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An AKT activity threshold regulates androgen-dependent and androgen-independent PSA expression in prostate cancer cell lines

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Abstract

The androgen receptor (AR) plays an important role in early prostate cancer by activating transcription of a number of genes participating in cell proliferation and growth and cancer progression. However, as the cancer progresses, prostate cancer cells transform from an androgen-dependent to an androgen-independent state. Androgen-independent prostate cancer can manifest itself in several forms, including a percentage of cancers that show reduced levels of prostate-specific antigen (PSA) and can progress without the need for the ligand or active receptor. Therefore, our goal was to examine the role of intracellular signaling pathways in an androgen-independent prostate cancer *in vitro* model. Using the cell line PC3(AR)$_2$, we stimulated cells with 5-α-dihydrotestosterone (DHT) and epidermal growth factor (EGF) and then analyzed PSA expression. We observed lower PSA expression when cells were jointly stimulated with DHT and EGF, and this was associated with an increase in AKT activity. We examined the role of AKT in AR activity and PSA expression by creating stable PC3(AR)$_2$ cell lines transfected with a PI3K-Ras-effector loop mutant. These cell lines showed lower DHT-stimulated PSA expression that correlated to changes in the phosphorylated state of AR. Therefore, we propose an *in vitro* androgen-independent model in which a PI3K/AKT activity threshold and subsequent AR transactivation regulate PSA expression.

Keywords: AKT activity; androgen-independent; androgen receptor; prostate cancer; prostate-specific antigen.

Introduction

Prostate cancer is the most frequently diagnosed cancer among men in North America and Europe. Several factors contribute the development of prostate cancer, but one highly influential protein is the androgen receptor (AR). The AR belongs to a superfamily of nuclear receptors and mediates the action of androgens such as testosterone and 5-α-dihydrotestosterone (DHT) (Kato et al., 2005). The AR and its activating ligands play an important role in prostate cancer progression by mediating the responses of androgens and activating gene transcription. Upon binding of the androgen to the cognate receptor, the receptor dimerizes and translocates into the nucleus and binds to specific DNA *cis*-elements known as androgen response elements (AREs). The RNA pol II complex is then recruited to the androgen-responsive gene to initiate transcription (Suzuki et al., 2003; Shand and Gelmann, 2006).

Of the androgen-responsive genes, the one most studied and associated with prostate cancer is prostate-specific antigen (PSA) (Pousette et al., 1999). PSA is the most commonly used biomarker for diagnosis of prostate cancer. Therapeutic approaches aim at reducing testosterone levels or blocking testosterone signaling through the AR, and thus blocking downstream gene activation (Trapman and Cleutjens, 1997; Balk et al., 2003; Kim and Coetzee, 2004). Prostate cancer can be separated into two hormone-associated classes (Shand and Gelmann, 2006). First is early stage cancer, also known as androgen-dependent cancer, in which gene expression is still associated with sensitivity to androgens and the action of the AR. Clinically, prostate cancer progression is associated with the patient's response to hormone therapy or lack thereof. Failure of hormone therapy is a characteristic of a more aggressive, hormone refractory stage or androgen-independent cancer that is often associated with loss of androgen sensitivity (Grossmann et al., 2001). The mechanism of progression from androgen dependence to androgen independence is poorly understood.

Currently, there are many definitions of androgen independence. The AR is still expressed in androgen-independent prostate cancer cells. However, gene activation profiles associated with loss of androgen sensitivity have prompted different definitions to describe the transition. The most common definition of androgen-independent prostate cancer is the activation of androgen-sensitive genes, such as PSA, by ligands other than testosterone that can associate with the AR and subsequently activate PSA. Such promiscuous, non-androgen ligand interactions can also be facilitated by mutations within the AR. Such mutations have been characterized in the most commonly used prostate cancer cell lines (such as LNCaP) and in patients in whom tumors can activate PSA expression under a wide variety of stimulants, including estrogens and anti-hormone agents (Taplin et al., 1995; Gelmann, 1996; Van Bokhoven et al., 2003; Taplin and Balk, 2004).

Intracellular signaling pathways can also influence AR activity. AR activation by intracellular signaling pathways

stimulated by growth factors and cytokines [such as epidermal growth factor (EGF), insulin-like growth factor (IGF), keratinocyte growth factor (KGF), forskolin, neuropeptides such as bombesin, and interlukin-6 (IL-6)] has been identified as an alternative to ligand binding and PSA gene expression (Trapman and Cleutjens, 1997; Grossmann et al., 2001; Yang et al., 2003; Taplin and Balk, 2004; Huang et al., 2006). These peptide mitogens signal through various pathways to stimulate AR phosphorylation. These pathways may also sensitize the magnitude of the AR response under low androgen levels. This cross-talk between AR response and cell signaling pathways was illustrated by Bakin et al. (2003), who showed that LNCaP cells stably transfected with a Ras-effector loop mutant driving constitutive activity of the MEK/extracellular signal-regulated kinase (ERK) pathway exhibited PSA expression that was sensitized upon stimulation with low androgen levels. Moreover, common genetic mutations found in prostate cancer such as in *PTEN* (phosphatase and tensin homolog deleted from chromosome 10 gene), and RAS or EGFR overexpression can all contribute to the transition of prostate cancer from androgen-dependent to androgen-independent (Li et al., 1997, 2005; Wang and Hung, 2001; Bonaccorsi et al., 2004b; Gao et al., 2006; McCubrey et al., 2006). However, paradoxically, in approximately 22% of prostate cancers exhibit another androgen-independent form, in which, rather than observing an increase in PSA expression, PSA levels are near normal or decreased (Hernandez and Thompson, 2004), as well as a wild-type form of the AR and normal circulating levels of androgens.

Therefore, in the present study we investigated the latter PSA paradox form of androgen-independent prostate cancer using an *in vitro* model. We manipulated cell signaling pathways using small-molecule inhibitors and stably transfected cell lines. Our results indicate that PSA expression is dependent on an AKT activity threshold that marks the difference between androgen-dependent and -independent forms of prostate cancer. Low AKT levels are characteristic of androgen-dependent PSA expression, whereas high levels are characteristic of androgen-independent prostate cancer.

Results

PI3K/AKT positively regulates androgen-dependent PSA expression

Previous work has shown that intracellular signaling pathways play an important role in regulating the androgen-dependent expression of PSA in breast cancer cell lines. Therefore, we further examined these pathways, with specific emphasis on the PI3K/AKT and MEK/ERK pathways in relation to PSA regulation and expression, using the prostate cancer cell line PC3(AR)$_2$. We selected PC3(AR)$_2$ instead of more commonly used prostate cancer cell lines such as LNCaP or 22Rv1 to study androgen-dependent PSA expression because PC3(AR)$_2$ was derived from PC3 cells by transfection with a wild-type AR; thus, PSA expression is tightly regulated by androgens (Heisler et al., 1997). LNCaP and 22Rv1 cell lines harbor mutations in the AR and accumulate PSA in conditioned media without the need for androgens, whereas PSA production by PC3(AR)$_2$ cell lines is tightly regulated, with untreated cells expressing 1–2 ng/l PSA over the 4-day stimulation period.

PC3(AR)$_2$ cells were stimulated with DHT and treated with 500 nM wortmannin, a PI3K inhibitor. Addition of wortmannin reduced the DHT-dependent stimulation of PSA. This suggests that the PI3K/AKT pathway exerts a positive influence on PSA expression in androgen-sensitive prostate cancer cells (Figure 1A). The changes in PSA expression appear to be a transcriptional event, as RNA levels also changed upon addition of wortmannin (Figure 1B). As PC3(AR)$_2$ cells possess a mutant (non-functional) PTEN protein (Li et al., 1997), we also examined whether a similar result could be obtained by stably transfecting a wild-type copy of the *PTEN* gene into these cells. PC3(AR)$_2$-PTEN stable transgenic cells were stimulated with DHT and PSA levels were compared against the parental line (Figure 2A).

Along with the reduced expression of PSA, the PC3(AR)$_2$-PTEN cell line also acquired other characteristics (see below) suggesting that the addition of wild-type PTEN could potentially target AKT activity. First, the PTEN transgenic cells showed reduced AKT activity compared to the parental cell line (Figure 2B). Second, since AKT is critical for cell survival through regulation of translation and apoptotic pathways, we used an MTT assay to analyze changes in cell survival between parental and PC3(AR)$_2$-PTEN lines (Figure 2C). In turn, we observed an earlier decrease in PC3(AR)$_2$-PTEN proliferation compared to the parental cells, which we have interpreted as a decrease in cell survival as a result of the addition of wild-type PTEN to PC3(AR)$_2$ cells. Since we did not observe any significant differences between vector-alone transfections and the PC3(AR)$_2$ parental line, data are expressed compared to the parental line. Similar

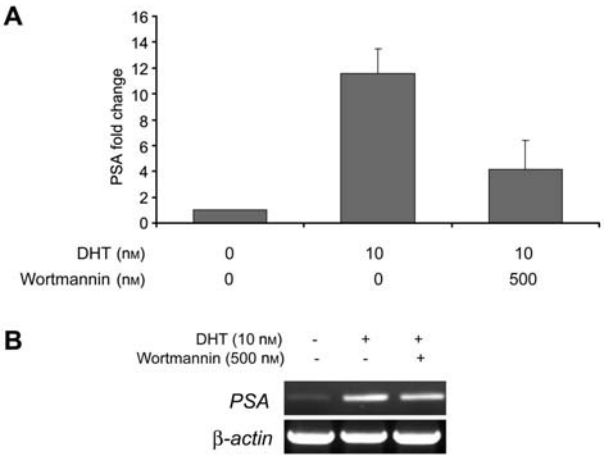

Figure 1 Inhibition of the PI3K/AKT pathway suppresses DHT-stimulated expression of PSA.
(A) PC3(AR)$_2$ cells were stimulated with 10 nM DHT and 500 nM wortmannin and PSA expression was assessed by ELISA in tissue culture supernatants. All changes in PSA levels are expressed as the fold change compared to unstimulated or untreated cells. (B) RT-PCR analysis of PSA expression (30 cycles) of cells treated with DHT and wortmannin; β-actin was used as a loading control (25 cycles).

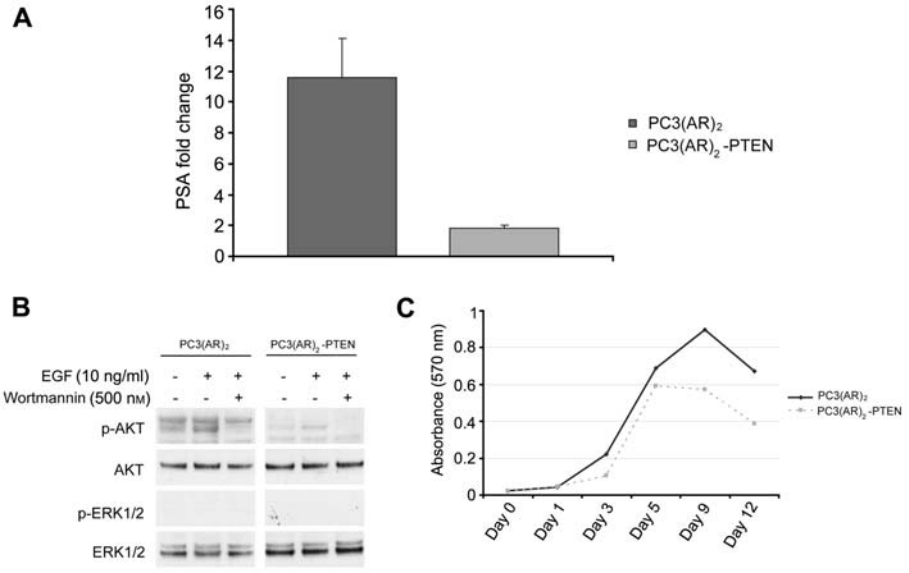

Figure 2 Characterization of PC3(AR)$_2$-PTEN versus the parental PC3(AR)$_2$ cell line.
(A) PSA expression profiles of DHT-stimulated parental versus PC3(AR)$_2$-PTEN transgenic cell lines. (B) Differences in AKT activity, shown by marked band intensity between the two cell lines PC3(AR)$_2$ and PC3(AR)$_2$-PTEN upon EGF stimulation. (C) MTT assay of the PC3(AR)$_2$-PTEN line versus parental cells. As described in the materials and methods section, the MTT assay represents the average of eight biological replicates of seeded unstimulated cells.

non-significant differences were also observed with vector-alone transfections used in Ras-effector loop transfection experiments.

EGF represses androgen-dependent PSA expression

To further assess the role of intracellular signaling pathways in PSA expression, PC3(AR)$_2$ cells were stimulated with DHT and EGF and PSA expression was analyzed by ELISA. The stimulation of PC3(AR)$_2$ cells with EGF reduced the DHT-dependent expression of PSA protein by 40% (Figure 3A). Cell stimulation with EGF alone did not alter PSA expression in PC3(AR)$_2$ cells (data not shown). The lower PSA expression induced by EGF in DHT-stimulated PC3(AR)$_2$ cells may be a transcriptional event, as demonstrated by RT-PCR (Figure 3B). As a mitogen, EGF is capable of activating both PI3K/AKT and MEK/ERK pathways (McCubrey et al., 2006; Rajalingam et al., 2007). Therefore, we checked the activation of these pathways in PC3(AR)$_2$ cells using Western blotting. We found that EGF was able to activate AKT, but not ERK1/2 (Figures 2B and 4A).

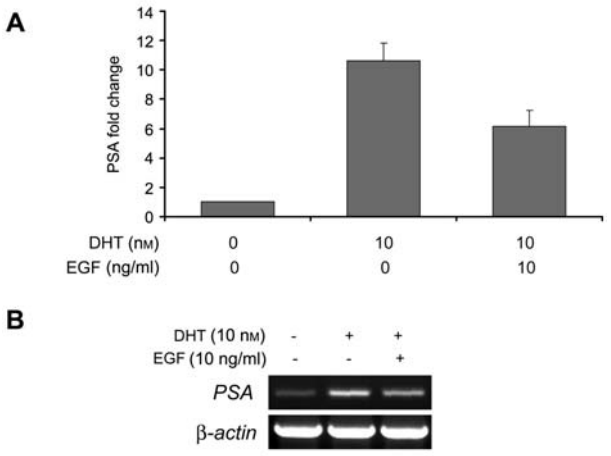

Figure 3 EGF stimulation of AKT suppresses PSA expression.
(A) PSA expression profile of EGF- and DHT-stimulated PC3(AR)$_2$ cells as measured by ELISA and expressed as fold changes. (B) RT-PCR analysis of PSA (30 cycles) expression and β-actin (25 cycles).

Characterization of PC3(AR)$_2$-Y40C stable transgenic cell lines

Since activation of the PI3K/AKT pathway by EGF reduced the PSA expression of DHT-stimulated PC3(AR)$_2$ cells, we followed a genetic approach to further confirm the negative regulation of PSA via activation of the PI3K/AKT pathway. The RAS oncoprotein is capable of binding to numerous effectors to trigger various signaling cascades. We created stable transgenic PC3(AR)$_2$ cells expressing the RAS-effector loop mutant-Y40C, which results in constitutive activity of the PI3K/AKT pathway (Rajalingam et al., 2007). Before analyzing PSA expression, we characterized some properties of the transfected and non-transfected cell lines. First, we stimulated both parental and transgenic Y40C cell lines with 10 ng/ml EGF for 10 min to identify differences in activity of the PI3K/AKT pathway (Figure 4A). PC3(AR)$_2$-Y40C cell lines exhibited higher AKT activation (based on our phospho-AKT Thr308 Western blots) compared to the parental line, indicating that the Ras-effector loop mutant confers increased sensitivity of the transgenic cell line to PI3K activation.

The PI3K/AKT pathway is a key survival pathway; therefore, we examined differences in either cell proliferation or survival between the two cell lines using the MTT assay over a 12-day period (Figure 4B). Although there was little difference initially in proliferation rates between the two cell lines, by days 9 and 12 the

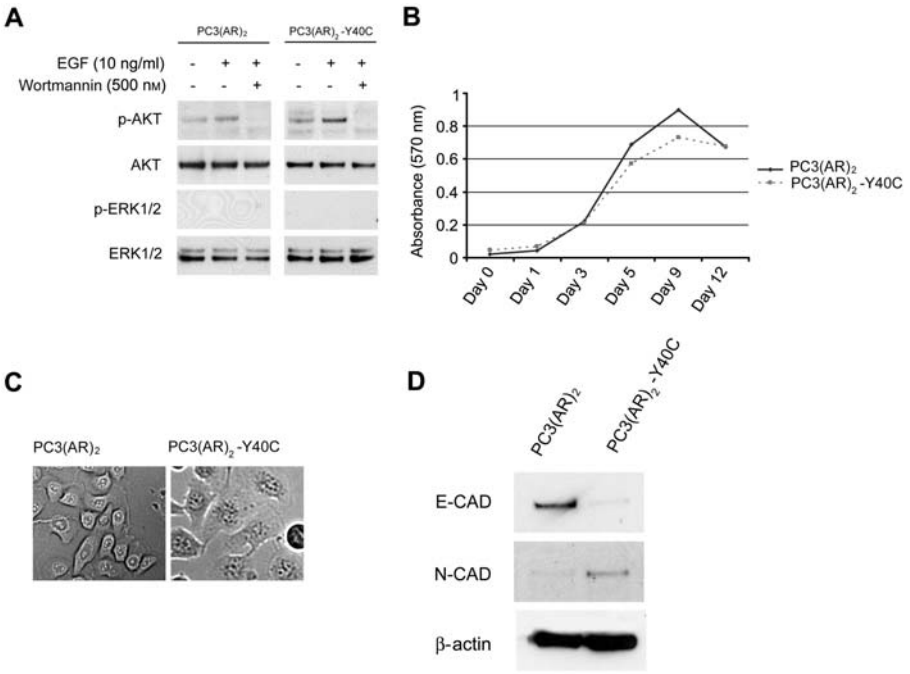

Figure 4 Characterization of the Ras-Y40C effector-loop mutant.
(A) Western blot analysis of AKT and ERK1/2 activity between parental and Ras-Y40C cell lines. (B) MTT proliferation assays of the $PC3(AR)_2$ parental line versus the $PC3(AR)_2$-Y40C transgenic line, averaged for eight biological replicates of unstimulated cells. (C) Morphological differences between parental and $PC3(AR)_2$-Y40C cell lines. (D) Western blot analysis of E-cadherin (E-CAD) and N-cadherin (N-CAD), markers for AKT-related EMT shift.

$PC3(AR)_2$ parental cell line started to die, as shown by a decrease in MTT absorbance. The $PC3(AR)_2$-Y40C stable cell line exhibited an increased survival rate.

There was a clear difference in overall morphology between the two cell lines. The $PC3(AR)_2$-Y40C stable cell line appeared to have undergone epithelial-mesenchymal transition (EMT) (Figure 4C). It has been reported that activation of AKT can promote EMT (Bakin et al., 2000; Boyer et al., 2000; Grille et al., 2003; Larue and Bellacosa, 2005; Lee et al., 2006; Pienta and Bradley, 2006). We confirmed the EMT shift in $PC3(AR)_2$-Y40C cell lines by Western blot analysis for E-cadherin and N-cadherin, two proteins that differentially change their expression patterns upon EMT (Figure 4D). $PC3(AR)_2$-Y40C cells showed a decrease in E-cadherin and subsequent increase in N-cadherin expression compared to the parental cell line. Such changes in E- and N-cadherin expression are indicative of AKT-dependent EMT.

Constitutive AKT activity results in reduced PSA expression

We compared the androgen-dependent expression of PSA between parental and $PC3(AR)_2$-Y40C cells. The cell lines were stimulated with 10 nM DHT for 3 days and PSA levels were measured by ELISA in the supernatants (Figure 5A). PSA levels were approximately 50% lower in $PC3(AR)_2$-Y40C cells than in the parental cell line upon DHT stimulation, a similar PSA expression pattern to that observed upon EGF stimulation of the $PC3(AR)_2$ parental line (as noted previously). We then examined whether blocking of the PI3K/AKT pathway can restore the androgen-dependent expression of PSA. Thus, $PC3(AR)_2$-Y40C cells were stimulated with DHT with or without 500 nM wortmannin. Indeed, inhibition of AKT by wortmannin did restore PSA expression to levels comparable to those in DHT-stimulated parental cells. Therefore,

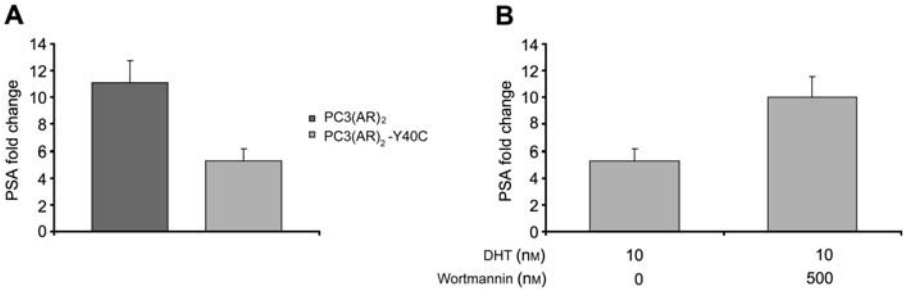

Figure 5 $PC3(AR)_2$-Y40 expression of PSA.
(A) PSA expression profiles of DHT-stimulated $PC3(AR)_2$ and $PC3(AR)_2$-Y40 cell lines. (B) PSA expression profiles of $PC3(AR)_2$-Y40 cells treated with DHT and wortmannin.

increased activation of the PI3K/AKT pathway exerts a negative influence on PSA expression.

Differential AKT-dependent phosphorylation of the AR

It has been shown that the AR can be phosphorylated at several residues. These phosphorylation events are regulated by several signaling pathways, including MEK/ERK, PKA, c-SRC and AKT. In particular, AKT can phosphorylate the AR at two serine residues, Ser210 and Ser790 (Gioeli et al., 2002; Xin et al., 2006). Since our results indicated that PI3K/AKT signaling is a key pathway for the regulation of PSA expression, we investigated differences in AR phosphorylation between the parental and PC3(AR)$_2$-Y40C cell lines. PC3(AR)$_2$ and PC3(AR)$_2$-Y40C cells were stimulated with EGF or DHT with and without wortmannin treatment. Cells were lysed and the AR was immunoprecipitated for Western blot analysis using a phospho-specific-Ser210 androgen receptor antibody (Figure 6). The AR of the PC3(AR)$_2$ parental cell line exhibited very little phosphorylation, with no apparent differences between any of the stimulations or wortmannin treatments. However, the basal level of Ser210 specific phosphorylation of the AR was much higher in PC3(AR)$_2$-Y40C than in parental cells, which we attribute to higher AKT activity. The PC3(AR)$_2$-Y40C line also showed sensitivity to wortmannin treatment, resulting in a decrease in Ser210 phosphorylation. These AKT-dependent phosphorylation patterns of the AR are consistent with our observations of a reduction in Ser210 phosphorylation of the AR in PC3(AR)$_2$-Y40C cells and restoration of PSA expression after wortmannin treatment. Thus, we speculate that above an AKT activity threshold, the PI3K/AKT pathway negatively regulates PSA expression via phosphorylation of the AR.

Discussion

In the present study we used a prostatic carcinoma cell line to show that PSA expression is regulated by an AKT activity threshold. This work addresses several previous suggestions and controversies indicating that AKT can both positively and negatively regulate PSA expression (Lin et al., 2001, 2003; Bonaccorsi et al., 2004a; Hakariya et al., 2006), and proposes a candidate model (Figure 7). Cell line selection is a critical issue for studying the role of PI3K/AKT in the regulation of AR activity and PSA expression. We were able to use a single cell line and a myriad of stimulations, inhibitor treatments, and genetics to reveal and explain both positive and negative influences of AKT activity.

We selected the cell line PC3(AR)$_2$, which was derived from PC3 cells, after stable transfection with wild-type AR. This cell line readily expresses PSA after stimulation with DHT. Addition of the PI3K inhibitor wortmannin to DHT-stimulated cells can block the expression of PSA (Figure 1). We further expanded on this observation by stably transfecting a wild-type copy of PTEN into PC3(AR)$_2$ cells. PTEN is a negative regulator of AKT activity. Since PC3(AR)$_2$ has a mutant form of PTEN, transfection of wild-type PTEN into these cells similarly induced a reduction in PSA expression (Figure 2). These results indicate that AKT activity positively influences DHT-dependent PSA expression. However, when cells were simultaneously stimulated with EGF and DHT, we also observed a decrease in PSA expression (Figure 3). Although the mitogen EGF can stimulate the activity of several intracellular pathways, we particularly observed activation of AKT rather than ERK upon EGF stimulation of PC3(AR)$_2$ cells. These results suggest that in this context, an increase in AKT activity could negatively regulate PSA expression.

To further characterize the role of increased AKT activity in the negative regulation of PSA, we created a transgenic cell line stably expressing a RAS-effector loop mutant that would constitutively drive PI3K activity. This PC3(AR)$_2$-Y40C stable cell line expresses PSA at much lower levels than the parental cell line upon DHT stimulation (Figure 5). This is consistent with the observation that EGF activation of AKT also results in reduced expression of PSA upon joint stimulation with DHT. Since an increase in AKT activity can negatively regulate PSA expression, we also showed that inhibition of this pathway by wortmannin could restore PSA expression. The

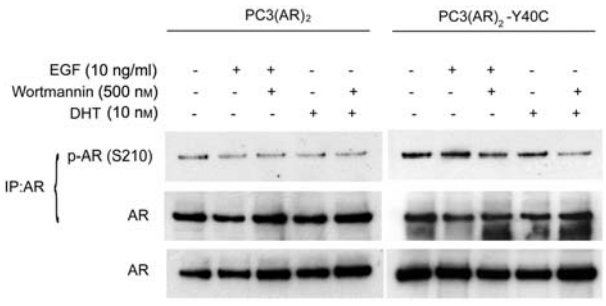

Figure 6 Differential AKT-dependent phosphorylation of the androgen receptor.
Phospho-specific AR Western blots of PC3(AR)$_2$ and PC3(AR)$_2$-Y40C cell lines from immunoprecipitated androgen receptor. Ser210 phosphorylation between the two cell lines is marked by differential band intensities. There were no overall changes in AR expression levels upon any of the stimulations or inhibitor treatments, as shown in the bottom panel.

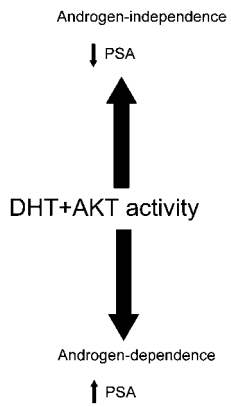

Figure 7 Schematic diagram of the putative relationship between AKT activity and androgen-dependent and -independent PSA expression.

changes in PSA expression as a result of the treatments were attributed to AR activity. We have shown that the increase in AKT activity in PC3(AR)$_2$-Y40C cells was associated with an increase in Ser210 phosphorylation of the AR. Decreased Ser210 phosphorylation in PC3(AR)$_2$-Y40C cells also correlated with an increase in PSA expression upon wortmannin treatment (Figure 6). Therefore, AKT-dependent phosphorylation of the AR negatively regulates the activity of the receptor and PSA transcription.

The resulting higher basal AKT activity induced EMT in PC3(AR)$_2$-Y40C cells, which exhibited increased cell survival in comparison to parental cells (Figure 4). The exact activity level of AKT that is required to cause this transition is unknown, and could be a result of amplification of the activity by other downstream factors. However, such phenomena are commonly observed in more aggressive forms of prostate cancer. An association between AKT activation and EMT has been described in model organisms such as *Xenopus*, *Caenorhabditis elegans*, and *Drosophila*; however, it is only recently that attempts have been made to understand the mechanism in mammals (Gioeli et al., 2002; Larue and Bellacosa, 2005). AKT is frequently activated in human epithelial cancers. However, the observation that EMT was induced by AKT was recently demonstrated by Grille et al. (2003), who showed that squamous carcinoma cell lines overexpressing activated mutants of AKT underwent EMT with morphological changes and downregulation of E-cadherin. A decrease in E-cadherin is often associated with EMT and is accompanied by upregulation of N-cadherin, similar to our observations for PC3(AR)$_2$ and transgenic PC3(AR)$_2$-Y40C cells. It has been hypothesized that the early steps of increased motility and cell invasiveness are associated with EMT and an overall more aggressive cancer.

Materials and methods

Cell line

The PC3(AR)$_2$ cell line was used for all experiments, including the development of stably transfected cell lines. The cell line was a gift from Dr. T.J. Brown (Mount Sinai Hospital, Toronto, Canada), and was developed as described elsewhere (Heisler et al., 1997).

Steroids and inhibitor compounds

DHT and EGF were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The inhibitor wortmannin was purchased from EMD Biosciences (San Diego, CA, USA).

Stable cell lines

Y40-Ras-effector loop mutants PC3(AR)$_2$ cells were seeded onto 10-cm dishes and transfected with 3 μg of pCMV-RasY40C using Fugene (Roche Diagnostics, Indianapolis, IN, USA). Stable clones were selected using G418 (Invitrogen, Carlsbad, CA, USA). Colonies were picked and screened by Western blot analysis using anti-Ras (Upstate, Charlottesville, VA, USA). The pCMV-RasY40C plasmid was a gift from Dr. M. White, University of Texas-Southwestern, Dallas, TX. Ras-Y40C stable cells are referred to as PC3(AR)$_2$-Y40C throughout the manuscript.

PTEN stable cell lines pOB7-PTEN cDNA was obtained from The American Type Culture Collection (ATCC, Rockville, MD, USA) and subcloned into the pMX-PIE expression vector. PC3(AR)$_2$ cells were seeded onto 10-cm dishes and transfected with 3 μg of pMX-PIE-PTEN construct using Fugene. Stable cell lines were screened using puromycin and screened for PTEN expression by Western blotting using anti-PTEN (Cell Signaling Technologies, Davers, MA, USA). PTEN-transfected stable cells are referred to as PC3(AR)$_2$-PTEN throughout the manuscript.

Cell culture: hormone stimulation, blocking studies, and determination of PSA concentrations

PC3(AR)$_2$, PC(AR)$_2$-Y40C and PC3(AR)$_2$-PTEN stable cell lines were cultured in phenol-red-free RPMI 1640 media supplemented with charcoal-dextran stripped fetal bovine serum (FBS) (11%) and 100 μg/ml hygromycin [plus 300 mg/ml G418 for PC(AR)$_2$-Y40 and 0.7 μg/ml puromycin for PC3(AR)$_2$-PTEN stable cell lines] at 37°C in 5% CO_2 in plastic culture flasks. Once confluent, 1×10^6 cells were seeded into 6-well plates with the same medium to allow the cells to adhere. The medium was changed 24 h after plating to RMPI supplemented with 10% charcoal-dextran stripped FBS and incubated for an additional 24 h. The following day, the medium was changed to fresh RMPI/charcoal-dextran stripped FBS for stimulation and inhibitor studies.

Stimulation experiments Cells were incubated with DHT (final concentration 10^{-8} M) for 24 h for RNA analysis and for 4 days for measurement of secreted PSA protein in cell supernatants (conditioned media). All stimulations were performed in triplicate. Cells were similarly stimulated with a final concentration of 10 ng/ml EGF.

Inhibitor studies Cells were cultured as described for the stimulation experiments. Selective inhibitors were added, either as a single dose for RNA and lysate analysis, or daily for 3 days and conditioned media were collected on the fourth day for PSA protein analysis.

Determination of PSA The concentration of PSA was measured using a quantitative specific immunofluorometric ELISA assay as described previously (Ferguson et al., 1996). The detection limit was 1 ng/l. Changes in PSA levels were calculated as the fold change compared to unstimulated or untreated parental cells.

RNA extraction and RT-PCR Total RNA was extracted from prostate cancer cells using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. The RNA concentration was determined spectrophotometrically and 5 μg of total RNA was reverse-transcribed into first-strand cDNA using a Superscript™ First Strand Synthesis kit (Invitrogen) with an Oligo(dT) primer. PCR was carried out using Qiagen HotStar Taq Polymerase (Qiagen, Valencia, CA, USA) on first-strand cDNA. Primers used for RT-PCR analysis were: PSA, forward 5′-CCC ACT GCA TCA GGA ACA AAA GCG-3′ and reverse 5′-GGT GCT CAG GGG TGG CCA C-3′; and β-actin, forward 5′-ATC TGG CAC CAC ACC TTC TA-3′ and reverse 5′-CGT CAT ACT CCT GCT TGC TG-3′. An equal amount of each PCR product was run on 0.9% agarose gel and visualized by ethidium bromide staining.

MTT proliferation assays PC3(AR)$_2$, PC3(AR)$_2$-PTEN, and PC3(AR)$_2$-Y40 cells (4000 cells) were seeded into 24-well plates with 8 wells per cell line over the noted time course. A 5 mg/ml

stock of MTT (3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) was prepared in 1× PBS, pH 7.8 and added at 1:10 dilution to cells. Cells were incubated with MTT for 2 h and developed with acidic isopropanol (0.1 M HCl in absolute isopropanol). The absorbance was read using a Wallac-Victor 1420 Multilable Counter (Perkin Elmer, Waltham, MA, USA).

Western blot analysis Cells were treated as described, then washed twice with 1× PBS and lysed using 1× cell lysis buffer supplemented with 1× complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Samples of 20 μg of cleared cell lysate were used for Western blot analysis. Antibodies used for Western blotting according to the manufacturers' protocols included β-actin (C4) and AR (N-20) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), E-cadherin and N-cadherin from BD Biosciences (San Jose, CA, USA), and phospho-Thr308-AKT and phospho-p44/42 (Thr202/Tyr204) ERK1/2 (E10), along with a-AKT (pan-11E7) and p44/42 ERK1/2 loading controls, from Cell Signaling Technologies. Immunoprecipitations of phosphorylated AR were carried out with PC3$(AR)_2$ cells pre-treated with 10 nM DHT and/or 200 nM wortmannin for 30 min, then stimulated for 10 min with 10 ng/ml EGF and lysed in immunoprecipitation lysis buffer (0.1 M Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1× complete protease inhibitors). A sample of 200 μg of cleared cell lysate was used per AR immunoprecipitation assay. AR immunoprecipitations were carried out overnight at 4°C. An 8% protein A Sepharose slurry was added to each sample and incubated at room temperature for 90 min. Beads were then washed three times with wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Tween 20) and resuspended in 100 μl of 1× SDS gel loading buffer. Samples were denatured by boiling, resolved on a 4–12% SDS acrylamide gel, and transferred to nitrocellulose membranes for Western blot analysis. A phosphoserine-specific AR antibody (p-AR 156C135.2, Santa Cruz Biotechnology) was used to determine the phosphorylated state of AR.

References

Bakin, A.V., Tomlinson, A.K., Bhowmick, N.A., Moses, H.L., and Arteaga, C.L. (2000). Phosphatidylinositol 3-kinase function is required for transforming growth factor β-mediated epithelial to mesenchymal transition and cell migration. J. Biol. Chem. *275*, 36803–36810.

Bakin, R.E., Gioeli, D., Sikes, R.A., Bissonette, E.A., and Weber, M.J. (2003). Constitutive activation of the Ras/mitogen-activated protein kinase signaling pathway promotes androgen hypersensitivity in LNCaP prostate cancer cells. Cancer Res. *63*, 1981–1989.

Balk, S.P., Ko, Y.J., and Bubley, G.J. (2003). Biology of prostate-specific antigen. J. Clin. Oncol. *21*, 383–391.

Bonaccorsi, L., Carloni, V., Muratori, M., Formigli, L., Zecchi, S., Forti, G., and Baldi, E. (2004a). EGF receptor (EGFR) signaling promoting invasion is disrupted in androgen-sensitive prostate cancer cells by an interaction between EGFR and androgen receptor (AR). Int. J. Cancer *112*, 78–86.

Bonaccorsi, L., Marchiani, S., Muratori, M., Carloni, V., Forti, G., and Baldi, E. (2004b). Signaling mechanisms that mediate invasion in prostate cancer cells. Ann. NY Acad. Sci. *1028*, 283–288.

Boyer, B., Valles, A.M., and Edme, N. (2000). Induction and regulation of epithelial-mesenchymal transitions. Biochem. Pharmacol. *60*, 1091–1099.

Ferguson, R.A., Yu, H., Kalyvas, M., Zammit, S., and Diamandis, E.P. (1996). Ultrasensitive detection of prostate-specific antigen by a time-resolved immunofluorometric assay and the Immulite immunochemiluminescent third-generation assay: potential applications in prostate and breast cancers. Clin. Chem. *42*, 675–684.

Gao, H., Ouyang, X., Banach-Petrosky, W.A., Gerald, W.L., Shen, M.M., and bate-Shen, C. (2006). Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer. Proc. Natl. Acad. Sci. USA *103*, 14477–14482.

Gelmann, E.P. (1996). Androgen receptor mutations in prostate cancer. Cancer Treat. Res. *87*, 285–302.

Gioeli, D., Ficarro, S.B., Kwiek, J.J., Aaronson, D., Hancock, M., Catling, A.D., White, F.M., Christian, R.E., Settlage, R.E., Shabanowitz, J., et al. (2002). Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. J. Biol. Chem. *277*, 29304–29314.

Grille, S.J., Bellacosa, A., Upson, J., Klein-Szanto, A.J., van Roy, F., Lee-Kwon, W., Donowitz, M., Tsichlis, P.N., and Larue, L. (2003). The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res. *63*, 2172–2178.

Grossmann, M.E., Huang, H., and Tindall, D.J. (2001). Androgen receptor signaling in androgen-refractory prostate cancer. J. Natl. Cancer Inst. *93*, 1687–1697.

Hakariya, T., Shida, Y., Sakai, H., Kanetake, H., and Igawa, T. (2006). EGFR signaling pathway negatively regulates PSA expression and secretion via the PI3K-Akt pathway in LNCaP prostate cancer cells. Biochem. Biophys. Res. Commun. *342*, 92–100.

Heisler, L.E., Evangelou, A., Lew, A.M., Trachtenberg, J., Elsholtz, H.P., and Brown, T.J. (1997). Androgen-dependent cell cycle arrest and apoptotic death in PC-3 prostatic cell cultures expressing a full-length human androgen receptor. Mol. Cell. Endocrinol. *126*, 59–73.

Hernandez, J. and Thompson, I.M. (2004). Prostate-specific antigen: a review of the validation of the most commonly used cancer biomarker. Cancer *101*, 894–904.

Huang, Y.W., Wang, L.S., Chang, H.L., Ye, W., Shu, S., Sugimoto, Y., and Lin, Y.C. (2006). Effect of keratinocyte growth factor on cell viability in primary cultured human prostate cancer stromal cells. J. Steroid Biochem. Mol. Biol. *100*, 24–33.

Kato, S., Sato, T., Watanabe, T., Takemasa, S., Masuhiro, Y., Ohtake, F., and Matsumoto, T. (2005). Function of nuclear sex hormone receptors in gene regulation. Cancer Chemother. Pharmacol. *56* (Suppl. 1), 4–9.

Kim, J. and Coetzee, G.A. (2004). Prostate specific antigen gene regulation by androgen receptor. J. Cell. Biochem. *93*, 233–241.

Larue, L. and Bellacosa, A. (2005). Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3′ kinase/AKT pathways. Oncogene *24*, 7443–7454.

Lee, J.M., Dedhar, S., Kalluri, R., and Thompson, E.W. (2006). The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J. Cell Biol. *172*, 973–981.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943–1947.

Li, L., Ittmann, M.M., Ayala, G., Tsai, M.J., Amato, R.J., Wheeler, T.M., Miles, B.J., Kadmon, D., and Thompson, T.C. (2005). The emerging role of the PI3-K-Akt pathway in prostate cancer progression. Prostate Cancer Prostatic Dis. *8*, 108–118.

Lin, H.K., Hu, Y.C., Yang, L., Altuwaijri, S., Chen, Y.T., Kang, H.Y., and Chang, C. (2003). 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. *278*, 50902–50907.

Lin, H.K., Yeh, S., Kang, H.Y., and Chang, C. (2001). Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc. Natl. Acad. Sci. USA *98*, 7200–7205.

McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., et al. (2006). Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv. Enzyme Regul. *46*, 249–279.

Pienta, K.J. and Bradley, D. (2006). Mechanisms underlying the development of androgen-independent prostate cancer. Clin. Cancer Res. *12*, 1665–1671.

Pousette, A., Grande, M., Carlstrom, K., and Stege, R. (1999). Tissue PSA is the best predicting variable for the outcome of endocrine treatment of prostatic carcinoma. Scand. J. Clin. Lab. Invest. Suppl. *229*, 27–32.

Rajalingam, K., Schreck, R., Rapp, U.R., and Albert, S. (2007). Ras oncogenes and their downstream targets. Biochim. Biophys. Acta *1773*, 1177–1195.

Shand, R.L. and Gelmann, E.P. (2006). Molecular biology of prostate-cancer pathogenesis. Curr. Opin. Urol. *16*, 123–131.

Suzuki, H., Ueda, T., Ichikawa, T., and Ito, H. (2003). Androgen receptor involvement in the progression of prostate cancer. Endocr. Relat. Cancer *10*, 209–216.

Taplin, M.E. and Balk, S.P. (2004). Androgen receptor: a key molecule in the progression of prostate cancer to hormone independence. J. Cell. Biochem. *91*, 483–490.

Taplin, M.E., Bubley, G.J., Shuster, T.D., Frantz, M.E., Spooner, A.E., Ogata, G.K., Keer, H.N., and Balk, S.P. (1995). Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N. Engl. J. Med. *332*, 1393–1398.

Trapman, J. and Cleutjens, K.B. (1997). Androgen-regulated gene expression in prostate cancer. Semin. Cancer Biol. *8*, 29–36.

Van Bokhoven, A., Varella-Garcia, M., Korch, C., Johannes, W.U., Smith, E.E., Miller, H.L., Nordeen, S.K., Miller, G.J., and Lucia, M.S. (2003). Molecular characterization of human prostate carcinoma cell lines. Prostate *57*, 205–225.

Wang, S.C. and Hung, M.C. (2001). HER2 overexpression and cancer targeting. Semin. Oncol. *28*, 115–124.

Xin, L., Teitell, M.A., Lawson, D.A., Kwon, A., Mellinghoff, I.K., and Witte, O.N. (2006). Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. Proc. Natl. Acad. Sci. USA *103*, 7789–7794.

Yang, L., Wang, L., Lin, H.K., Kan, P.Y., Xie, S., Tsai, M.Y., Wang, P.H., Chen, Y.T., and Chang, C. (2003). Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells. Biochem. Biophys. Res. Commun. *305*, 462–469.

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