferation was determined by WST1 assay (Roche Applied Science, Indianapolis, IN) as described.[29]

Xenografts

Six-week-old male athymic mice (Charles River Laboratories, Wilmington, MA) were injected subcutaneously into the hind flank with 2×10^6 vector or PAGE4:LNCaP (subline #8) cells resuspended in 50 μL sterile PBS and an equal volume of Matrigel (BD Biosciences). At the time of inoculation, mice were either castrated or sham-castrated (each subgroup, $n = 17$). Harvested tumors were halved, with one half snap-frozen in liquid nitrogen for RNA isolation and the second half placed into 4% formaldehyde in PBS for immunohistochemistry (IHC). Experiments were approved by the Austrian Animal Experimentation Ethics Committee.

Immunofluorescence, IHC, TMA, and Microscopy

Immunofluorescence was performed as described.[29] Use of the clinical samples for tissue microarray (TMA) construction was approved by the Ethical Committee of the University of Basel (Switzerland). The PCa progression TMA and scoring system have been described.[30,31] Briefly, the arrays were reviewed by an experienced pathologist (L.B.) and the score was calculated by multiplying the given intensity (between 0 and 3) by the percentage of cells within this intensity. Thus, a score between 0 and 300 was possible. The advanced PCa TMA comprises 305 surgical specimens from palliative transurethral resections of the prostate (TURP) from patients with local advanced obstructive PCa (see Supplemental Table S1 at *http://ajp.amjpathol.org*) that typically infiltrate into the transition zone. Thus, a distinction between peripheral or transition zone PCa no longer applies, enabling direct comparison of epithelial PAGE4 levels in TURP resections from patients with benign prostatic hyperplasia (BPH) (without PCa) versus patients with PCa. Each specimen is represented by three to four cores. For PAGE4 analysis, the average value of evaluable spots per specimen was calculated (denoted as mean PAGE4). IHC was performed as described.[27] Where indicated, PAGE4 antibodies were pre-incubated overnight at 4°C in 1% bovine serum albumin/PBS containing 40 μg/mL blocking peptide (residues 45 to 60) or nonblocking peptide corresponding to residues 77 to 92 of the PAGE4 aa sequence. Sections were counterstained with hematoxylin. Peroxidase-stained TMAs were visualized using a Zeiss AXIO Imager.A1, using Axio Vision software version 4.6 (Carl Zeiss AG, Feldbach, Switzerland). Peroxidase-stained xenograft tumor sections were visualized using an inverted Nikon Eclipse TE300, using Nikon Plan Fluor ELWD objectives and images acquired using NIS Elements Basic Research software (Nikon Instruments, Inc., Amsterdam, The Netherlands). Fluorescent microscopy was performed using a confocal laser microscope with constant laser intensity settings (Zeiss Axiophot, μ-Radiance scanning system; Carl Zeiss Laser Optics/Laser Sharp Software from Bio-Rad).

Statistical Analysis

Numeric data are presented as mean $\pm$ SEM from at least three independent experiments. Statistical differences between treatments were calculated using a paired Student's *t*-test. TMAs were analyzed using the statistical test indicated using JMP 8.0 software (SAS Corporation, Cary, NY). For patient survival analyses, the third quartile of mean epithelial PAGE4 staining intensity was set as the cut-off with negative/low PAGE4 levels defined as less than 130 and high PAGE4 levels defined as 130 or higher. Time point zero for survival statistics was TURP for local advanced obstructive PCa. *P* values were calculated using log-rank tests for univariate analysis and the Cox proportional hazard model for multivariate analysis.

Results

Dysregulation of Epithelial PAGE4 during Prostate Cancer Progression

Consistent with previous reports,[32] IHC of benign prostate tissue sections using an in-house anti-PAGE4 mono-

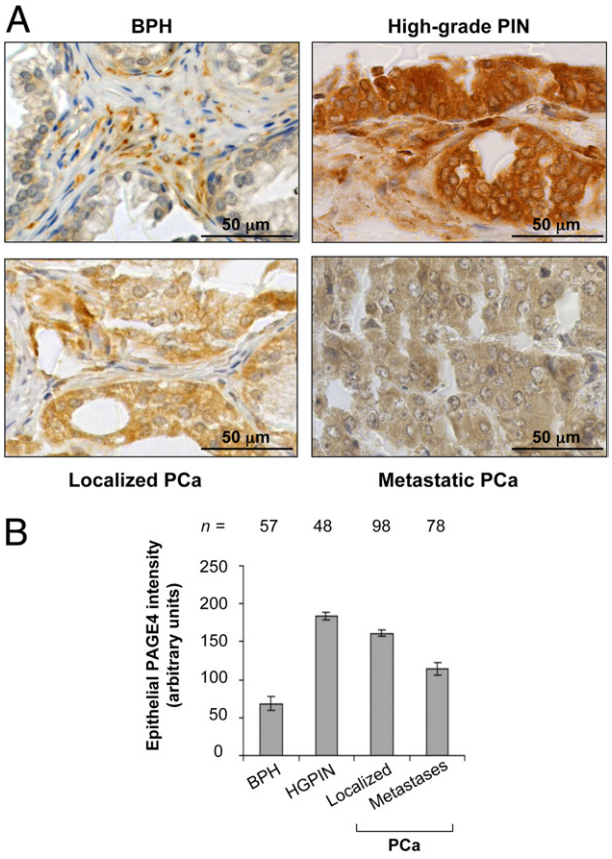

Figure 1. Dysregulation of epithelial PAGE4 during prostate cancer development and progression. **A** and **B:** IHC analysis of PAGE4 on the PCa progression TMA. Representative images are shown, including a pleural metastasis (**A**). **B:** Mean epithelial PAGE4 staining intensity (±SEM) was quantified as described in *Materials and Methods* [high-grade PIN (HGPIN)]. All pairwise comparisons were statistically significant ($P < 0.001$) by Wilcoxon rank sum test.

clonal antibody (see Supplemental Figures S1 and S2 at *http://ajp.amjpathol.org*) revealed intense nucleocytoplasmic PAGE4 staining of smooth muscle cells and to a lesser extent in the stromal cells of the prostate. Either none or only weak PAGE4 was detectable in endothelial cells and luminal epithelial cells of benign glands (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). PAGE4 was not detectable in basal epithelial cells. On a PCa progression TMA, however, high levels of epithelial PAGE4 were observed in preneoplastic high-grade prostatic intraepithelial neoplasia (PIN) lesions with progressively lower levels in localized PCa and metastases (Figure 1). In contrast to the nucleocytoplasmic localization of PAGE4 in benign tissue (see Supplemental Figure S1 at *http://ajp.amjpathol.org*), PAGE4 was confined mostly to the cytoplasm in high-grade PIN and PCa (Figure 1A).

Attenuation of AR and GR Activity in PAGE4-Positive Cells

Because PAGE4 spatially is restricted to steroidogenic tissues and aberrant AR signaling is implicated in PCa development/progression, we investigated whether PAGE4 modulates AR activity. In the presence but not absence of wild-type AR, basal and ligand-induced androgen-responsive luciferase activity was reduced in PAGE4-expressing COS7 cells (see Supplemental Figure S3A at *http://ajp.amjpathol.org*). Similar attenuated activity was observed for the structurally related GR from a glucocorticoid-responsive reporter (see Supplemental Figure S3B at *http://ajp.amjpathol.org*), but not for the endogenous progesterone receptor in LNCaP cells (see Supplemental Figure S3C at *http://ajp.amjpathol.org*).

In contrast to a recent study,[22] we and others observed very low endogenous *PAGE4* mRNA levels in prostate-derived primary cells and PCa cell lines with PAGE4 undetectable at the protein level (not shown and G. Jenster, unpublished data). Thus, it was not possible to perform siRNA-mediated PAGE4 silencing in prostatic cells. However, consistent with high expression of *PAGE* genes in placenta,[19] endogenous PAGE4 was readily detectable in JEG3 (GR^+) choriocarcinoma cells (Figure 2A), localizing to the nucleocytoplasm (Figure 2B) and consistent with the earlier-described findings (Figure 1; see also Supplemental Figure S1 at *http://ajp.amjpathol.org*). PAGE4-specific siRNA decreased endogenous PAGE4 mRNA by 6.3 ± 0.8-fold, markedly reduced PAGE4 protein levels, and significantly potentiated basal and ligand-induced activity of a glucocorticoid-responsive luciferase reporter relative to scrambled control (Figure 2, C–E). Moreover, PAGE4 silencing significantly potentiated basal expression of the primary glucocorticoid-responsive genes *GILZ*, *TAT*, and *RGS2* with the expression of *GILZ* and *TAT* potentiated to levels comparable with those in dexamethasone-treated scrambled control cells (Figure 2F). In the presence of dexamethasone, PAGE4 knockdown significantly potentiated dexamethasone-mediated induction of *RGS2* and *GILZ* (Figure 2F). Similar results were obtained for a second siRNA targeting a distinct region of the *PAGE4* mRNA transcript (not shown).

Attenuation of Endogenous AR in PAGE4-Expressing PCa Cells

Subsequent experiments investigated the functional significance of increased epithelial PAGE4 in PCa with respect to AR activity using polyclonal LNCaP (AR^+ GR^-) sublines overexpressing different levels of native PAGE4 (PAGE4:LNCaP) relative to empty vector control (vector:LNCaP; Figure 3A). Similar to the earlier description (see Supplemental Figure S3 at *http://ajp.amjpathol.org*), basal and ligand-induced transactivation of endogenous AR was dose-dependently attenuated in PAGE4:LNCaPs (Figure 3B), with relative fold induction by DHT ($P = 0.0006$) also significantly reduced. Although *AR* mRNA levels were not altered, expression of the primary androgen-responsive genes *PSA*, *CAV1*, and *IGFBP3* was reduced significantly in PAGE4:LNCaPs but not in parallel-generated LNCaP sublines overexpressing *Dickkopf homolog 3*, a member of the Dickkopf family of wingless-type MMTV integration site (Wnt) antagonists (see Supplemental Figure S4, A and B, at *http://ajp.amjpathol.org*), indicating that these effects are PAGE4-specific and not an artifact of the overexpression system. Interestingly,

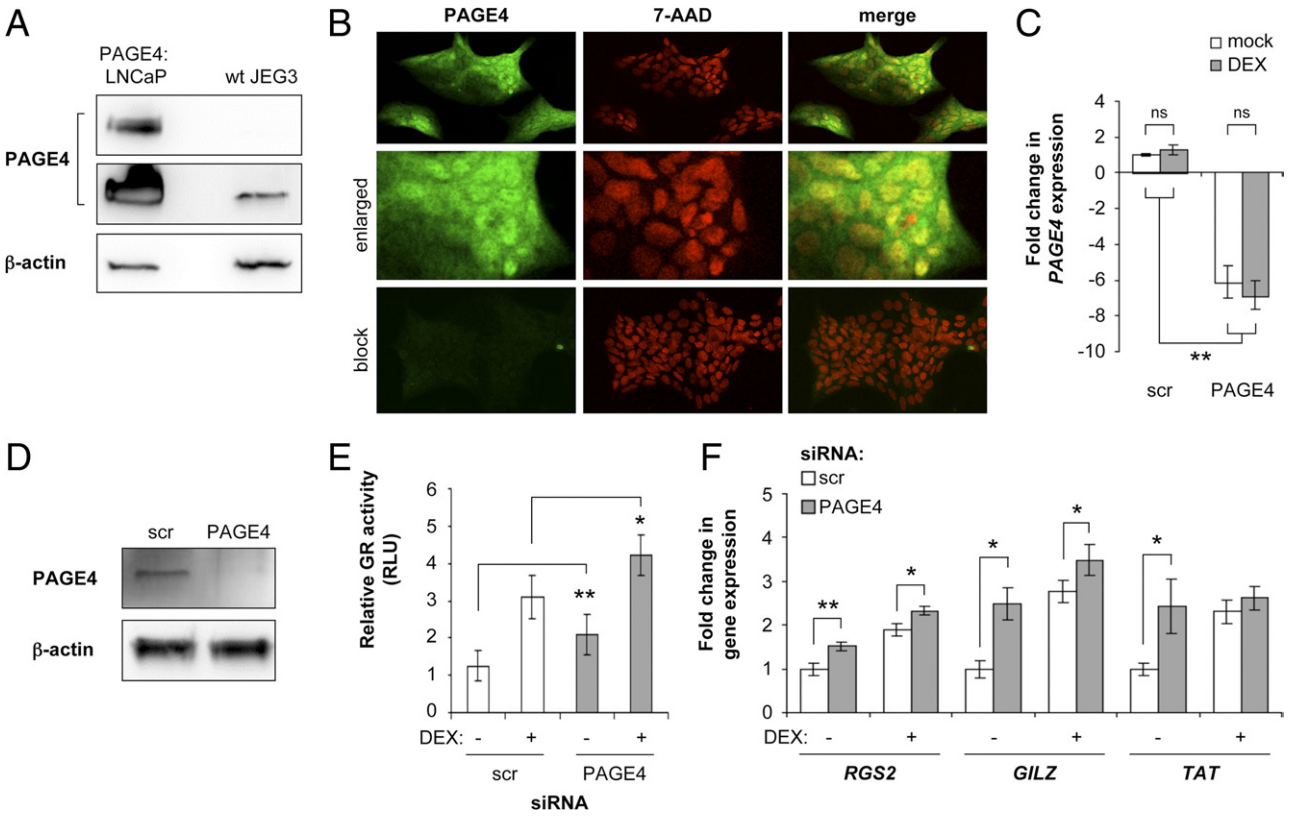

Figure 2. Potentiation of endogenous GR activity on PAGE4 knockdown. **A:** Immunoblotting of endogenous PAGE4 in wild-type (wt) JEG3 cell lysates. PAGE4-overexpressing LNCaP subline #8 served as positive control (**top panel**, short exposure; **middle panel**, longer exposure). **B:** Immunofluorescence of endogenous PAGE4 in JEG3 cells using anti-PAGE4 monoclonal antibody (green) pre-incubated with or without blocking peptide. Nuclei were counterstained with 7-aminoactinomycin D (7-AAD, red). Merged images are shown (**right**). Quantitative PCR (**C**) or Western blotting (**D**) of endogenous PAGE4 in JEG3 cells transiently transfected with scrambled (scr) or PAGE4 siRNA for 72 hours before stimulation with 10 nmol/L dexamethasone (DEX) or vehicle equivalent (mock) for 24 hours. **E:** Luciferase activity from the glucocorticoid-responsive reporter pGREtkLuc in JEG-3 cells treated as in **C** and stimulated with 10 nmol/L DEX (+) or vehicle equivalent (−) for 24 hours. Values represent mean relative light units (RLU) ± SEM of triplicate wells ($n = 4$). **F:** Quantitative PCR of glucocorticoid-responsive genes in JEG3 cells treated as in **C**. **A**, **B**, and **D:** Images are representative of three independent experiments. **C** and **F:** Values represent mean fold-change in expression ± SEM relative to mock-treated scrambled control from three independent experiments. **C**, **E**, and **F:** Statistical significance is indicated: NS, not significant where $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

PAGE4 expression did not influence that of two other primary androgen-responsive genes (*NKX3-1*, *TMPRSS2*; see Supplemental Figure S4A at *http://ajp.amjpathol.org*), suggesting that PAGE4 may exert its effects in a promoter-specific manner. *CAV1* and *PSA* expression was investigated further in the presence or absence of 10 nmol/L DHT (Figure 3C). Relative to vector control, basal expression and relative fold induction of *CAV1* and *PSA* by DHT were attenuated significantly in a PAGE4 dose-dependent manner (Figure 3C). Similar findings were observed for PSA secretion (Figure 3C).

To investigate whether the androgenic proliferative response also was attenuated, vector or PAGE4:LNCaPs were incubated in the presence or absence of proliferation-inducing concentrations of DHT (0.1 nmol/L). Relative to vector control, basal proliferation of PAGE4:LNCaPs was reduced marginally in sublines 7 and 8 whereas DHT induction of proliferation was attenuated across all PAGE4:LNCaP sublines in a dose-dependent manner (Figure 3D). These effects on proliferation appear to be AR-dependent because stable overexpression of PAGE4 in AR^{-} PC3 PCa cells had no significant effect on cell proliferation (see Supplemental Figure S4C at *http://ajp.amjpathol.org*). Collectively, these data indicate that AR signaling is attenuated in both a ligand-dependent and ligand-independent manner in PAGE4-expressing PCa cells.

Potentiated AR Inhibition by the Clinical AR Antagonist Bicalutamide in PAGE4-Expressing PCa Cells

We next compared the level of AR inhibition in PAGE4:LNCaPs with that achieved by the nonsteroidal anti-androgen bicalutamide by treating vector and PAGE4:LNCaPs with DHT in the presence or absence of bicalutamide. DHT-mediated induction of *PSA* served as a molecular readout for AR activity (Figure 3E). In PAGE4:LNCaPs treated with DHT alone, *PSA* mRNA levels were either comparable with (sublines 7 and 9), or lower than (subline 8), bicalutamide and DHT combined treated vector:LNCaPs (Figure 3E). In bicalutamide and DHT combined treated PAGE4:LNCaP sublines 7 and 9, *PSA* mRNA levels were reduced significantly further. Bicalutamide had no synergistic effect on *PSA* expression in PAGE4:LNCaP#8 (Figure 3E), suggesting that under these conditions AR inhibition in this subline (which expresses the

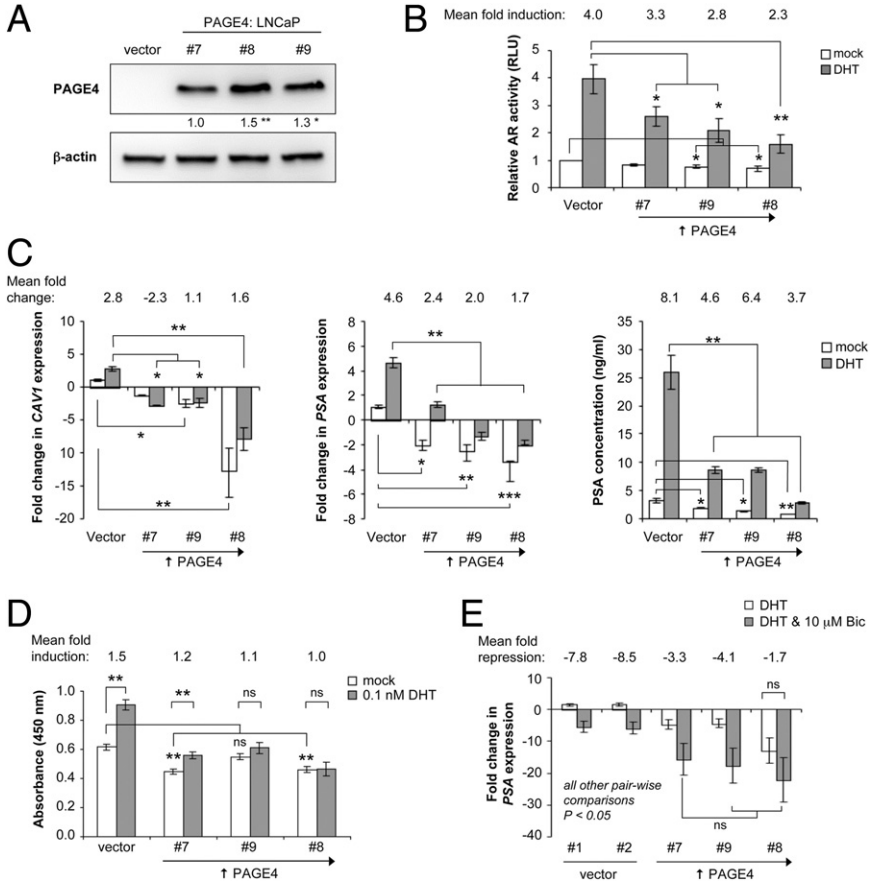

Figure 3. Attenuation of endogenous AR activity in PAGE4-expressing PCa cells. **A:** PAGE4 immunoblotting in polyclonal LNCaP sublines stably overexpressing PAGE4 (#7 to 9) or vector control. Values denote mean PAGE4 densitometric intensity (±SEM) relative to subline 7 normalized against β-actin ($n = 3$). **B:** Androgen-responsive luciferase reporter activity (pARE2tataLuc) in vector and PAGE4:LNCaPs stimulated with 10 nmol/L DHT or ethanol equivalent (mock) for 24 hours. Values represent mean relative light units (RLU) ± SEM relative to mock-treated vector:LNCaP from triplicate wells normalized against renilla luciferase activity and total protein content ($n = 3$). **C:** Vector and PAGE4:LNCaPs stimulated with vehicle (mock) or 10 nmol/L DHT for 24 hours (**left** and **center**) or 72 hours (**right**) before quantitative PCR of *CAV1* and *PSA*. Enzyme-linked immunosorbent assay quantification of PSA in 72-hour conditioned medium (**right**). Data represent mean fold-change ± SEM relative to mock-treated vector:LNCaPs ($n = 4$). **D:** Proliferation of vector and PAGE4:LNCaPs treated with 0.1 nmol/L DHT or vehicle equivalent (mock) for 24 hours. Mean absorbance ± SEM of triplicate wells is shown ($n = 3$). **E:** Quantitative PCR of *PSA* in vector and PAGE4:LNCaPs preblocked for 1 hour with 10 μmol/L bicalutamide (Bic) or vehicle equivalent (mock) before stimulation with 10 nmol/L DHT in the presence or absence of Bic for 24 hours. Bars represent mean fold-change in *PSA* expression (±SEM) relative to DHT-treated vector:LNCaP#1 in the absence of Bic ($n = 3$). NS, not significant where $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

highest levels of PAGE4) had reached maximal levels with respect to AR-dependent regulation of *PSA*.

Ligand-Mediated AR Protein Stabilization Is Attenuated in PAGE4-Expressing PCa Cells

We next sought to investigate the mechanism by which PAGE4 attenuates AR activity. Subsequent experiments used PAGE4:LNCaP#8; however, similar findings were observed for sublines 7 and 9 (not shown). Although PAGE4 did not alter *AR* mRNA levels (see Supplemental Figure S4 at *http://ajp.amjpathol.org*) or basal AR protein levels, ligand-mediated AR stabilization was attenuated in PAGE4:LNCaPs (see Supplemental Figure S5, A and B, at *http://ajp.amjpathol.org*). Consistent with previous reports,[33] the proteasome inhibitor MG132 significantly increased AR protein levels both in the presence and absence of DHT in vector:LNCaPs. In PAGE4:LNCaPs, however, MG132 did not rescue suppression of ligand-induced AR protein stabilization, indicating a proteasome-independent mechanism. DHT-mediated AR stabilization was investigated further in the presence of cycloheximide (10 μg/mL) to inhibit *de novo* protein synthesis (see Supplemental Figure S5, C and D, at *http://ajp.amjpathol.org*). Compared with vector:LNCaP cells, AR levels decreased at a faster rate in PAGE4:LNCaPs (relative $t_{1/2}$, 2.6 ± 0.3-fold; $P < 0.05$), with significantly lower AR levels detected at 12 hours (see Supplemental Figure S5, C and D, at *http://ajp.amjpathol.org*).

Attenuation of AR Phosphorylation in PAGE4-Expressing PCa Cells

AR phosphorylation at serine (Ser) 81 and Ser213 is implicated in regulating AR protein stabilization.[34–36] In addition, ligand-mediated AR phosphorylation at Ser81 increases AR chromatin binding and transcriptional activity in a promoter-specific manner.[34,37–39] Consistent with the well-documented cross-talk between AR and IGF1 signaling pathways,[40–42] AR phosphorylation at Ser213 after IGF1-induced AKT activation also is implicated in regulating hormone-binding, AR transcriptional activity, and is associated with decreased survival of CR-PCa patients.[35,36,43] In accordance with previous observations,[34] DHT increased pAR-Ser81 levels 1.8 ± 0.1-fold in vector:LNCaPs (Figure 4, A and B). Although basal levels of pAR-Ser81 were unaltered in PAGE4:LNCaPs relative to vector control, DHT-mediated induction of pAR-Ser81 was attenuated (Figure 4, A and B). Similarly, the synergistic induction of pAR-Ser213 after combined treatment of vector:LNCaP cells with IGF1 and DHT (2.1 ± 0.3-fold) was attenuated significantly in PAGE4-expressing cells (0.9 ± 0.2-fold; $P = 0.027$ relative to combined treated vector:LNCaPs; Figure 4, C and D).

Ligand-induced AR-Ser81 phosphorylation is temporally significantly slower than the kinetics of AR nuclear import.[34,44] In addition, pAR-Ser213 appears to be localized predominantly to the nucleus.[45] We thus investigated whether AR nuclear translocation also was atten-

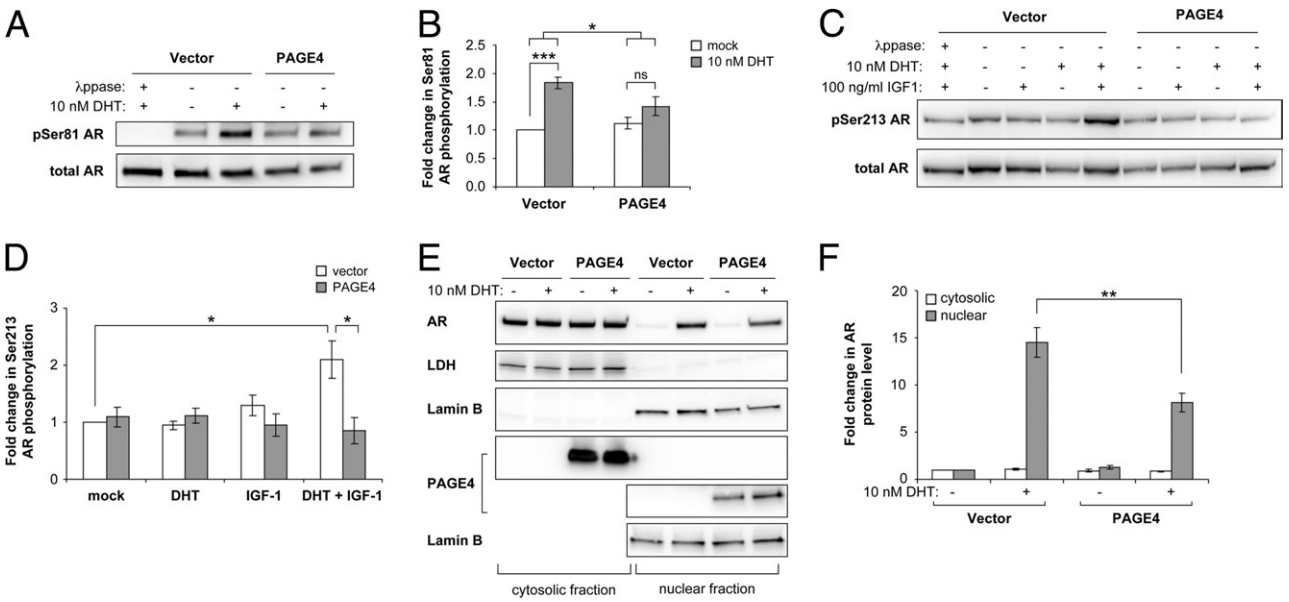

Figure 4. Ligand-mediated AR nuclear translocation and phosphorylation are attenuated in PAGE4-expressing PCa cells. Immunoblotting of AR phosphorylated at serine 81 (**A**) or serine 213 (**C**) in vector and PAGE4:LNCaP#8 stimulated for 24 hours with 10 nmol/L DHT (+) or vehicle equivalent (−). For pSer213-AR, IGF-1 (100 ng/mL) subsequently was added to existing media as indicated for a further 4 hours. Membranes were stripped and reprobed for total AR. Lysates from vector:LNCaPs treated as indicated were incubated with lambda phosphatase before loading as specificity control. **B** and **D:** Densitometric quantification of assays in A and C, respectively, relative to mock-treated vector control normalized against total AR ($n = 4$). **E:** Immunoblotting with the indicated antibodies in nuclear and cytosolic extracts from vector and PAGE4:LNCaP cells stimulated for 1 hour with 10 nmol/L DHT (+) or vehicle equivalent (−). Nuclear fractions were reloaded for longer exposure (5×) enabling detection of nuclear PAGE4 (**lower two panels**). **F:** Mean fold-change in AR densitometric intensity (±SEM) in extracts prepared as in **F** from four independent experiments relative to mock-treated vector control and normalized against lactate dehydrogenase (LDH, cytosolic fractions) or lamin B (nuclear fractions). **A**, **C**, and **E:** Representative images are shown. **B**, **D**, and **F:** NS, not significant where $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$.

uated in PAGE4-expressing cells (Figure 4, E and F). In the absence of DHT, AR was confined predominantly to the cytosol at comparable levels in both PAGE4 and vector:LNCaP cells. However, after 1 hour of treatment with DHT (when total AR protein levels remain comparable between PAGE4 and vector:LNCaPs; see Supplemental Figure S5, C and D, at *http://ajp.amjpathol.org*), nuclear AR levels increased 14.5-fold in vector:LNCaP cells but were increased only 8.1-fold in PAGE4:LNCaPs (−1.8-fold; $P = 0.008$; Figure 4, E and F). Under these conditions, nuclear and cytosolic PAGE4 levels were not altered significantly in the presence of DHT relative to mock-treated cells (Figure 4E and data not shown). Collectively, these data suggest that PAGE4 may impair AR activity by attenuating AR phosphorylation and subsequently transcriptional activity, perhaps as an indirect consequence of reduced AR nuclear translocation.

Attenuation of CR-PCa Xenograft Development of PAGE4-Expressing PCa Cells

To investigate the significance of increased PAGE4 in PCa (Figure 1) on tumor development and progression to CR-PCa, PAGE4 and vector:LNCaP xenografts were established in sham-castrated or castrated animals. In intact mice, there was no significant difference in vector or PAGE4:LNCaP xenografts with respect to tumor incidence or volume (Figure 5, A and B). In agreement with the earlier-described *in vitro* observations, PSA mRNA and protein levels were significantly lower in PAGE4 versus vector:LNCaP xenografts from sham-castrated animals (Figure 5, C and D). Thus, despite attenuation of AR in PAGE4:LNCaPs *in vivo*, tumorigenicity was not altered in androgen-replete animals, probably owing to residual AR activity because serum PSA still was detectable in mice harboring PAGE4:LNCaP xenografts (2.0 ± 0.7 ng/mL compared with 8.2 ± 3.2 ng/mL in vector control mice; $P = 0.04$).

In castrated animals the tumor incidence was reduced markedly by PAGE4 compared with vector control (Figure 5A). Initially, growth of the PAGE4:LNCaP tumors that successfully developed was retarded significantly relative to the corresponding vector:LNCaP tumors (Figure 5B). After day 43, however, PAGE4:LNCaP tumors in castrated mice underwent a marked growth surge, reaching a mean volume comparable with their vector control counterparts by day 57 (Figure 5B). Consistent with progression to CR-PCa, *AR* and *PSA* mRNA and protein levels were increased significantly in vector:LNCaP tumors from castrated hosts relative to intact animals (Figure 5, C and D). Similar up-regulation of AR and PSA also was observed in PAGE4:LNCaP xenografts in castrated mice, in contrast to AR inhibition in intact hosts with PAGE4:LNCaP tumors (Figure 5, C and D), raising the possibility that AR inhibition was subverted in these successful PAGE4:LNCaP tumors. Consistently, although unchanged at the mRNA level (not shown), PAGE4 protein levels were reduced in PAGE4:LNCaP tumors from castrated animals compared with the co-harvested and parallel-stained PAGE4:LNCaP tumors from sham-castrated hosts (Figure 5D).

A

	Maximal tumor incidence (%)*	
	sham	castrated
Vector	83.3	73.5
PAGE4	78.9	44.4

* at day 57 post inoculation ($n \geq 17$ per subgroup)

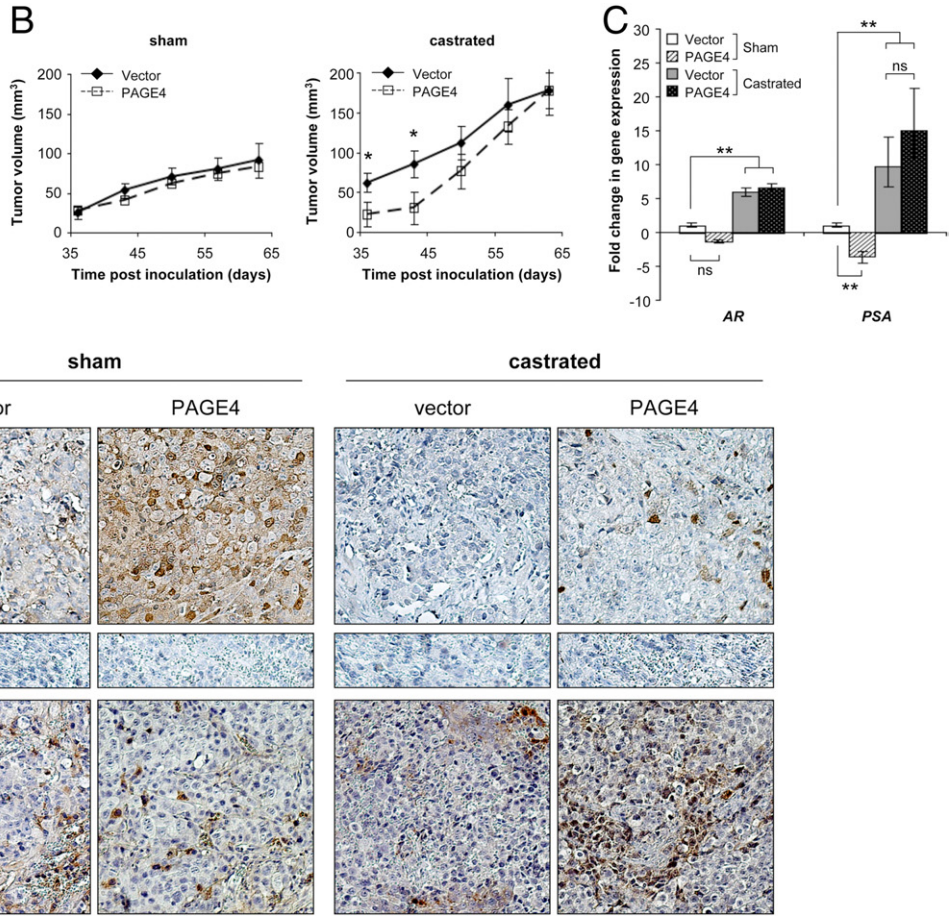

Figure 5. PAGE4 inhibits PCa xenograft development in androgen-depleted hosts. Xenografts from vector and PAGE4:LNCaP#8 were established as described in *Materials and Methods* ($n \geq 17$ mice per subgroup). **A:** Maximal tumor incidence expressed as the percentage of animals inoculated. **B:** Mean tumor volume ± SEM at the indicated interval after inoculation. **C:** *AR* and *PSA* quantitative PCR in vector and PAGE4:LNCaP tumors harvested at day 63 from sham or castrated animals. Mean fold-change in expression ± SEM is shown relative to vector:LNCaP tumors from sham-castrated hosts ($n = 6$). **D:** IHC of the indicated xenografts isolated at day 63 and processed in parallel. Images are representative of four tumors for each subgroup. **B** and **C:** NS, not significant where $P > 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$.

PAGE4 Levels Inversely Correlate with AR Status in PCa Clinical Specimens

By using a second independently stained TMA (advanced PCa TMA), we investigated whether PAGE4 levels were associated with AR inhibition and progression to advanced PCa in clinical specimens. Epithelial PAGE4 levels did not differ between hormone-naive PCa or CR-PCa (see Supplemental Table S2 at *http://ajp.amjpathol.org*) and correlated neither with Gleason pattern nor proliferation index (see Supplemental Tables S3 and S4 at *http://ajp.amjpathol.org*). However, PAGE4 levels inversely correlated with total AR and pAR-Ser213 levels in both hormone-naive PCa and CR-PCa with the inverse correlation more strongly associated with hormone-naive PCa (Table 2). Unfortunately, the reagents to address pAR-Ser81 by IHC are lacking to date. Epithelial PAGE4 levels also inversely correlated with serum PSA in hor-

Table 2. PAGE4 Inversely Correlates with AR Status in Clinical PCa

	Total AR*		pSer213-AR*		Serum PSA†	
	Coefficient‡	*P* value	Coefficient‡	*P* value	Coefficient‡	*P* value
PAGE4						
Hormone-naive PCa	−0.16	0.0162	−0.32	<0.001	0.32	0.0034
$n = 253$	($n = 236$)		($n = 212$)		($n = 81$)	
CR-PCa	−0.13	0.0394	−0.18	0.006	−0.19	0.121
$n = 271$	($n = 248$)		($n = 246$)		($n = 67$)	

*Spot-by-spot analysis of epithelial PAGE4 immunohistochemistry staining intensity with immunostaining levels of total AR and AR phosphorylated at Ser213.
†Comparison of serum PSA with PAGE4 immunohistochemistry scores using the mean epithelial PAGE4 staining intensity score per biopsy specimen in cases in which multiple spots were derived from a single patient biopsy specimen.
‡Values represent Spearman ρ correlation coefficients. Numbers in parentheses denote sample number (n) for which both parameters were available.

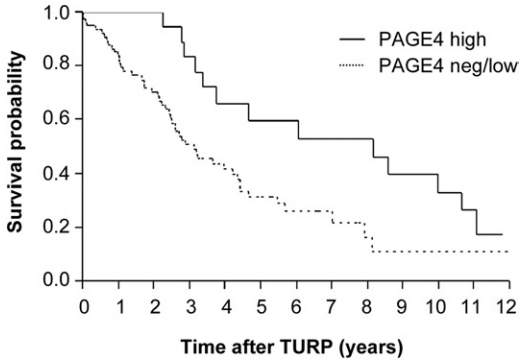

Figure 6. PAGE4 levels correlate with survival of patients with hormone-naive PCa. Overall survival of patients with hormone-naive PCa after TURP for local advanced obstructive PCa stratified for high versus negative/low (neg/low) epithelial PAGE4 levels on the advanced PCa TMA (third quartile of mean epithelial PAGE4 intensity was set as the cut-off level).

mone-naive PCa ($P = 0.003$; Table 2) but not in CR-PCa, where PSA and PAGE4 levels showed an inverse but nonsignificant trend (Table 2).

PAGE4 Levels Correlate with and Predict Survival of Patients with Hormone-Naive PCa

Epithelial PAGE4 levels were analyzed with respect to overall patient survival after TURP for local advanced obstructive PCa. In CR-PCa, PAGE4 was not associated with patient survival ($P = 0.8851$). In hormone-naive PCa, however, median survival of patients with tumors expressing high PAGE4 levels was 8.2 years (95% CI, 3.4 to 10.6) compared with 3.1 years (95% CI, 2.4 to 4.3) for patients with tumors expressing negative/low levels of PAGE4 ($P = 0.0096$; Figure 6). In univariate analyses, significant associations with hormone-naive patient survival were also observed for non-metastatic versus metastatic PCa (cM stage M0/M1, respectively), patient age at TURP (cut-off = median), and Gleason pattern (Table 3). A multivariate analysis with the same parameters revealed that high epithelial PAGE4 levels significantly predicted patient survival independently of Gleason pattern, age, and cM stage (Table 3).

Discussion

This study aimed to investigate the function of PAGE4, a prostate-associated member of CTAs, and whether PAGE4 dysregulation may play a role in PCa development/progression. Collectively, *in vitro* and *in vivo* studies herein indicate that signaling by AR and structurally related GR are attenuated in PAGE4-expressing cells, findings consistent with the spatial restriction of PAGE4 to steroidogenic tissues and with observations that intrinsically disordered proteins, including AR/GR, often are involved in regulatory processes and cell signaling.[25] Attenuation of AR/GR is unlikely to be a nonspecific artifact of PAGE4 overexpression because (i) no effect on progesterone receptor activity (which also is related closely to AR/GR) was observed on PAGE4 overexpression, (ii) attenuated proliferation was observed in PAGE4-expressing AR^+ LNCaP cells but not AR^- PC3 cells, (iii) AR activity was unaltered in parallel-generated LNCaP sublines overexpressing *Dickkopf homolog 3*, (iv) attenuation of total/phospho-AR levels in PAGE4-expressing cells *in vitro* similarly was observed in clinical specimens, and (v) PAGE4 silencing in JEG3 cells (the only cell line identified that expresses detectable protein levels of endogenous PAGE4) potentiated endogenous GR activity, a finding analogous to attenuated GR activity in COS7 cells on PAGE4 overexpression. The structural and functional similarity of GR with AR was exploited as the only means, to our knowledge, of examining the effect of endogenous PAGE4 knockdown on steroid hormone receptor signaling. Further investigation into the significance of dysregulated epithelial PAGE4 with respect to GR signaling in PCa development/progression will be the subject of future investigations.

Despite very low *PAGE4* mRNA levels (our observations and that of G. Jenster, unpublished data), a recent study reported detectable levels of endogenous PAGE4 protein in LNCaP cells, siRNA-mediated silencing that induced apoptosis, and attenuated xenograft growth in intact mice, whereas PAGE4 overexpression in HEK293T and CWR22rv1 cells (both strongly GR^+) enhanced proliferation.[22] On the basis of data herein, it is possible that enhanced proliferation on PAGE4 overexpression in these cell lines reflects attenuation of antiproliferative, pro-apoptotic GR signaling. Supportively, PAGE4 over-

Table 3. PAGE4 Levels Are an Independent Predictor of Survival for Patients with Hormone-Naive PCa

Parameter	Univariate *P* value	Multivariate			
		Hazard ratio	95% CI		*P* value
			Lower	Upper	
cM	0.030*	1.55	0.82	2.92	0.173
Gleason pattern	0.005*	1.68	1.04	2.80	0.033*
Age at surgery	0.003*	1.32	0.96	1.80	0.087
High PAGE4	0.009*	0.67	0.45	0.95	0.024*

Univariate and multivariate analyses assessed by log-rank test or Cox proportional hazard ratio, respectively, for the indicated parameters with respect to hormone-naive PCa patient survival. Cut-off scores were set as M0 versus M1 for cM tumor stage, ≥median patient age after TURP for local advanced obstructive PCa, and high epithelial PAGE4 staining intensity on the advanced PCa TMA with the cut-off value set as the third quartile of the mean epithelial PAGE4 intensity.

*$P < 0.05$.

expression in PC3 cells (AR^- GR^+) modestly increased cell proliferation at day 3, although this was not significant by day 5 (see Supplemental Figure 4C at *http://ajp.amjpathol.org*). We were unable to detect endogenous PAGE4 in LNCaP or other PCa cell lines using commercial polyclonal or our in-house developed monoclonal PAGE4 antibodies (Figure 3A), which show high specificity by signal quenching with blocking but not nonblocking PAGE4 peptides (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). It also may be noted that the authors of the aforementioned study[22] previously identified PAGE4 protein in rat prostate,[46] an unexpected observation because many CTAs, including *PAGE4*, lack identifiable orthologs in rodent genomes.[47] Thus, it is possible that apoptosis induction by PAGE4 siRNAs in PCa cell lines[22] that express little or no detectable PAGE4 may represent off-target effects. Supportively, *PAGE4* siRNAs had no significant effect on *PSA* mRNA levels in wild-type LNCaP cells (our observation, not shown). However, it also is plausible that very low basal levels of PAGE4 are required to maintain cell viability, whereas at increased concentrations (as observed in high-grade PIN/localized PCa and mimicked in PAGE4:LNCaPs) PAGE4, as an intrinsically disordered protein, may engage in low affinity but specific protein-protein interactions to modulate AR/GR activity.

PAGE4 expression was associated with impaired ligand-induced AR nuclear translocation, reduced phosphorylation at Ser81 and Ser213, and decreased AR stability. Consistently, total/pSer213-AR levels inversely correlated with PAGE4 levels in clinical PCa. AR phosphorylation at Ser81 and Ser213 is directly mechanistically linked to AR stabilization and activity, the former regulating AR in a promoter-specific manner[37–39] and likely accounting for AR attenuation at selective androgen-responsive genes in PAGE4:LNCaPs. This is consistent with studies showing that *cis*-acting AR regulators confer promoter specificity of androgen signaling.[11–16] However, AR phosphorylation at Ser81 is temporally slower than the kinetics of nuclear import, whereas Ser213 phosphorylation is rapid but apparently confined to the nucleus.[34,45] These observations, together with the absence of PAGE4 sequence homology to known phosphatase or enzyme domains, suggest that impaired AR phosphorylation, stabilization, and activity are likely downstream consequences of attenuated nuclear translocation.

Precisely how PAGE4 attenuates AR nuclear translocation remains to be determined. In the absence of androgens, AR is sequestered in the cytoplasm via association with multiprotein complexes of chaperones.[9] On ligand binding, AR undergoes a conformational change resulting in the sequential loss of chaperones and unmasking of the nuclear localization signal resulting in AR nuclear translocation. Although PAGE4 is present in the nucleus, a large fraction localizes to the cytosol, both *in vitro* and *in vivo*. Thus, it is plausible that PAGE4 may directly sequester the AR in the cytoplasm. However, co-immunoprecipitation experiments failed to identify an interaction between PAGE4 and AR (not shown), although it cannot be excluded that as an intrinsically disordered protein, PAGE4 interacts with AR in a specific, but weak and/or transient, manner. Future studies thus will focus on identifying PAGE4-interacting partners and whether PAGE4 associates with AR kinases/phosphatases or proteins known to modulate AR subcellular localization, for example, heat shock protein chaperones, phosphatase and tensin homolog deleted on chromosome 10, p21-activated kinase 6, and protein interacting with amyloid precursor protein tail 1 (PAT1/ARA67).[48–50]

Up-regulation of epithelial PAGE4 in high-grade PIN possibly reflects increased epigenetic remodeling and/or transforming growth factor-β1 production, changes that are apparent in PIN and induce *PAGE4* mRNA in prostatic epithelial cells *in vitro*[17,51,52] (and data not shown). In localized PCa and metastases, epithelial PAGE4 levels subsequently were decreased. This is the first study reporting dysregulation of PAGE4 at the protein level during PCa development/progression and is in agreement with mRNA-based studies.[53–57] Given that PAGE4 appears to attenuate AR in a promoter-selective manner, we hypothesize that increased PAGE4 in PIN may result in attenuated AR signaling, favoring tumor initiation. It should be noted that although established tumors are AR-dependent, epithelial AR can suppress or promote tumorigenesis depending on the cancer-initiating signal.[58,59] AR regulates distinct transcriptional programs during PCa development/progression owing, at least in part, to dysregulation of AR coregulators.[11–16,60,61] Thus, the subsequent down-regulation of epithelial PAGE4 in localized PCa and metastases may facilitate AR reactivation and transcriptional programs required for progression to lethal advanced PCa. This hypothesis is supported by the following observations: i) PAGE4 impaired the development of CR-PCa xenografts; ii) the few CR-PCa xenografts that did establish, showed posttranscriptional PAGE4 silencing; iii) PAGE4 levels inversely correlated with AR status in clinical PCa; and iv) low PAGE4 levels correlated with decreased patient survival in hormone-naive PCa. Such promoter-specific regulation of AR by PAGE4 and the distinct AR transcriptional program in PCa versus CR-PCa also may explain why PAGE4 impaired xenograft development in androgen-depleted but not replete animals.

In contrast to impaired development of CR-PCa xenografts by PAGE4:LNCaPs and reduced survival of hormone-naive patients with high PAGE4-expressing tumors, epithelial PAGE4 protein levels did not correlate with serum PSA or patient survival in clinical CR-PCa, even though total/pSer213-AR levels inversely correlated with epithelial PAGE4, albeit less strongly than in hormone-naive PCa. Interestingly, PAGE4 protein levels were comparable in CR-PCa and hormone-naive PCa, suggesting that in these clinical CR-PCa specimens AR bypass pathways were major determinants of tumor progression (eg, BCL2, cyclooxygenase-2, or neuroendocrine differentiation)[62] and/or PAGE4-mediated AR inhibition may have been subverted, perhaps via altered PAGE4 posttranslational modification(s) required for AR inhibition, but not detected by the current monoclonal antibody. Further studies are underway to investigate this possibility and to determine whether restoring PAGE4-

mediated AR inhibition may offer therapeutic benefit for patients with CR-PCa.

Loss of PAGE4 correlated with poor overall survival in hormone-naive PCa patients. Contemporary survival data for hormone-naive PCa are somewhat higher than those herein, but typically derive from asymptomatic patients with low-grade PCa, whereas the hormone-naive PCa patient cohort in the current study underwent TURP for local advanced obstructive PCa. In support of our findings, *PAGE4* mRNA levels recently were reported to be lower in biochemical recurrent PCa, but although positively associated with delayed biochemical recurrence, *PAGE4* mRNA levels were not independent predictors of biochemical recurrence.[57] At the protein level, however, multivariate analyses showed that PAGE4 positivity predicted favorable survival of patients with hormone-naive PCa independently of Gleason pattern, age at surgery, and cM tumor stage. Thus, PAGE4 represents a potential independent prognostic molecular marker to identify patients at risk of developing lethal advanced PCa.

In summary, these data are consistent with an AR/GR repressor function of PAGE4, which, when dysregulated, may play a functional role during tumor progression to advanced lethal PCa, and highlight the potential value of PAGE4 as a prognostic/therapeutic target.

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References

1. Sampson N, Untergasser G, Plas E, Berger P: The ageing male reproductive tract. J Pathol 2007, 211:206–218
2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ: Cancer statistics, 2009. CA Cancer J Clin 2009, 59:225–249
3. Denis LJ, Griffiths K: Endocrine treatment in prostate cancer. Semin Surg Oncol 2000, 18:52–74
4. Basch EM, Somerfield MR, Beer TM, Carducci MA, Higano CS, Hussain MH, Scher HI: American Society of Clinical Oncology endorsement of the Cancer Care Ontario Practice Guideline on nonhormonal therapy for men with metastatic hormone-refractory (castration-resistant) prostate cancer. J Clin Oncol 2007, 25:5313–5318
5. Molina A, Belldegrun A: Novel therapeutic strategies for castration resistant prostate cancer: inhibition of persistent androgen production and androgen receptor mediated signaling. J Urol 2011, 185: 787–794
6. Chen CD, Welsbie DS, Tran C, Baek SH, Chen R, Vessella R, Rosenfeld MG, Sawyers CL: Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004, 10:33–39
7. Ruizeveld de Winter JA, Janssen PJ, Sleddens HM, Verleun-Mooijman MC, Trapman J, Brinkmann AO, Santerse AB, Schroder FH, van der Kwast TH: Androgen receptor status in localized and locally progressive hormone refractory human prostate cancer. Am J Pathol 1994, 144:735–746
8. Lamont KR, Tindall DJ: Minireview: alternative activation pathways for the androgen receptor in prostate cancer. Mol Endocrinol 2011, 25:897–907
9. Heemers HV, Tindall DJ: Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. Endocr Rev 2007, 28:778–808
10. Wolf IM, Heitzer MD, Grubisha M, DeFranco DB: Coactivators and nuclear receptor transactivation. J Cell Biochem 2008, 104:1580–1586
11. Heemers HV, Schmidt LJ, Sun Z, Regan KM, Anderson SK, Duncan K, Wang D, Liu S, Ballman KV, Tindall DJ: Identification of a clinically relevant androgen-dependent gene signature in prostate cancer. Cancer Res 2011, 71:1978–1988
12. Jia L, Berman BP, Jariwala U, Yan X, Cogan JP, Walters A, Chen T, Buchanan G, Frenkel B, Coetzee GA: Genomic androgen receptor-occupied regions with different functions, defined by histone acetylation, coregulators and transcriptional capacity. PloS One 2008, 3:e3645
13. Massie CE, Adryan B, Barbosa-Morais NL, Lynch AG, Tran MG, Neal DE, Mills IG: New androgen receptor genomic targets show an interaction with the ETS1 transcription factor. EMBO Rep 2007, 8:871–878
14. Wang Q, Li W, Liu XS, Carroll JS, Janne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, Brown M: A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell 2007, 27:380–392
15. Wang Q, Li W, Zhang Y, Yuan X, Xu K, Yu J, Chen Z, Beroukhim R, Wang H, Lupien M, Wu T, Regan MM, Meyer CA, Carroll JS, Manrai AK, Janne OA, Balk SP, Mehra R, Han B, Chinnaiyan AM, Rubin MA, True L, Fiorentino M, Fiore C, Loda M, Kantoff PW, Liu XS, Brown M: Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer. Cell 2009, 138:245–256
16. Comstock CE, Revelo MP, Buncher CR, Knudsen KE: Impact of differential cyclin D1 expression and localisation in prostate cancer. Br J Cancer 2007, 96:970–979
17. Sampson N, Untergasser G, Lilg C, Tadic L, Plas E, Berger P: GAGEC1, a cancer/testis associated antigen family member, is a target of TGF-beta1 in age-related prostatic disease. Mech Ageing Dev 2007, 128:64–66
18. Untergasser G, Gander R, Lilg C, Lepperdinger G, Plas E, Berger P: Profiling molecular targets of TGF-beta1 in prostate fibroblast-to-myofibroblast transdifferentiation. Mech Ageing Dev 2005, 126: 59–69
19. Brinkmann U, Vasmatzis G, Lee B, Yerushalmi N, Essand M, Pastan I: PAGE-1, an X chromosome-linked GAGE-like gene that is expressed in normal and neoplastic prostate, testis, and uterus. Proc Natl Acad Sci U S A 1998, 95:10757–10762
20. Gjerstorff MF, Ditzel HJ: An overview of the GAGE cancer/testis antigen family with the inclusion of newly identified members. Tissue Antigens 2008, 71:187–192
21. Fratta E, Coral S, Covre A, Parisi G, Colizzi F, Danielli R, Marie Nicolay HJ, Sigalotti L, Maio M: The biology of cancer testis antigens: putative function, regulation and therapeutic potential. Mol Oncol 2011, 5:164–182
22. Zeng Y, He Y, Yang F, Mooney SM, Getzenberg RH, Orban J, Kulkarni P: The cancer/testis antigen prostate-associated gene 4 (PAGE4) is a highly intrinsically disordered protein. J Biol Chem 2011, 286:13985–13994
23. Hellman M, Tossavainen H, Rappu P, Heino J, Permi P: Characterization of intrinsically disordered prostate associated gene (PAGE5) at single residue resolution by NMR spectroscopy. PloS One 2011, 6:e26633
24. Chen J, Cheng J, Dunker AK: Intrinsically disordered proteins: analysis, prediction, simulation, and biology. Pac Symp Biocomput 2012, 17:67–69
25. Tantos A, Han KH, Tompa P: Intrinsic disorder in cell signaling and gene transcription. Mol Cell Endocrinol 2012, 348:457–465
26. Berger P, Panmoung W, Khaschabi D, Mayregger B, Wick G: Antigenic features of human follicle stimulating hormone delineated by monoclonal antibodies and construction of an immunoradiomometric assay. Endocrinology 1988, 123:2351–2359
27. Sampson N, Koziel R, Zenzmaier C, Bubendorf L, Plas E, Jansen-Durr P, Berger P: ROS signaling by NOX4 drives fibroblast-to-myofibroblast differentiation in the diseased prostatic stroma. Mol Endocrinol 2011, 25:503–515
28. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−delta delta C(T)) method. Methods 2001, 25:402–408
29. Zenzmaier C, Sampson N, Pernkopf D, Plas E, Untergasser G, Berger P: Attenuated proliferation and trans-differentiation of prostatic stromal cells indicate suitability of phosphodiesterase type 5 inhibitors for prevention

and treatment of benign prostatic hyperplasia. Endocrinology 2010, 151:3975–3984
30. Zellweger T, Ninck C, Bloch M, Mirlacher M, Koivisto PA, Helin HJ, Mihatsch MJ, Gasser TC, Bubendorf L: Expression patterns of potential therapeutic targets in prostate cancer. Int J Cancer 2005, 113: 619–628
31. Ruiz C, Holz DR, Oeggerli M, Schneider S, Gonzales IM, Kiefer JM, Zellweger T, Bachmann A, Koivisto PA, Helin HJ, Mousses S, Barrett MT, Azorsa DO, Bubendorf L: Amplification and overexpression of vinculin are associated with increased tumour cell proliferation and progression in advanced prostate cancer. J Pathol 2011, 223:543–552
32. Prakash K, Pirozzi G, Elashoff M, Munger W, Waga I, Dhir R, Kakehi Y, Getzenberg RH: Symptomatic and asymptomatic benign prostatic hyperplasia: molecular differentiation by using microarrays. Proc Natl Acad Sci U S A 2002, 99:7598–7603
33. Sheflin L, Keegan B, Zhang W, Spaulding SW: Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. Biochem Biophys Res Commun 2000, 276:144–150
34. Gioeli D, Ficarro SB, Kwiek JJ, Aaronson D, Hancock M, Catling AD, White FM, Christian RE, Settlage RE, Shabanowitz J, Hunt DF, Weber MJ: Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J Biol Chem 2002, 277:29304–29314
35. Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY, Chang C: Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. J Biol Chem 2003, 278:50902–50907
36. Palazzolo I, Burnett BG, Young JE, Brenne PL, La Spada AR, Fischbeck KH, Howell BW, Pennuto M: Akt blocks ligand binding and protects against expanded polyglutamine androgen receptor toxicity. Hum Mol Genet 2007, 16:1593–1603
37. Hsu FN, Chen MC, Chiang MC, Lin E, Lee YT, Huang PH, Lee GS, Lin H: Regulation of androgen receptor and prostate cancer growth by cyclin-dependent kinase 5. J Biol Chem 2011, 286:33141–33149
38. Gordon V, Bhadel S, Wunderlich W, Zhang J, Ficarro SB, Mollah SA, Shabanowitz J, Hunt DF, Xenarios I, Hahn WC, Conaway M, Carey MF, Gioeli D: CDK9 regulates AR promoter selectivity and cell growth through serine 81 phosphorylation. Mol Endocrinol 2010, 24:2267–2280
39. Chen S, Gulla S, Cai C, Balk SP: Androgen receptor serine 81 phosphorylation mediates chromatin binding and transcriptional activation. J Biol Chem 2012, 287:8571–8583
40. Wang Y, Kreisberg JI, Ghosh PM: Cross-talk between the androgen receptor and the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer. Curr Cancer Drug Targets 2007, 7:591–604
41. Zhu ML, Kyprianou N: Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocr Relat Cancer 2008, 15: 841–849
42. Liu X, Choi RY, Jawad SM, Arnold JT: Androgen-induced PSA expression requires not only activation of AR but also endogenous IGF-I or IGF-I/PI3K/Akt signaling in human prostate cancer epithelial cells. Prostate 2011, 71:766–777
43. McCall P, Gemmell LK, Mukherjee R, Bartlett JM, Edwards J: Phosphorylation of the androgen receptor is associated with reduced survival in hormone-refractory prostate cancer patients. Br J Cancer 2008, 98:1094–1101
44. Black BE, Vitto MJ, Gioeli D, Spencer A, Afshar N, Conaway MR, Weber MJ, Paschal BM: Transient, ligand-dependent arrest of the androgen receptor in subnuclear foci alters phosphorylation and coactivator interactions. Mol Endocrinol 2004, 18:834–850
45. Taneja SS, Ha S, Swenson NK, Huang HY, Lee P, Melamed J, Shapiro E, Garabedian MJ, Logan SK: Cell-specific regulation of androgen receptor phosphorylation in vivo. J Biol Chem 2005, 280:40916–40924
46. Shah US, Arlotti J, Dhir R, Lu S, Pirozzi G, Prakash K, Getzenberg RH: Androgen regulation of JM-27 is associated with the diseased prostate. J Androl 2004, 25:618–624
47. Liu Y, Zhu Q, Zhu N: Recent duplication and positive selection of the GAGE gene family. Genetica 2008, 133:31–35
48. Lin HK, Hu YC, Lee DK, Chang C: Regulation of androgen receptor signaling by PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor through distinct mechanisms in prostate cancer cells. Mol Endocrinol 2004, 18:2409–2423
49. Schrantz N, da Silva Correia J, Fowler B, Ge Q, Sun Z, Bokoch GM: Mechanism of p21-activated kinase 6-mediated inhibition of androgen receptor signaling. J Biol Chem 2004, 279:1922–1931
50. Zhang Y, Yang Y, Yeh S, Chang C: ARA67/PAT1 functions as a repressor to suppress androgen receptor transactivation. Mol Cell Biol 2004, 24:1044–1057
51. Cardillo MR, Petrangeli E, Perracchio L, Salvatori L, Ravenna L, Di Silverio F: Transforming growth factor-beta expression in prostate neoplasia. Anal Quant Cytol Histol 2000, 22:1–10
52. Nelson WG, De Marzo AM, Yegnasubramanian S: Epigenetic alterations in human prostate cancers. Endocrinology 2009, 150:3991–4002
53. Yu YP, Landsittel D, Jing L, Nelson J, Ren B, Liu L, McDonald C, Thomas R, Dhir R, Finkelstein S, Michalopoulos G, Becich M, Luo JH: Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. J Clin Oncol 2004, 22:2790–2799
54. LaTulippe E, Satagopan J, Smith A, Scher H, Scardino P, Reuter V, Gerald WL: Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. Cancer Res 2002, 62:4499–4506
55. Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C, Tamayo P, Renshaw AA, D'Amico AV, Richie JP, Lander ES, Loda M, Kantoff PW, Golub TR, Sellers WR: Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 2002, 1:203–209
56. Suyama T, Shiraishi T, Zeng Y, Yu W, Parekh N, Vessella RL, Luo J, Getzenberg RH, Kulkarni P: Expression of cancer/testis antigens in prostate cancer is associated with disease progression. Prostate 2010, 70:1778–1787
57. Shiraishi T, Terada N, Zeng Y, Suyama T, Luo J, Trock B, Kulkarni P, Getzenberg RH: Cancer/testis antigens as potential predictors of biochemical recurrence of prostate cancer following radical prostatectomy. J Transl Med 2011, 9:153
58. Niu Y, Altuwaijri S, Lai KP, Wu CT, Ricke WA, Messing EM, Yao J, Yeh S, Chang C: Androgen receptor is a tumor suppressor and proliferator in prostate cancer. Proc Natl Acad Sci U S A 2008, 105:12182–12187
59. Memarzadeh S, Cai H, Janzen DM, Xin L, Lukacs R, Riedinger M, Zong Y, DeGendt K, Verhoeven G, Huang J, Witte ON: Role of autonomous androgen receptor signaling in prostate cancer initiation is dichotomous and depends on the oncogenic signal. Proc Natl Acad Sci U S A 2011, 108:7962–7967
60. Chmelar R, Buchanan G, Need EF, Tilley W, Greenberg NM: Androgen receptor coregulators and their involvement in the development and progression of prostate cancer. Int J Cancer 2007, 120:719–733
61. Hendriksen PJ, Dits NF, Kokame K, Veldhoven A, van Weerden WM, Bangma CH, Trapman J, Jenster G: Evolution of the androgen receptor pathway during progression of prostate cancer. Cancer Res 2006, 66:5012–5020
62. Bonkhoff H, Berges R: From pathogenesis to prevention of castration resistant prostate cancer. Prostate 2010, 70:100–112