Crosstalking between Androgen and PI3K/AKT Signaling Pathways in Prostate Cancer Cells

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Running Title: Interplay between androgen and PI3K/AKT pathways

Key Words: androgen receptor, PTEN, PI3K, AKT, prostate cancer, mouse models

Background: An interaction between androgen and PI3K/AKT pathways has been implicated in prostate cancer cells. Conditional expression of AR transgene represses PI3K/Akt activation, and Pten loss results in reduced AR expression and transcriptional activity.

Results: Conditional expression of AR transgene represses PI3K/Akt activation, and Pten loss results in reduced AR expression and transcriptional activity.

Conclusion: Both androgen and PI3K/AKT pathways inversely regulate each other in prostate cancer cells.

Significance: Interplay between androgen and PI3K/AKT pathways may directly contribute to prostate tumorigenesis.

ABSTRACT

Both androgen action and PI3K medicated signaling pathways have been implicated in prostate tumorigenesis. Our androgen receptor (AR) conditional transgenic mice developed murine prostatic intraepithelial neoplasia (mPIN) and prostatic adenocarcinoma lesions recapitulating human prostate cancer development and progression. Role of transgenic AR contributing to malignancy was demonstrated by high degree of transgenic AR expression in atypical and tumor cells in mPIN as well as prostatic adenocarcinoma lesions of the transgenic mice, but not in adjacent normal tissue. Interestingly, reduced PI3K/Akt activation also appeared in these mouse atypical and tumor cells, suggesting an interaction between androgen and PI3K/AKT pathways. In this study, we further investigated this interaction. We showed that the androgen depletion or knockdown of AR expression results in elevated levels of active phosphorylated AKT in prostate cancer cells. Castration of conditional Pten knockout mice showed increased Akt, phosphorylated Akt and pS6 expression in the mouse prostate. Using a series of newly generated Ar reporter and Pten knockout compound mice, we showed that Pten loss directly represses endogenous Ar expression in prostatic epithelial cells. Moreover, Pten loss and PI3K/Akt activation reduced Ar mediated transcription in purified Pten null cells. This study provides novel evidence demonstrating interplay between androgen and PI3K pathways, as well as introduces unique and relevant mouse models for further studies of PI3K and AR pathways in the context of prostate tumorigenesis.

INTRODUCTION

The androgen-signaling pathway is essential for normal prostate growth and differentiation and for prostate cancer initiation and progression (1,2). It is mediated through the androgen receptor (AR) and its ligands, testosterone and 5α-dihydrotestosterone (DHT) (3,4). It is believed that AR is critical for prostate cancer cell proliferation as most prostate cancers are androgen-dependent and androgen ablation therapy (ADT) can result in prostate cancer cell apoptosis (1,2). Androgen receptor is expressed in most prostate cancer samples before and after androgen ablation therapy (5). AR gene amplification appears mainly in post ADT prostate cancer samples (6). Global gene expression profiling further shows that the AR is one of several genes to be consistently up-regulated in castration resistant prostate cancer (CRPC) (7,8).
which directly underscores the significance of androgen signaling in disease progression. Additionally, recent integrative genomic analysis revealed a significant enrichment of androgen signaling in early-onset prostate cancers, but not in elderly-onset prostate cancers (9), further highlighting the significance of AR across the prostate tumorigenesis spectrum.

The phosphatidylinositol (3,4,5)-phosphate-kinase (PI3K) signaling pathway plays a critical role in human tumorigenesis, including prostate cancer (10). The phosphatase and tensin homolog chromosome 10 (PTEN) is one of the most commonly mutated and/or deleted tumor suppressors (11,12) and its somatic mutations were frequently detected in many sporadic human tumors, including glioblastoma, endometrial cancer, and prostate cancer (13). PTEN functions as a negative regulator of the PI3K pathway by blocking the activation of the kinase AKT/PKB (14,15). Complete PTEN inactivation has been found in 15% of primary prostate tumors, and in up to 60% of prostate cancer metastases (8,16-18). The biological significance of Pten in prostate tumorigenesis has been further confirmed in mouse models (19-22). In particular, conditional deletion of Pten in mouse prostate tissues results in invasive prostate cancers (20,23).

We have previously shown that conditional expression of human AR transgene in the mouse prostate induces the development of prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma lesions in R26hAR/Cre:Osrl-Cre mice (24). In this model, transgenic AR expression was detected in atypical and tumor cells in both murine PIN (mPIN) and murine prostatic adenocarcinomas. Interestingly, we also observed decreased Akt activation in atypical and tumor cells of these transgenic mice, suggesting an interaction between the PI3K/Akt and androgen/AR signaling pathways in murine prostate tumorigenesis. In this study, we used a series of in vitro and in vivo experimental approaches to scrutinize this interaction. We showed that the depletion of androgens, presence of antiandrogens, or knockdown of endogenous AR expression results in elevated phosphorylated AKT (pAKT) expression. Castration of conditional Pten knockout mice elevated total Akt levels, as well as levels of phosphorylated Akt (pAkt), and phosphorylated S6 (pS6) expression in prostate cancer cells. Using the newly developed Ar-IRESPten-loxp/LoxP:PB-Cre4 (Ar-IPInL:Pten+/LoxP::PB-Cre) and Ar-IRESPten-loxp/LoxP:Osrl-Cre (Ar-IPInL:Pten+/LoxP::Osrl-Cre) mice, we further demonstrated the effect of decreased endogenous Ar expression in Pten null prostate cells, suggesting a repressive role for Ar over activated PI3K signaling under endogenous AR expression conditions. Using another new mouse line, mT/mGloxP/loxP::Pten-loxp/loxP::PB-Cre4, in which Pten deletion and membrane-targeted green fluorescent protein (mG) expression are controlled simultaneously through Cre-mediated recombination (25), we observed increased Akt activation and downregulation of Ar’s downstream target gene expression in prostatic Pten null cells from these mice. Taken together, these data demonstrate a functional interaction between PI3K/AKT and androgen/AR signaling pathways in prostate cancer cells.

**EXPERIMENTAL PRODUCERES**

**Mouse Breeding and Castration.** Mice homozygous for floxed Pten exon 5, Pten-loxp/loxP, on a 129/Balb/c background, were obtained from the Jackson Laboratory (Strain#: 004597, Bar Harbor, ME). They were crossed with the Osrl-Cre strain, a kind gift of Dr. Gail Martin at the University of California at San Francisco (26), and PB-Cre4 mice, driven by a modified probasin promoter (ARR2PB) on a C57BL/6xDBA2 background (27). Pten-loxp/loxP::Osrl-Cre mice were backcrossed more than five times with C57BL/6J mice and then used for mating to produce homozygous (Pten-loxp/loxP::Osrl-Cre) mice. Using similar mating strategies, we also generated Pten conditional knockout mice with PB-Cre4 mice. Female mice homozygous for Ar reporters, Ar-IPInL on a C57BL/6 background, in which both nuclear targeted LacZ (nLacZ) and placental alkaline phosphatase (PLAP) were inserted into the 3’ untranslated region of endogenous Ar gene with the internal ribosome entry sites (IRESs) (28), were obtained from Jackson Laboratory (Strain#: 012374, Bar Harbor, ME). Ar-IPInL::Pten-loxp/loxP::PB-Cre4 or Ar-IPInL::Pten-loxp/loxP::Osrl-Cre mice were generated by crossing Pten-loxp/loxP::PB-Cre4 or Pten-loxp/loxP::Osrl-Cre males and Ar-IPInL::Pten-loxp/loxP females. The
double-fluorescent mT/mG reporter strain was kindly provided by Dr. Liqun Luo at Stanford University (25), and used it to generate mT/mGloxp/loxP::PtenloxP/loxP:PB-Cre4 compound mice. R26hARloxP::Rosa26hARloxP Cre mice were generated as described in our previous report (24). For castration, the mice were anesthetized by IP injection of Ketamine and Xylazine. Both testicles and epididymis were removed through a scrotal approach. The distal end of the spermatic cord was ligated with silk thread as described previously (29). We then examined castrated mice with age-matched intact mice as controls after 8 weeks. All animal experiments performed in this study were approved by the ethics committee of the Administrative Panel on Laboratory Animal Care at Stanford University.

**Histological Analyses, Immunohistochemistry, Immunofluorescence, and β-gal, staining.** Mouse tissues were fixed in 10% neutral-buffered formalin and processed into paraffin, and 5 µm serial sections were cut and processed from xylene to water through a decreasing ethanol gradient followed by 0.1 M PBS for histological, immunohistochemical and immunofluorescence analyses (23,24). For immunohistochemistry and immunofluorescence, tissue slides were blocked with 0.3% hydrogen peroxide in methanol for 15 min and 5% goat serum in PBS for 30 minutes at room temperature. They were then exposed to different first antibody in PBS with 1% goat serum at 4°C overnight, including anti-human AR (sc-7305, Santa Cruz), anti-mouse/human AR (sc-816, Santa Cruz), anti-pAKT (4060, Cell Signaling), anti-AKT (9272, Cell Signaling), anti-ki67 (NCL-ki67, Novacastra), anti-pS6 (2211, Cell Signaling), anti-FKBP5 (ab46002, Abcam), anti-PHLPP (ab71972, Abcam), and anti-PTEN (9559, Cell Signaling) antibodies. For immunohistochemistry, slices were then incubated with biotinylated anti-rabbit or antimouse secondary antibody (BA-1000 or BA-9200, Vector Laboratories) for 1 hour, horseradish peroxidase streptavidin (SA-5004, Vector Laboratories) for 30 min, and then visualized by DAB kit (SK-4100, Vector Laboratories) at room temperature. All samples were subsequently counterstained with 5% (w/v) Harris Hematoxylin. For immunofluorescence analyses, slides were incubated with appropriate Alexa Fluor 488- or 594-conjugated secondary antibodies diluted 1:500 in blocking solution for 2 hours at room temperature, washed, and mounted on slides with VECTASHIELD Mounting Medium with DAPI (H-1200, Vector Laboratories). Coverslips were mounted using Permount Mounting Medium (Cat#SP15-500, Fisher Scientific, Pittsburg, PA). All images were obtained using a Nikon Eclipse E800 Epi-fluorescence microscope and Adobe Photoshop CS5.

For β-gal staining, mouse prostate tissues were embedded in OCT compound (Tissue-Tek) for snap freezing. Frozen blocks were sectioned at 7 µm intervals and fixed in 0.2% glutaraldehyde solution at 4°C for 10 min (30). Slides were washed three times at room temperature for 15 min in buffer (0.1 M phosphate buffer, 0.02% Nonidet P-40, 0.01% sodium deoxycholate) and stained with 1 mg/ml X-gal staining solution (washing buffer with 5 mM potassium ferrocyanide and 5 mM potassium ferricyanide) at room temperature for overnight. All slides were subsequently counterstained with the Nuclear Fast Red (H-3404, Vector Laboratories).

**Prostatic Epithelial Cell Isolation and Purification.** Prostate tissues from mT/mGloxp/loxP::Pten+/+;PB-Cre4 and mT/mGloxp/loxP::PtenloxP/loxP:PB-Cre4 mice were minced into 1 mm3 pieces, digested in DMEM/Collagenase/FBS for 3 hours at 37°C, and 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA) on ice for 1 hour. Digested cells were dissociated with pipetting and then passed through 70 µm cell strainers (BD Biosciences, San Jose, CA) to make single cell suspensions. GFP-positive cells were sorted through a FACS AriaII cytometer (BD Biosciences).

**RNA Isolation and Reverse Transcription (RT)-Quantitative Real-Time PCR (qRT-PCR) Assays.** Single cell suspensions prepared above were lysed in RNA-Bee (TEL-TEST, Inc., Friendswood, TX) and total RNA was isolated as recommended by the manufacturer (31). For reverse transcription, cDNA was synthesized from 10 µg of total RNA with 15 units of avian myeloblastosis virus reverse transcriptase (M5108, Promega) with 0.5 µg of random primer (C1181, Promega) in a total volume of 20 µl. For qRT-PCR, cDNA samples were mixed with SYBR
qPCR Super Mix Universal (11762, Invitrogen) with specific primers in the MX 3005P thermocycler (Stratagene). Relative mRNA levels were calculated by Delta Delta C(T) method (32). Reactions were done in triplicate and the values were normalized by the GAPDH expression levels. Primers for FKBPs (5'-TGAGGGCACCAGTAACAATGG-3'; 5'-CAACATCCCTTTGATGCGACAT-3'), Probasin (5'-ATTGAGAACCTACTTCCGTACA-3'; 5'-CAGTTGGCAGTCTAGTACTCTTCA-3'), Nkx3.1 (5'-CCGGGAGCACACCAAGAT-3'; 5'-CCTGGATATTGTTCAGATCTAA-3'), Msmb (5'-TGGCTGGGACTTCTTATATC-3'; 5'-CAGGGAGTGTTAAGGAAATGCTT-3'), Phlp (5'-CCGGGAAGACCTCGGCTTTAC-3'; 5'-CTGCCATCGCCTTATCGTCTC-3'), Ar (5'-TGCCCCGATACGAAAGTGTC-3'; 5'-TTGGCGTAACTCCCTTGGAA-3'), and GAPDH (5'-AGGTCTGGGTGTAAGGAAATGCTT-3'; 5'-TCACGTACATGATGTAGTGGACAT-3') were used in the above qRT-PCR reactions, respectively.

**Cell Cultures and Lentivirus Production/Infection.** The human embryonic kidney cell line, HEK293, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) (HyClone, Denver, CO). LNCaP and LNCaP C4-2 cell lines were maintained as described previously (33,34). For 5α-dihydrotestosterone (DHT) and Flutamide, an antiandrogen, treatments, the cells were incubated in DMEM supplemented with 5% charcoal stripped fetal bovine serum (CS-FBS) for 40 hours, then further grown in the presence of 10 nM DHT with or without 1 μM Flutamide for 8 hours. The AR shRNA constructs were created by inserting double-stranded oligonucleotides corresponding to the human AR cDNA sequences 5'-GGACACTTTGAAACTGCGTCTC-3' [amino acids (aa) 335–342, AR shRNA1], and 5'-GGTGTCACTATGGAGCTTCCTC-3' (aa 568–575, AR shRNA2) downstream of U6 promoter in the pB5/U6 vector (35). To generate shRNA lentiviruses, pLenti-shRNA vectors, pCMV-dR8.91, and pMD2.G-VSVG plasmids were co-transfected into HEK293T cells at a ratio of 3:2:1 using a Lipofectamine kit (Invitrogen, Carlsbad, CA). The media were replaced at 6 hours post-transfection and then collected after 36 to 40 hours. The viral supernatant was centrifuged briefly to remove cellular debris and stored at -80 °C. Lentivirus infection was carried out in the presence of 6 mg/ml Polybrene for 8 hours.

**Western Blotting.** Cells were harvested and then lysed in a buffer containing 0.5% Nonidet P-40, 150 mM NaCl, 2 mM MgCl2, 50 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25 mM NaF, and 1 mM sodium orthovanadate. After SDS-PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.08% Tween 20) with 5% dry nonfat milk. Membranes were probed with AR (sc-816, Santa Cruz), pAKT (4060, Cell Signaling) or AKT (9272, Cell Signaling) antibody. Anti-rabbit IgG conjugated to horseradish peroxidase were used as secondary antibodies (Promega). Detection was performed with ECL reagents according to the manufacturer’s protocol using ECL Hyperfilm (Amersham Biosciences). Densitometry of the protein bands was performed using ImageJ software, and the relative numbers were reported as OD units of pAKT/OD units of AKT.

**Statistical Analyses.** Data are shown as the mean ± SD. Differences between groups were examined by 2-tailed Student’s t test or 2-way ANOVA for comparisons among multiple groups. For all analyses, P < 0.05 was considered statistically significant.

**RESULTS**

Conditional expression of AR transgene results in decreased Akt activation in mouse prostatic tumor cells. The Osr1 (odd-skipped-related 1) promoter activates at E11.5 in urogenital sinus epithelium and retains its activity in prostatic epithelium throughout development (26). Conditional expression of human AR transgene in the prostates of R26hAR<sup>Cre</sup>:Osr1-<sup>Cre</sup> mice displayed PIN and prostatic adenocarcinoma lesions (24). In order to fully understand the role of transgenic AR in prostate oncogenesis, we performed a series of experiments to establish additional cell signaling pathways that promote...
oncogenic transformation in the prostate of \( R26\alpha\text{AR}^{\text{Int}}:\text{Osr1-Cre} \) mice. We observed that transgenic AR expression in prostatic adenocarcinoma cells in \( R26\alpha\text{AR}^{\text{Int}}:\text{Osr1-Cre} \) mice (Fig. 1A to 1B') have robust Ki67 expression (Fig. 1C and 1C') and expression of Akt also appears in these cells (Fig. 1D and D'). However, these tumor cells show very weak or no immunoreactivity with antibodies against either pAkt or pS6, a downstream target of the Akt pathway (Fig. 1E to 1F'), suggesting inactivated Akt signaling in prostatic adenocarcinoma cells of \( R26\alpha\text{AR}^{\text{Int}}:\text{Osr1-Cre} \) mice. A reciprocal regulation between PI3K and androgen signaling pathways has been demonstrated in prostate cancer cells and in the prostates of conditional \( \text{Pten} \) knockout mice (36,37). Recent studies demonstrated that an androgen-regulated gene FKBP5 promotes PHLPP dephosphorylation of AKT and as a result suppresses AKT activity (37,38). To establish whether transgenic AR expression also increases the expressions of FKBP5 and PHLPP proteins, we examined both protein expressions in prostate cancer cells of \( R26\alpha\text{AR}^{\text{Int}}:\text{Osr1-Cre} \) mice. We observed high levels of Fkbp5 and Phlpp proteins in prostate cancer cells of \( R26\alpha\text{AR}^{\text{Int}}:\text{Osr1-Cre} \) mice (Fig. 1G1 to 1H2'). Our data are consistent with the previous observation and suggest that AR-induced Fkbp5 and Phlpp expression is involved in repressing pAkt expression.

**Depletion of androgens elevates AKT activity in prostate cancer cells.** We next evaluated the role of androgen in the regulation of AKT signaling activation in prostate cancer cells. We first analyzed the effects of androgens on AKT expression and activation in LNCaP cells, an AR positive and \( \text{Pten} \) null prostate cancer cell line (11,12). LNCaP cells were cultured in the presence or absence of \( \text{5a-dihydrotestosterone} \) (DHT), or in the presence of DHT and Flutamide, an antiandrogen. Western blotting analyses showed no significant change in both endogenous AR and AKT expression in the whole cell lysates isolated from LNCaP cells among different treatments (Fig. 2A & 2E1). However, decreased expression of pAKT was detected in cells cultured in the presence of DHT, suggesting a repressive role of androgens in AKT activation. We then analyzed the effect of androgens on AKT expression and activation in LNCaP C4-2 cells, which are derived from LNCaP cells passaged in a castrated host (39). Consistent with previous reports we found a slightly higher AR expression in samples in the presences of DHT (Fig. 2B) (39). However, there is no significant change in either AKT or pAkt expression between samples with different treatment (Fig. 2B, 2E2). To further explore the effects of AR on AKT expression and activation, we performed knockdown experiments using two short hairpin RNAs against AR. Both LNCaP and LNCaP C4-2 cells were transduced with either AR shRNA or Swabled shRNA lentiviruses as a control. A significant reduction of endogenous AR expression was shown in cells transduced with AR shRNA lentiviruses (Fig. 2C and 2D). While there is no notable change in AKT expression levels between samples transduced with either AR shRNA or controls in both LNCaP and C4-2 cells, we observed an increase of pAKT in LNCaP cells transduced with AR shRNA viruses (bottom panel, Fig. 2C). Densitometry of both AKT and pAKT protein bands in each sample was performed and the relative numbers were reported as OD units of pAKT/OD units of AKT (Fig. 2E1-4). Interestingly, addition of antiandrogen, and knockdown of AR expression increase AKT activation in LNCaP but not C4-2 cells, suggesting an alternative role for AR in these prostate cancer cells.

As a tumor suppressor, PTEN can counteract PI3 kinase in repressing AKT signaling in cell growth and tumor formation (14,15). Conditional deletion of Pt\( \text{ten}^{\text{L/L}}:\text{Osr1-Cre} \) mice resulted in high-grade PIN and invasive prostatic tumor development (23). We further evaluated the effect of androgen signaling on Akt expression and activation in prostate tissues isolated from castrated mice for 8 weeks and age-matched intact controls. As shown in Figure 3A and 3A', deletion of Pt\( \text{ten} \) was confirmed in both intact and castrated prostate tissues isolated from Pt\( \text{ten}^{\text{L/L}}:\text{Osr1-Cre} \) mice. Using specific antibodies against Akt and pAkt, we assessed expression and activity of Akt in the above prostate tissues. While both Akt and pAkt staining appears in the lesions of both intact and castrated mice, more intense immunoreactivity to Akt and pAkt antibodies is observed in castrated mice (Fig. 3B' and 3C' versus 3B and C). Staining of the above samples with an antibody
against pS6, also showed strong immunoreactivity in the lesions of castrated mice (Fig. 3D’ versus 3D). We also observed a typical nuclear staining pattern of AR in tumor cells of intact mice (Fig. 3E), and in contrast a diffuse and cytoplasmic staining pattern of AR in castrated mice (Fig. 3E’). There is no significant change in the staining of Ki67 between castrated and intact mice (Fig. 3F and 3F’). These data demonstrate that castration elevates Akt and pAkt expression in prostate cancer cells of Pten<sup>L/L</sup>:Osr1-Cre mice.

**Reduced Ar expression in Pten null prostate tumor cells.** Previous studies have shown that Pten loss attenuates androgen signaling (36,37). However, the effect of Pten loss on endogenous Ar expression has not been fully investigated in a biologically relevant system. For this reason, we intercrossed Pten<sup>L/L</sup>:Osr1-Cre or Pten<sup>L/L</sup>:PB-Cre mice to Ar-IRE-PLAP-IRE-MAcZ (Ar-IPIL) mice (Fig. 4A), in which both nuclear targeted LacZ (nLacZ) and placental alkaline phosphatase (PLAP) were inserted into the 3′ untranslated region of endogenous Ar gene with the internal ribosome entry sites (IRESs) (28). These new mouse models enabled us to directly assess the effect of Pten loss on Ar expression. We first analyzed 6-week-old Ar-IPIL/Pten<sup>L/L</sup>:PB-Cre4 mice. As shown in Figure 4B and 4C, strong LacZ staining was observed in normal prostate glands. In contrast, adjacent atypical prostate glands showed much weaker staining (Fig. 4B’ and 4C’). At high magnification, robust nuclear and cytoplasmic LacZ staining was observed in normal prostatic luminal cells (blue arrows, Fig. 4B and 4C) in comparison to few scattered blue atypical cells in mPIN areas (pink arrows, Fig. 4B’ and 4C’). With immunohistochemistry, we analyzed endogenous Ar and Pten expression using adjacent sections of the above tissues. We observed stronger nuclear expression of Ar protein in normal luminal cells than atypical cells (Fig. 4D and 4D’). In contrast, Pten expression appears much weaker in atypical cells than normal luminal epithelial cells (Fig. 4E and 4E’). Consistently, we detected much stronger pAkt staining in atypical cells of PIN lesions than in prostatic luminal cells of adjacent normal glands (Fig. 4F-G’). Taken together, these data demonstrate that Pten loss significantly reduces endogenous Ar expression in prostatic atypical cells.

The PB-Cre4 mice have been frequently used in the field of prostate cancer research, in which a composite promoter, ARR2PB, drives Cre transgene expression, which is androgen dependent and derived from the rat prostate specific probasin gene (27). This introduces a possibility that the PB-Cre4 induced Pten loss may be influenced diversely by autonomous Ar expression. For this reason, we further assessed the effect of Pten loss in endogenous Ar expression using another prostatic conditional Pten deletion mouse model, Ar-IPIL/Pten<sup>L/L</sup>:Osr1-Cre. The Osr1 promoter is not androgen dependent and is activated at E11.5 in the urogenital sinus epithelium (26). Pten<sup>LoxP/LoxP</sup>:Osr1-Cre mice developed high-grade PINs with high penetrance as early as one-month of age, and locally invasive prostatic tumors after 12-months of age (23). Histological analyses showed intracystic carcinoma lesions in the prostates of Ar-IPIL/Pten<sup>LoxP/LoxP</sup>:Osr1-Cre mice (Fig. 5A and 5A’), which were very similar to the changes that we previously observed in Pten<sup>LoxP/LoxP</sup>:Osr1-Cre mice (23). Consistently, Pten staining is much weaker in tumor areas than in normal prostatic glands (Fig. 5F and 5F’). To evaluate endogenous Ar expression in the mice, we first examined LacZ expression and observed a robust staining in normal prostatic cells but no or very weak staining in tumor cells (Fig. 5B and 5B’). Immunohistochemical analyses also showed a strong Ar signal in normal prostatic cells in comparison to tumor cells (Fig. 5C and 5C’). Again, in contrast, strong expression of pAkt and pS6 appears in prostatic intracystic tumor cells but weak or no expression is observed in normal prostatic epithelial cells (Fig. 5D to E’). Taken together, our observation of decreased expression of endogenous Ar in Pten null prostatic tumor cells in Ar-IPIL/Pten<sup>LoxP/LoxP</sup>:Osr1-Cre mice further demonstrates a role of Pten loss in endogenous Ar expression.

**Repressing Ar transcriptional activity in Pten null tumor cells.** In the double-fluorescent mT/mG Cre reporter mouse strain, expression of membrane targeted tandem dimer Tomato (mT) or membrane-targeted green fluorescent protein (mG) is controlled by Cre-mediated recombination (25). Therefore, with an appropriate Cre expression, mT/mG expression can be employed to trace and isolate specific cell populations that have
undergone Cre-driven recombination (25). In this study, we developed mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 mice that enable us to label and isolate mG positive and Pten null prostatic tumor cells (Fig. 6A). We first analyzed mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 and mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 mice as controls using co-immunofluorescence microscopy. We observed specific mG protein expression in normal prostatic epithelial cells and atypical cells in PIN lesions in mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 or mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 mice, respectively (Fig. 6B1 and 6C1). However, robust expression of pAkt was only observed in atypical cells in PIN lesions of mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 mice (Fig. 6C2), which fully overlapped with mG expression (Fig. 6C3). However, we did not detect pAkt staining in the prostate of mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 mice (Fig. 6B2 and 6B3). We proceeded to isolate these mG positive epithelial cells from both mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 and mT/mG\textsuperscript{loxP/+}/Pten\textsuperscript{loxP/loxP}::PB-Cre4 mice and collected total RNA to determine the effect of Pten loss on androgen signaling. Approximately 180,000 mG positive cells were obtained through cell sorting (Fig. 6D and 6E). Subsequent qRT-PCR analysis revealed that endogenous Ar expression was reduced in samples isolated from mG positive and Pten null cells in comparison to ones from control mG positive cells (Fig. 6F). Expression of Fkbp5, Nkx3.1, Probasin, Msmb, and Phlp1, all established Ar downstream targets, was significantly less in mG positive and Pten null cells than in control mG positive cells (Fig. 6F). These data further reveal that Pten loss results in PI3K/Akt activation and subsequently represses endogenous Ar expression and its transcriptional activity in prostatic cells.

**DISCUSSION**

The androgen-signaling pathway is essential in the pathogenesis of prostate cancer (40,41). AR is a member of the nuclear hormone receptor superfamily (3,4) and regulates its downstream target expression in a ligand-dependent manner (42,43). This in turn controls prostate development and tumorigenesis. Thus, androgen deprivation therapy has been developed and routinely used to treat prostate cancer (44). Despite substantial effort that has been devoted in past decades, the precise molecular mechanisms by which androgen signaling promotes prostate cancer initiation and progression still remain largely unknown. As reported previously, we developed a conditional AR transgenic mouse line, R26hAR\textsuperscript{loxP/loxP}::Osr1-Cre, in which the human AR transgene with a LoxP-stop-loxP (LSL) cassette was activated through Osr-1 promoter mediated Cre activity (24). These transgenic mice developed both PIN and prostatic adenocarcinoma lesions in a manner akin to human prostate cancer. In the process of searching for additional cell signaling players in the context of AR mediated oncogenic transformation in this mouse model, we observed that transgenic AR expression inactivated Akt signaling in both prostatic atypical and tumor cells. Our observation demonstrated a possible role of androgen signaling in repressing PI3K/AKT activation.

Within the in vitro scope of this work, we examined the effect of AR/androgen signaling in phosphorylated AKT in two Pten null prostate cell lines, LNCaP and LNCaP C4-2. Interestingly, we observed that either depleting androgens or adding Flutamide, an antiandrogen, induces phosphorylation of AKT in LNCaP, but not in LNCaP C4-2 cells, which are derived from LNCaP cells passaged in a castrated host (39). Furthermore knockdown of endogenous AR expression with two different AR shRNAs also increased phosphorylated AKT in LNCaP cells, but not in LNCaP C4-2 cells. Our findings suggest a novel mechanism for prostate cancer progression and CRPC development in which AR signaling is also a mechanism of PI3K/AKT pathway repression.

We further supported our in vitro findings and explored the effect of androgen signaling in Akt activation in vivo. We analyzed Akt expression and activation in the prostates of 10 month old castrated Pten\textsuperscript{loxP/loxP}::Osr1-Cre mice that contain invasive prostatic tumors (23). Interestingly, although there was no significant regression of tumor lesions in castrated mice, an increase in Akt, pAkt, and pS6 expression was observed in tumor cells of castrated mice. Although the precise mechanisms of how AR/androgens regulate Akt expression and activation in prostate cancer cells are currently unclear, decreased Ar in the nucleus in tumor cells
of castrated mice suggests the action mediated through nuclear AR protein may contribute to this cellular event. Because AR expression has been observed in most androgen-insensitive prostate cancer cells (7), it would be of great value to establish AR’s role in androgen insensitive tumor cells. Data generated from these lines of research should further clarify an important but unclear question regarding the interaction between androgen and PI3K/AKT pathways during prostate cancer initiation and progression, leading toward identifying new therapeutic targets for future prostate cancer treatments.

A reciprocal regulation between PI3K and androgen signaling pathways has been implicated in prostate tumorigenesis (36,37). However, the molecular mechanism underlying this regulation remains unclear. In this study, we used a series of technically advanced and biologically relevant mouse models to further determine whether and how Pten loss regulates AR/androgen action in the mouse prostate. We first generated Ar-IPIL/PtenloxPloxP:PB-Cre and Ar-IPIL/PtenloxPloxP:Osr1-Cre mice (28). Because both nuclear targeted LacZ (nLacZ) and placental alkaline phosphatase (PLAP) reporter genes were inserted into the 3’ untranslated region of the endogenous Ar gene with internal ribosome entry sites (IRESs) (28), we could evaluate endogenous Ar expression by examining LacZ reporter expression in either Pten containing or Pten null prostatic cells in a direct and sensitive way. We observed robust LacZ staining in normal prostatic epithelial cells in both of the transgenic mice (Figures 4 and 5). In contrast, only a few scattered LacZ positive atypical or tumor cells appeared in PIN or prostatic adenocarcinoma lesions of PtenloxPloxP:PB-Cre4 or PtenloxPloxP:Osr1-Cre mice, respectively indicative of reciprocal Ar suppression accompanying Pten loss. Using immunohistochemical approaches, we further confirmed reduced endogenous Ar staining in atypical and tumor cells in those mice. As expected, Akt, pAkt, and pS6 staining appeared much stronger in Pten null atypical and tumor cells than in adjacent normal prostatic epithelial cells. Given that nuclear targeted LacZ was inserted into the 3’ untranslated region of the endogenous Ar gene in the above mouse models (28), our observation that both endogenous Ar and LacZ reporter expression was reduced in Pten null prostatic atypical and tumor cells suggests that Pten loss/Akt activation likely suppress endogenous Ar expression at transcriptional level. These newly generated mouse models present a unique opportunity to further interrogate the mechanisms and pathways involved in Pten/Akt mediated endogenous Ar expression and vice versa.

To support our assertions on transcriptional level we developed another new mouse model, mT/mGloxPloxP/PtenloxPloxP:PB-Cre4 mice, to directly and precisely determine the effect of Pten loss on endogenous Ar mediated transcription. In this elegant double-fluorescent mT/mG Cre reporter mouse strain, expression of membrane targeted tandem dimer Tomato (mT) or membrane-targeted green fluorescent protein (mG) is controlled by Cre-mediated recombination (25). Therefore, with the probasin promoter driven Cre expression, mT/mG expression and Pten deletion can occur simultaneously in prostatic epithelial cells, which enables us to trace and isolate Pten null cells. In these mice we observed a significant decrease in the expression of a series of AR downstream target genes in purified mG positive and Pten null prostatic epithelial cells in comparison to mG positive only prostatic epithelial cells (Fig. 6). These data clearly demonstrated that Pten loss represses AR-mediated transcription. Our findings complement published observations in prostate cancer cell lines and human prostate tissues showing that Pten loss and PI3K/AKT activation can repress AR transcriptional activity through either AR co-regulators, EGR1, c-Jun, and EZH2, or HER3 mediated pathways (36,37). However, our data suggest an additional mechanism by which Pten loss may directly reduce AR expression, directly resulting in decreased AR transcriptional activity. More investigation using the above newly developed mouse models should be pursued to define the mechanisms underlying Pten loss and PI3K/AKT activation in AR expression and transcriptional activation as well as other potential synergistic mechanisms that contribute to prostate tumorigenesis.

In this study, we used a series of relevant and advanced mouse models to investigate the interaction between androgen and PI3K/Akt signaling pathways in Pten null prostatic epithelial
cells and animal models. Our data provide several lines of novel evidence demonstrating a reciprocal regulation between PI3K and androgen signaling pathways during prostate cancer initiation and progression. We also provided more comprehensive evidence pointing to a direct role of Pten loss and PI3K/AKT activation in repressing AR expression and activation using biologically relevant mouse models. These mouse models will be further utilized to investigate cellular and molecular events by which the loss of Pten contributed to prostate tumorigenesis. Indubitably, further study of the regulation of the interaction between PI3K and androgen signaling pathways in prostate cancer cells is necessary and should provide fresh insight into the pathogenesis of prostate cancer. This and subsequent work in these presented animal models may enable the identification of novel pathways that can be targeted for prostate cancer prevention and treatment treatment.

Footnotes: We are especially grateful for the Sun lab members for their technical assistance and scientific inputs. This work was supported by Public Health Service grants R01CA070297, R01CA151623, U01CA166894, and R21CA190021 from the National Cancer Institute.

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were analyzed with different antibodies as marked, including the human AR (Arg)) and Ki67 (C), pAkt (E), pS6 (F), FKBP5 (G1 and H1), and PHLPP (G2 and H2). Corresponding high power images (400X) are shown in A'-H'.

Figure 2. Androgen signaling negatively regulates AKT phosphorylation in prostate cancer cells. A-B. LNCaP (A) and LNCaP C4-2 (B) cells were grown in hormone-deprived media supplemented with 5% charcoal stripped fetal bovine serum for 40 hours, then incubated for further 8 hours in the presence of 10 nM DHT with or without 1 μM Flutamide, then harvested for Western blotting assays with anti-AR, anti-AKT, and anti-pAKT antibodies. C-D. LNCaP (C) and LNCaP C4-2 (D) cells were infected with lentivirus encoding shRNA against AR (AR shRNA1 or shRNA2). The infected cells were cultured for 48 hours, then harvested for Western blotting assays with AR, AKT and pAKT antibodies. E. Densitometry of AKT and pAKT protein bands was performed using ImageJ software, and the relative numbers were reported as OD units of pAKT/OD units of AKT.

Figure Legends:

Figure 1. Reduced Akt phosphorylation in prostatic epithelial cells with conditionally expressed transgenic AR. H&E staining (A) and immunohistochemistry (B-H) were performed on prostate tissues from prostatic adenocarcinoma regions of R26hARΔL:Osrl-Cre mice. Adjacent prostate tissue sections were analyzed with different antibodies as marked, including the human AR (B), Ki67 (C), Akt (D), pAkt (E), pS6 (F), FKBP5 (G1 and H1), and PHLPP (G2 and H2). Corresponding high power images (400X) are shown in A'-H'.

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Figure 3. Androgens affect Akt expression and activation in Pten null prostatic cancer cells. 
*Pten*^loxP/loxP*:Osr1-Cre* mice were castrated at 12 month for 8 weeks, and then analyzed with age-matched intact controls. Immunohistochemistry was carried out to address PI3K signaling in Pten null prostate tumors isolated from intact (A-F) and castrated (A'-F') mice. Adjacent prostate tissue slides were analyzed with the antibodies as labeled in the figure.

Figure 4. *Pten* loss represses endogenous Ar expression in the mouse prostate. A. Scheme of the PLAP and nLacZ expression at the AR locus in *Ar-IPInL* mice is shown on an upper figure. A first IRES was inserted in the 3' UTR of the *AR* gene, addressing expression of PLAP. A second IRES was placed 3' of the *PLAP* gene to activate expression of nLacZ. Diagram for conditional *Pten* knockout strategy is shown on a bottom figure. In *Pten*^loxP/loxP* mice, LoxP sites were placed into the endogenous *Pten* locus flanking exon 5. Two different Cre transgenic lines carrying *Osr1-Cre* or *PB-Cre4* transgene were crossed to *Pten*^loxP/loxP* mice for the generation of *Pten*^loxP/loxP*:Osr1-Cre and *Pten*^loxP/loxP*:PB-Cre4 mice. B-C. Frozen sections of prostate tissues isolated from 6-weeks-old *Ar-IPInL*:Pten^loxP/loxP*:Osr1-Cre and *Ar-IPInL*:Pten^loxP/loxP*:PB-Cre4 mice were stained for β-gal activity. Corresponding high power images (400X) are shown in B'-C'. D-G. Serially adjacent sections from each mouse were stained with the antibodies against AR, pAkt, pS6 and Pten. Corresponding high power images (400X) are shown in D'-G'. “N” indicates normal glands. “P” indicates PIN lesions.

Figure 5. Analysis of endogenous Ar expression and Akt expression and activation in the mouse prostate. Serially adjacent frozen sections of prostate tissues isolated from 5-weeks-old *Ar-IPInL*:Pten^loxP/loxP*:Osr1-Cre mice were stained with hematoxylin and eosin (A), stained for β-gal activity (B), and stained with the antibodies against AR, pAkt, pS6 and Pten (C-F). Corresponding high power images (400X) are shown in A'-F'. “N” indicates normal glands. “T” indicates tumor areas.

Figure 6. Decreased AR transcriptional activity in Pten null prostatic cells. A. Schematic illustration of labeling *Pten*^loxP/loxP*:PB-Cre4 positive cells with the mT/mG reporter. B-C. Immunofluorescence analysis was performed on prostate tissues from 6-weeks-old *mT/mG*^Ltrans*:Pten^+/+*:PB-Cre4 (B1-B3) or *mT/mG*^Ltrans*:Pten^loxP/loxP*:PB-Cre4 (C1-C3) mice with the GFP and pAkt antibodies. D-E. Dissociated prostatic cells from 6-weeks-old *mT/mG*^Ltrans*:Pten^+/+*:PB-Cre4 or *mT/mG*^Ltrans*:Pten^loxP/loxP*:PB-Cre4 mice were analyzed for FACS sorting of GFP-expressing cells. The dot-plots show the prostatic cell population before (D) and after FACS sorting (E). F. The sorted GFP-positive cells from *mT/mG*^Ltrans*:Pten^+/+*:PB-Cre4 or *mT/mG*^Ltrans*:Pten^loxP/loxP*:PB-Cre4 prostates were harvested for real-time qRT-PCR. The levels of Ar, Fkbp5, Nkx3.1, Probasin, Msmb and Phlpp1 were normalized to that of GAPDH mRNA. The relative mRNA levels from each sample are presented as the mean ± S.D. of the triplicate reactions.
Figure 1

*R26AR<sup>loxP/Wt</sup>:Osr1-Cre*
Figure 2
Figure 4  

Ar-IPInl/\textit{Pten}^{L/L-}:PB-Cre
Figure 6
Crosstalking between Androgen and PI3K/AKT Signaling Pathways in Prostate Cancer Cells
Suk Hyung Lee, Daniel Johnson, Richard Luong and Zijie Sun

J. Biol. Chem. published online December 19, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M114.607846

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