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Cell Autonomous Role of PTEN in Regulating Castration-Resistant Prostate Cancer Growth

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SUMMARY

Alteration of the PTEN/PI3K pathway is associated with late-stage and castrate-resistant prostate cancer (CRPC). However, how PTEN loss is involved in CRPC development is not clear. Here, we show that castration-resistant growth is an intrinsic property of *Pten* null prostate cancer (CaP) cells, independent of cancer development stage. PTEN loss suppresses androgen-responsive gene expressions by modulating androgen receptor (AR) transcription factor activity. Conditional deletion of *Ar* in the epithelium promotes the proliferation of *Pten* null cancer cells, at least in part, by downregulating the androgen-responsive gene *Fkbp5* and preventing PHLPP-mediated AKT inhibition. Our findings identify PI3K and AR pathway crosstalk as a mechanism of CRPC development, with potentially important implications for CaP etiology and therapy.

INTRODUCTION

Prostate cancer (CaP) is the most common male malignancy and a leading cause of mortality in western countries (ACS, 2010). Androgens are critical both for the development and function of the normal prostate gland and for the maintenance of CaP cells that arise from the secretory epithelium of the prostate. Androgens function through their cognitive receptor, the androgen receptor (AR). Therefore, therapies for advanced CaP usually involve either reducing or blocking the production of androgens or antagonizing the AR and its target genes (Chen et al., 2008). However, all men with metastatic disease develop resistance to these therapies and will progress to castration-resistant prostate cancer (CRPC).

AR is expressed in CRPC and may function in an androgen-independent manner through autocrine signaling or crosstalk with other prosurvival and proliferative pathways (Attard et al., 2009a; Montgomery et al., 2008). However, levels of AR are also heterogeneous and, in some instances, absent from late-stage diseases (Roudier et al., 2003). Such clinical observations raise the possibility that loss of AR expression or activity may serve as an alternative means of escaping androgen withdrawal or AR-targeted therapies, possibly through concomitant activation of compensatory signaling pathways. One of the possible survival and proliferative pathways is the PI3K/AKT/mTOR pathway, which is negatively regulated by the PTEN tumor suppressor (Hill and Wu, 2009; Taylor et al., 2010). *PTEN* loss occurs frequently during human CaP progression, with up to 70% of late stage samples exhibiting loss of PTEN function or activation of the PI3K pathway (Taylor et al., 2010). PTEN loss or activation of the PI3K/AKT pathway leads to enhanced cell

Significance

Resistance to androgen-deprivation therapy is the major hurdle for managing patients with advanced CaP. Therefore, understanding the molecular mechanisms underlying CRPC will be helpful for the design of therapeutic strategies to overcome resistance. We found that CaPs initiated by PTEN loss result in suppression of AR transcription output and can progress to CRPC independent of epithelial AR, providing a mechanism of escaping the requirement of the androgen/AR axis for castration-resistant growth. AR loss or inhibition, on the other hand, can further activate AKT activity via downregulation of the FKBP5 scaffold protein and PHLPP phosphatase. Our study suggests that cotargeting both AR and PTEN/PI3K pathways may enhance therapeutic efficacy for CaPs initiated by PI3K pathway alterations.

proliferation, survival, and migration (Stiles et al., 2004; Vivanco and Sawyers, 2002) as well as castration-resistant growth (Gao et al., 2006; Jiao et al., 2007; Wang et al., 2003).

Progress in defining the mechanisms of CRPC has been limited due to a paucity of xenograft models and scarcity of matched human clinical specimens representing castration-sensitive and castration-resistant disease. Several well-established transgenic CaP models, i.e., TRAMP (Greenberg et al., 1995) and *hi-Myc* (Ellwood-Yen et al., 2003), have the limitation that the oncogene is driven by an androgen-dependent promoter. Therefore, the effect of androgen ablation on CaP growth is confounded by its effect on transgene expression. Previously, we established the *Pten* null CaP model by conditional deletion of *Pten* in the murine prostatic epithelium and showed that *Pten* null CaPs progress with defined kinetics that mimic histopathological features of human disease (Wang et al., 2003). We also tested whether the *Pten* null CaP model could be used for studying CRPC by surgically castrating mutant mice at 16 weeks, when invasive adenocarcinoma had already developed. Despite activation of prosurvival AKT signaling, *Pten* null cancer cells are sensitive to androgen withdrawal, and the cell death index is higher than that of age- and genetic background-matched WT controls and persists for 5–10 weeks after castration. However, the cell proliferation index is not changed in comparison to intact *Pten* null mice. Such androgen-independent growth overrides androgen-dependent cell death and causes castration-resistant growth and invasive adenocarcinoma (Wang et al., 2003). Therefore, the *Pten* conditional murine CaP model provides a unique opportunity to address the mechanism of resistance to androgen ablation therapy where the oncogenic event is androgen independent. Using this defined genetic model, we now test whether CRPC development is cancer stage dependent and whether CRPC remains dependent on AR signaling in the epithelium.

RESULTS

Early Castration Cannot Prevent *Pten* Null CaP Initiation and Castration-Resistant Growth

In our previous work (Wang et al., 2003), we castrated *Pten* conditional knockouts after the development of invasive adenocarcinoma. However, the androgen-independent proliferative signal could be either intrinsic to Pten loss or due to other molecular/genetic alterations accumulated during cancer progression. To separate these two possibilities, we tested whether CRPC development depends on cancer stage by castrating the mutant mice at time points corresponding to prostatic epithelial *Pten* deletion, hyperplasia, and PIN lesion stages, respectively (Wang et al., 2003) (Figure 1A). Age- and genetic background-matched *Pten* null intact and WT castrated mice were used as controls, and all animals were sacrificed at 10 weeks of age. Early castration, particularly when performed at 2 weeks old, significantly reduced the total prostate volume (data not shown). However, E-cadherin-positive PIN lesions and localized invasion, judged by loss of smooth muscle actin (SMA) staining, were clearly evident in the dorsolateral lobes of the mutants (Figure 1B, arrow). Elevated P-AKT (S473) and P-S6 (S240) were also observed, coinciding with Pten loss, in mutant mice castrated at 2 or 6 weeks old (Figure 1B; see Figure S1 available online). Proliferation index (percentage [%] of Ki67+ cells and Cyclin D1+ cells) was significantly increased in mutant prostates, although counts were less in mice castrated at 2 weeks compared to those castrated at 6 weeks (Figure 1C). This suggests that other molecular or genetic events may occur between 2 and 6 weeks after *Pten* deletion and collaborate with PI3K pathway to promote cell proliferation. Nevertheless, these results suggest that androgen-independent proliferation is intrinsic to Pten loss, which is consistent with previous studies (Jiao et al., 2007; Gao et al., 2006).

Pten Null CaPs Are Less Dependent on AR Signaling, and Pten Loss Can Suppress Androgen-Responsive Gene Expression

That *Pten* null CaP cells can proliferate under castrate conditions suggests that Pten loss may sensitize AR to castrate androgen levels or even obviate the requirement of androgens. To test this, we compared the AR-signaling status, defined by the expression of androgen-responsive genes, in age- and genetic background-matched *Pten* null and WT prostates at 0, 3, 6, and 14 days post-castration (n = 3). To determine the AR-responsive genes in normal, noncancerous prostate, we analyzed a previously published data set (Wang et al., 2007) with two criteria: gene expressions were upregulated or downregulated at least 2-fold post-castration (castration/intact), and their expressions were reverted upon hormone replacement (hormone replacement/castration). This led to the selection of 148 androgen-responsive genes, including 47 activated and 101 suppressed genes (Table S1). When compared with published human androgen-responsive gene lists (Chauvin and Griswold, 2004; Nelson et al., 2002), we found many overlaps (data not shown).

Surprisingly, Pten loss in the epithelium does not sensitize AR signaling, and to the contrary, it suppresses androgen-responsive gene expression or AR transcriptional output in the intact mice (Figure 2A, orange brackets). Although the expressions of androgen-responsive genes are significantly changed in WT mice after castration, the expressions of the majority of these genes are similar in *Pten* null prostates before and after castration (Figures 2A and 2B; Figure S2A). Strikingly, the overall AR transcriptional output of 14 day castrated WT prostates is similar to that of intact *Pten* null prostates (Figure 2C; Figure S2A). Using a rank-rank analysis and a hypergeometric overlap algorithm (Plaisier et al., 2010), we also found this similarity between the global expression profiles of genes altered in *Pten* null CaPs (*Pten* null cancer, WT control) with those enriched after castration (castration, intact or day 0 control, Figures S2B and S2C). Together, these results indicate that Pten loss not only suppresses AR transcriptional output but also drives the overall gene expression profiles toward a castration-like phenotype.

To test the relevance of our finding to human CaPs, we analyzed two public data sets (Lapointe et al., 2004; Taylor et al., 2010). Human samples were first stratified according to their *PTEN* DNA copy number status (PTEN CN; Figure 2D) and then tested if the murine-derived *Ar* signature genes are differentially expressed according to the *PTEN* CN status. We found 81 of 148 murine androgen-responsive genes in both human data sets, including 26 activated and 55 suppressed

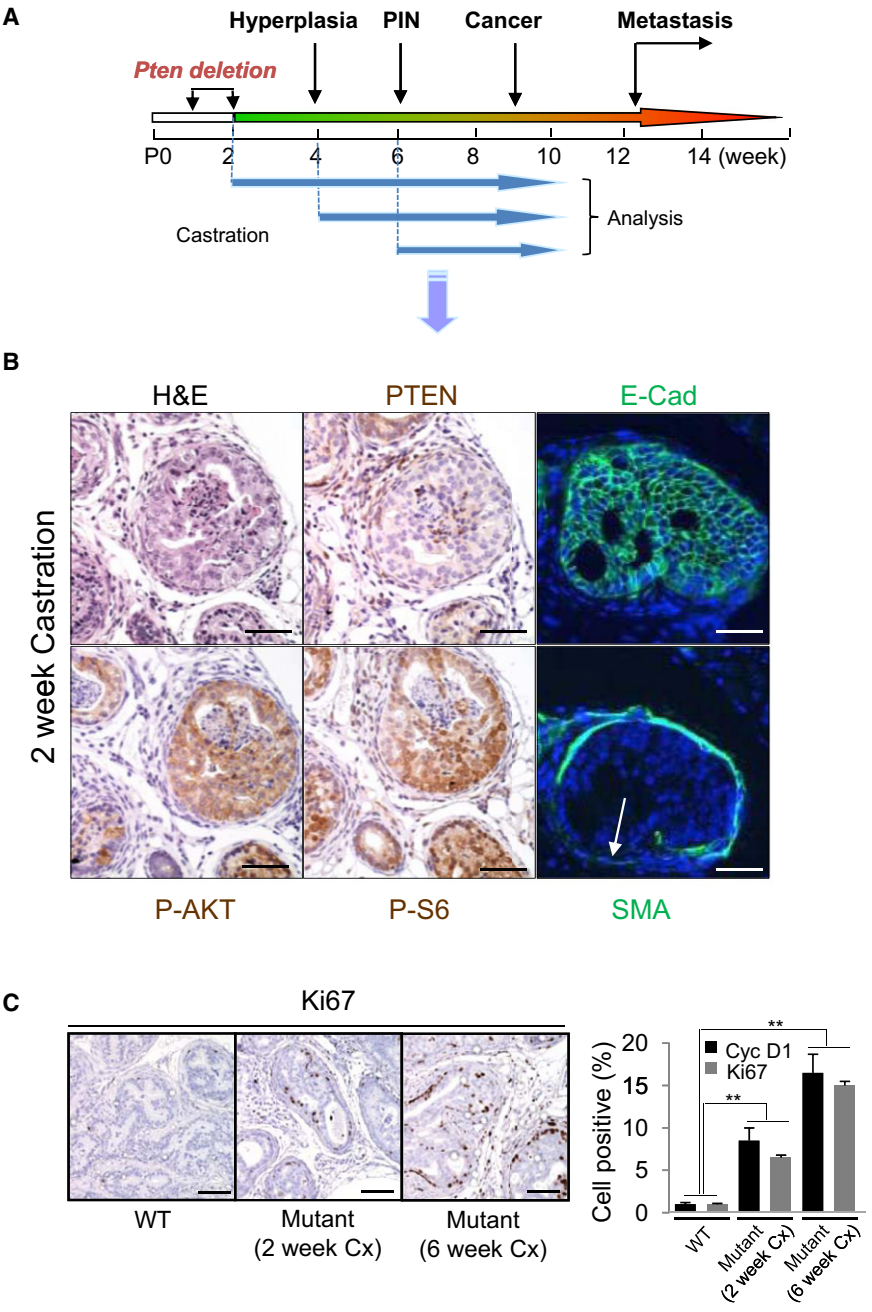


Figure 1. Early Castration Does Not Prevent Initiation of *Pten* Null CaP

(A) *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ mutants were castrated (Cx) at 2, 4, or 6 weeks and aged to 10 weeks.

(B) Mutants Cx at 2 weeks were evaluated for carcinoma, PI3K (P-AKT, P-S6) activation coinciding with PTEN loss. Prostates were also evaluated for invasiveness based on SMA loss but maintained E-Cadherin expression (arrow). Scale bars represent 100 μm.

(C) Cell proliferation, Ki67+; Cyclin D1+ (Cyc D1) cells, in 2 and 6 week Cx cohorts in comparison to WT Cx controls (**p < 0.01). Scale bars represent 150 μm. Error bars represent mean ± SD.

See also Figure S1.

genes (Figure 2D). The two-dimensional comparison illustrates the consistency between the two human data sets for the differential expression of the murine-derived androgen-responsive genes in *PTEN* CN mutant versus normal cohorts (p value of the correlation coefficient =2.2e-6), suggesting that overall suppression of AR output in human CaPs with *PTEN* CN variation, is similar to our observation in *Pten* null mutants. In contrast, cancers with abnormal copy number (CN) of the *MYC* oncogene showed less agreement between the two data sets (p value of the correlation coefficient =1.1e-4) (Figure S2D), indicating that suppression of AR transcriptional output may depend on specific oncogenic events.

PTEN Controls AR-Transcription Factor Activity by Regulating EGR1, c-JUN, and EZH2 Expression

To determine how PTEN loss leads to repression of AR output, we used an inducible system in which *PTEN* expression is controlled in a doxycycline-dependent manner in *Pten* null, *Pten*$^{\Delta loxp/\Delta loxp}$ murine cells (Chang et al., 2004). Although *Ar* expression is not influenced by *PTEN* reexpression, Egr1 and c-Jun transcription factor expressions are downregulated within 24 hr upon induced PTEN reexpression, which were confirmed in a PTEN-inducible human CaP line, PC3 (Figure 2E; data not shown). Both EGR1 and c-JUN are upregulated in CaPs, especially CRPC, and can physically interact with AR, leading to downregulated AR-targeted gene expression and CaP cell growth in an androgen-depleted environment (Gitenay and Baron, 2009; Sato et al., 1997; Yang et al., 2006; Yuan et al., 2010). *Egr1* deletion impairs prostate tumorigenesis (Abdulkadir et al., 2001). Therefore, we hypothesized that by negatively regulating EGR1 and c-JUN expression, PTEN upregulates AR function. Using network component analysis (NCA) (Tran et al., 2010), we deduced the TFAs from the expressions of their target genes. Both EGR1 and c-JUN TFAs are reduced upon *PTEN* reexpression, whereas AR TFA is increased 24 hr after *PTEN* reexpression and EGR1/c-JUN activity changes (Figure 2E), supporting our hypothesis. The activity of AR was further examined using two murine CaP models: our *Pten* conditional knockout (Wang et al., 2003); and the transgenic mice overexpressing *mAKT* (Majumder et al., 2004). The AR TFAs are significantly reduced in both models, which can be reverted by rapamycin treatment (Figure 2E), suggesting that the mTOR pathway is involved in PTEN regulation of AR activity.

Besides EGR1 and c-JUN, AR target gene expression is also inhibited by the ERG transcription factor, a member of the ETS

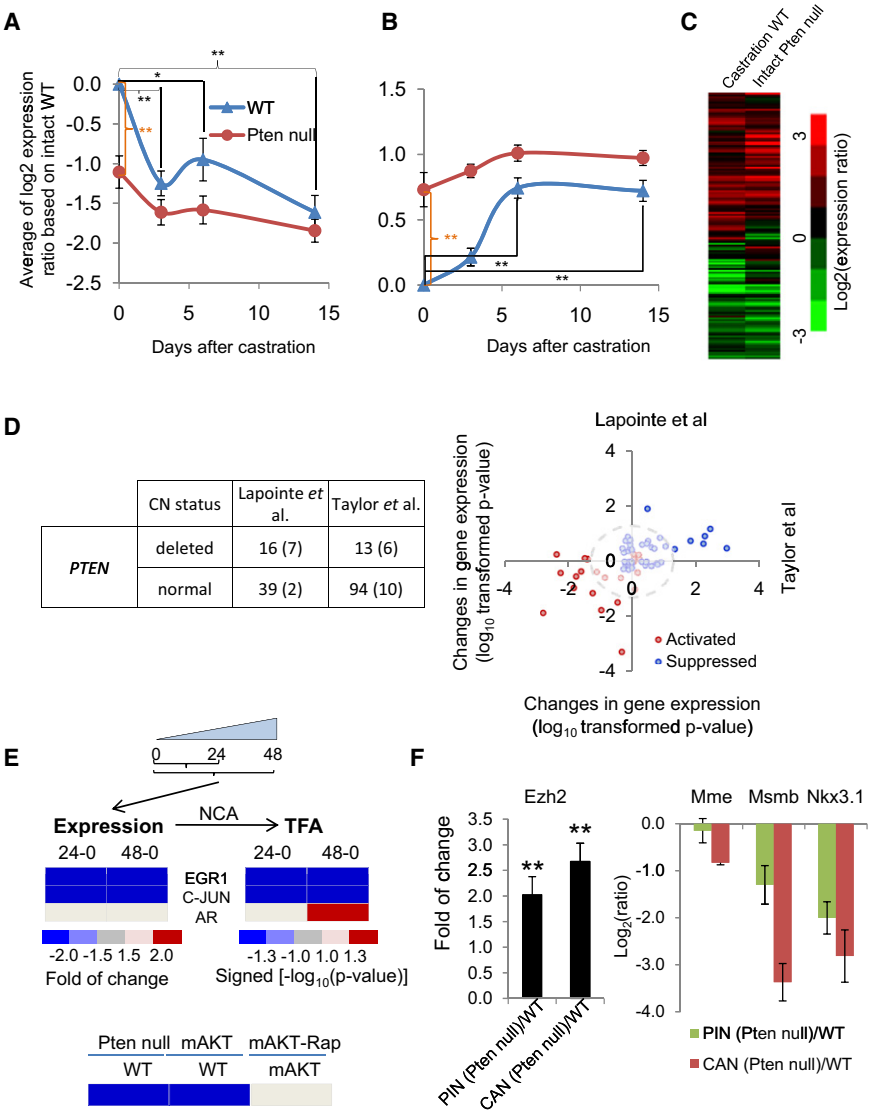


Figure 2. PTEN Loss Can Suppress Androgen-Responsive Gene Expression

(A and B) Expression profile (mean ± SEM) of AR-activated (A) and -suppressed (B) genes in WT and *Pten*-null murine prostates after castration.

(C) Heat map of expression ratios of androgen-responsive genes in WT (14 day post-castration) and intact *Pten* null mutants with respect to intact WT mice.

(D) Variation in expression of androgen-responsive genes based on *PTEN* CN in human CaP samples. Left view is a summary of human samples based on *PTEN* CN (the numbers inside parentheses indicate the number of metastatic cases); right is a comparative analysis of AR-activated (red circles) and -suppressed (blue circles) gene expression values in two human CaP data sets.

(E) Top view shows gene expression and NCA-derived activities of EGR1, JUN, and AR transcription factors in induced PTEN expression in $Pten^{-/-}$ cells. Bottom view illustrates the activity of AR in murine models when the PTEN/AKT/mTOR pathway was manipulated genetically or pharmacologically.

(F) Expression (mean ± SD) of *Ezh2* (left), and AR and EZH2 cotarget genes (right) in PIN and cancer (CAN) stages of *Pten* null prostate. *$p < 0.05$; **$p < 0.005$.

See also Figure S2 and Table S1.

family whose gene is frequently translocated to the AR-responding TMPRSS2 gene (Yu et al., 2010). ERG activates EZH2 expression, a member of the polycomb complex associated with human CaP (Sellers and Loda, 2002; Varambally et al., 2002), and the EZH2-mediated dedifferentiation program (Yu et al., 2010). *Ezh2* expression is upregulated in *Pten* null CaP (Figure 2F; Figure S2E), and the expressions of several AR and ERG-cotargeted genes, including *Nkx3.1*, *Mme*, and *Msmb* (Kunderfranco et al., 2010), are also downregulated in *Pten* null prostate at PIN and cancer stages (Figure 2F). Our analyses suggest that PTEN loss suppresses androgen-responsive genes by regulating AR activity through multiple coregulators, thereby rendering *Pten* null CaP cells less dependent or completely independent of signaling provided by androgens, hence promoting CRPC growth.

Epithelial AR Is Not Required for the Initiation and Progression of CaPs Caused by Pten Loss

Having demonstrated that *Pten* null CaP can proliferate independent of androgens, we considered whether CaP can develop in the absence of AR in the epithelium. We crossed *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ (*Pten* null) mutants with mice carrying the *AR* conditional allele (*Ar*^{L}) (De Gendt et al., 2004). *Ar* deletion led to minimal effects on prostatic epithelium (data not shown), similar to previous publications (Simanainen et al., 2007, 2009; Wu et al., 2007). The resulting *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* (*Pten* null;*Ar* null) mutants were then analyzed at the ages of 4–6, 10–14, and >20 weeks (n = 4) and compared to their age- and genetic background-matched *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ mutants. *Pten* and *Ar* deletion promoted robust in situ, latent adenocarcinoma development in the dorsolateral lobes. Overall cancer progression in the double knockouts was similar to that of *Pten* single knockout, as evaluated by localized loss of SMA staining (Figure 3C; Figure S3A).

The anterior and ventral lobes often showed mosaic patterns of conditional deletion (Figure S3A), likely due to lower levels of Cre expression in these lobes (Wu et al., 2001) and less efficient recombination of the *Ar*^{loxP} allele. This allowed us to directly compare the effect of *Ar* deletion on *Pten* null CaP cells. In Pten- and AR-deleted regions, we found enhanced nuclear atypia, cell proliferation (Ki67 staining), and cell death (arrow in the H&E section) in contrast to AR intact regions (comparing areas above and below the red dashed lines in the lower panels of Figure 3A). In *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants, Pten^{-};AR^{-} adenocarcinomas also appeared less differentiated than Pten^{-};AR^{+} regions.

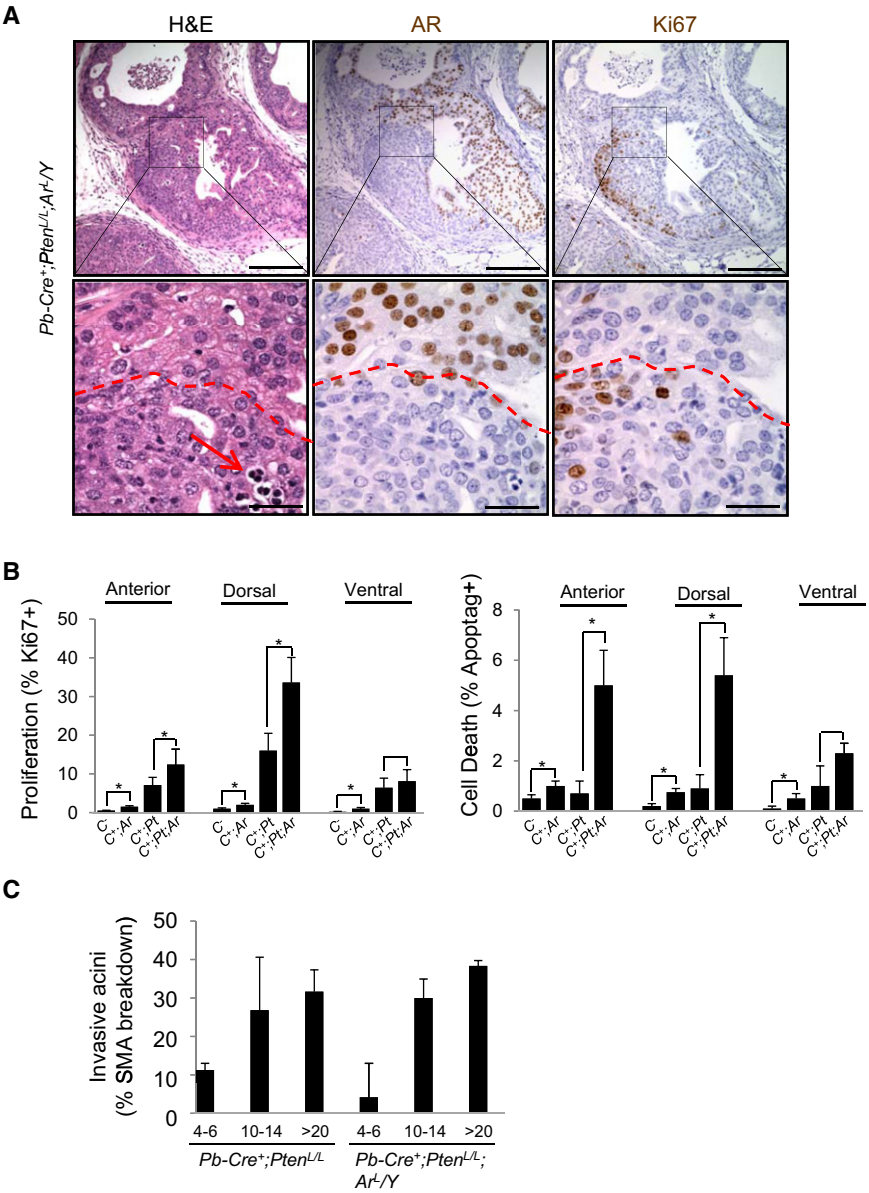


Figure 3. Epithelial AR Is Not Required for the Initiation of *Pten* Null CaP

(A) Deletion of epithelial AR in the anterior lobe of *Pten* null CaP (*Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y*) mutants and the impact on cell proliferation (Ki67+ cells), apoptosis (red arrow), and cancer formation (scale bars, 200 μm [top] and 50 μm [bottom]).

(B) Cell proliferation (left) and apoptotic indexes (right) in *Cre*$^-$ (*C*$^-$), *Pb-Cre*$^+$;*Ar*^{L}/*Y* (*C*$^+$;*Ar*); *Pb-Cre*$^+$;*Pten*$^{L/L}$ (*C*$^+$;*Pt*) and *Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* (*C*$^+$;*Pt;Ar*) mutants.

(C) Frequency of invasiveness based on SMA breakdown in *Pb-Cre*$^+$;*Pten*$^{L/L}$ and *Cre*$^+$;*Pten*$^{L/L}$; *Ar*^{L}/*Y* mutants during progression. Error bars represent mean ± SD.

See also Figure S3.

Cell Autonomous Role of PTEN and PTEN-Controlled Pathway in Regulating AR-Independent Growth

The mosaic patterns of AR deletion in *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* prostates can potentially complicate our conclusions because AR$^+$ epithelium may supply paracrine factors to "feed" the adjacent AR$^-$ cells. Thus, we employed prostate tissue regeneration assays. We used FACS to enrich for prostate epithelium from *Pten*$^{L/L}$ (n = 6) and *Pten*$^{L/L}$;*Ar*^{L}/*Y* (n = 4) mice, then infected them with either Cre lentivirus (Cre-FUCRW) or control lentivirus (FUCRW). Infected prostate epithelium combined with WT urogenital mesenchyme (UGSM) was grafted under the kidney capsule of NOD;SCID;IL2rγ null mice (Figure 4A). Recombinants were examined for the presence of pathology coinciding with Cre-FUCRW or FUCRW infection, as visualized by the expression of viral-associated RFP reporter gene (Figure S4A). *Pten*$^{L/L}$;*Ar*^{L}/*Y* cells infected with Cre-FUCRW generated neoplastic pathology with high levels of P-AKT and P-S6 (Figure 4B), similar to the *Pten*$^{L/L}$ Cre virus infection control (Figures S4A and S4B), whereas *Pten*$^{L/L}$; *Ar*^{L}/*Y* cells infected with control FUCRW virus displayed normal acinar structure (Figure S4C).

One explanation for our observation is that epithelial cells isolated from the *Pten*$^{L/L}$;*Ar*^{L}/*Y* prostate have been "primed" by paracrine factors secreted by AR$^+$ stroma cells (Cunha et al., 2002). To further affirm the cell autonomous role of PTEN in CaP initiation in the absence of epithelial AR, we carried out prostate tissue regenerations using prostate epithelium isolated from E16–17 *Pten*$^{L/L}$;*Tfm* mutant mice obtained from crossing the *Pten*$^{L/L}$ conditional line with the *Tfm* mutant mice. *Tfm* mutant mice express a rapidly degraded AR mutant resulting in complete blockage of prostate development (Cunha and Chung, 1981). Therefore, prostate epithelium of E16–17 *Pten*$^{L/L}$;*Tfm*

We then examined the content of cells positive for the basal cell markers, p63 and cytokeratin 5 (CK5). We found that Pten$^-$;AR$^-$ cancers had a significant expansion of p63$^+$ cells in the proximal region, known to be enriched for stem/progenitor cells (Figure S3B, bottom) (33.5% versus 15.5% of total cells; *, $p < 0.05$), accompanied by increased CK5$^+$;CK8$^+$ transient-amplifying and p63$^+$;Ki67$^+$ double-positive cells (Figure S3B). Conversely, the distal regions of *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants, which compose the bulk of the tumor mass, contained low or no p63$^+$ cells, similar to human CaP (Figure S3C). Proliferation and apoptotic indexes were also increased in Pten null;AR null cancers (*$p < 0.05$), most notably in the proximal regions of the dorsal-lateral lobe (Figure 3B). Despite this, AR-independent cell proliferation appeared to override AR-dependent cell death, similar to Pten null cancers under castration conditions (Wang et al., 2003), resulting in overall AR-independent tumor growth.

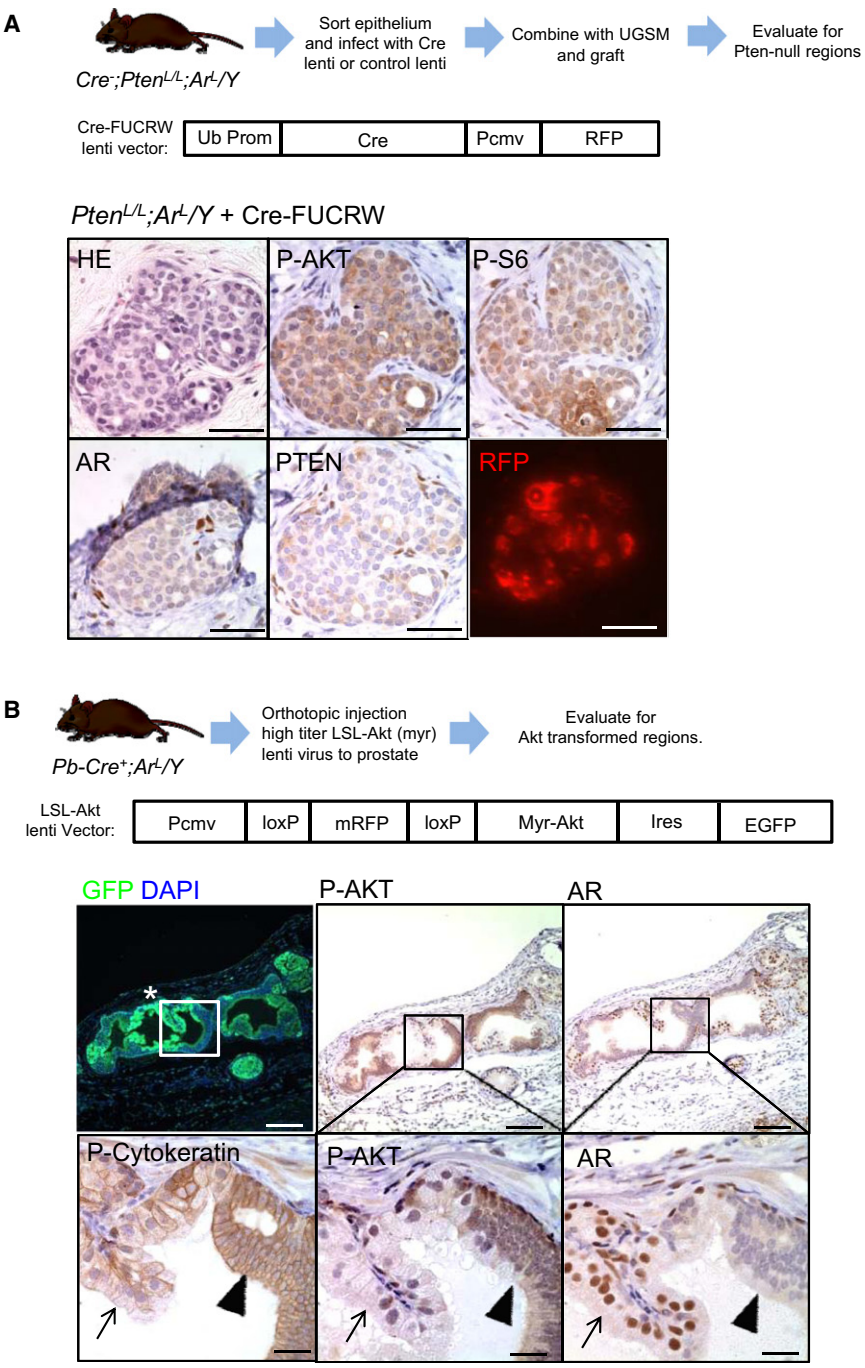


Figure 4. Epithelial AR Is Not Required for Transformation by *Pten* Deletion or Myristoylated Akt in Regeneration Assays

(A) Evaluating the impact of Cre-mediated deletion of *Pten* and *Ar* on histopathology and PI3K signaling. The top panel shows the outline of the experiment, and the bottom panels show results of tissues stained as indicated. Scale bars represent 100 μm.

(B) Evaluating AR deletion and myristoylated AKT expression in primary *Pb-Cre*$^+$;*Ar*^{L}/*Y* mutants and the impact on prostate histopathology and PI3K signaling. The top panel shows the outline of the experiment, and the bottom panels show results of tissues stained as indicated. Scale bars represent 200 μm [top] and 50 μm [bottom].

See also Figure S4.

mice should have never been exposed to such potential paracrine factors. We found that *Pten*$^{\Delta/\Delta}$;*Tfm* epithelial cells formed PIN lesions with activated AKT (Figure S4D; n = 3). Therefore, using two in vivo tissue recombinant models, our data indicate that AR-independent carcinogenesis is intrinsic to PTEN inactivation.

To further assess whether it is PTEN or the PTEN-controlled signaling pathway that renders CaP development without epithelial AR, we orthotopically injected LSL-AKT virus directly to the anterior lobe of *Pb-Cre*$^+$;*Ar*^{L}/*Y* prostates (n = 3) (Figure 4B). Using this strategy, only regions expressing Cre and infected by lentivirus (indicated by GFP$^+$ expression in Figure 4B and Figure S4E) will coordinately delete AR and express Myr-AKT. Although AR$^+$ regions did not show AKT hyperactivation and abnormal phenotypes (Figure 4B, arrows in lower panels), AR-deleted neoplastic regions were pan-cytokeratin positive and showed higher levels of P-AKT (Figure 4B, arrowheads in lower panels). These data support our hypothesis that neoplastic transformation of the prostate by activated cell autonomous PI3K/AKT signaling can occur in the absence of epithelial AR.

AR Downregulates AKT Activity by Stimulating FKBP5 and PHLPP-Mediated AKT Dephosphorylation

Postnatal epithelial AR deletion sensitizes prostate epithelium to paracrine signaling mediated by AR$^+$ stroma, leading to androgen-induced epithelial hyperproliferation (Cunha, 1994; Cunha et al., 2002; Niu et al., 2008a; Simanainen et al., 2009). To block potential stroma-derived androgen-induced mitogenic signals, we castrated *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants at 6 weeks (n = 4) and analyzed their response. Similar to *Pten* null mutants, *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* prostates did respond to castration but developed castration-resistance when analyzed 4 weeks later. Histopathological analysis indicated that castration-resistant cancer outgrowths in the dorsolateral lobes are composed mainly of Pten$^-$;AR$^-$ epithelium (data not shown), whereas anterior and ventral lobes contained both AR$^+$ and AR$^-$ regions (Figure 5A). Strikingly, in comparison to AR$^+$ regions (arrowheads), AR$^-$ regions (arrows) contain much higher levels of membranous P-AKT and intracellular P-S6 as well as elevated cell proliferation and cell death, whether assessed at 4 weeks

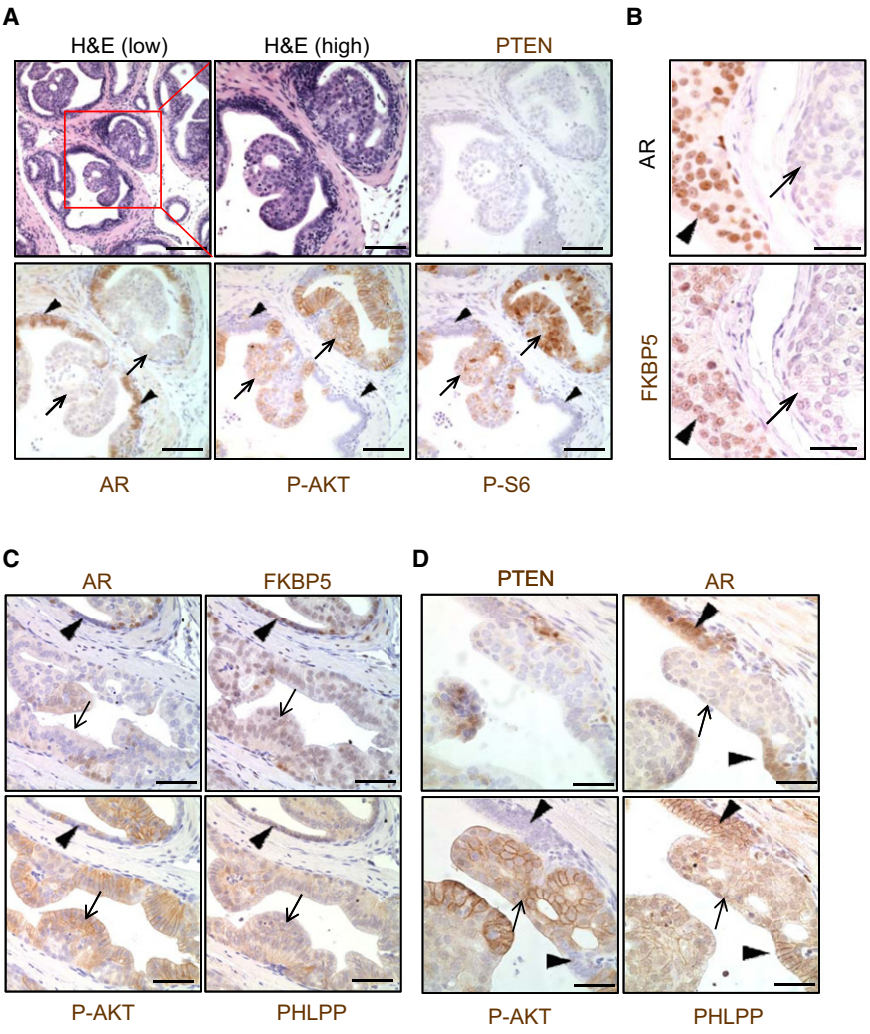


Figure 5. AR Downregulates AKT Activity by Stimulating FKBP5 and PHLPP-Mediated, AKT Dephosphorylation

(A) PI3K activation (P-AKT, P-S6) in AR$^+$ regions (arrows) versus AR$^-$ regions (arrowheads) in castrated *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants (scale bars, 150 μm [low magnification] and 75 μm [high magnification]).

(B) Effect of AR deletion on FKBP5 expression in *Pb-Cre*$^+$*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants (scale bars, 50 μm).

(C and D) Expression of FKBP5, PHLPP, and P-AKT in AR$^+$ regions (arrowheads) compared to AR null regions (arrows) in castrated *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants at 2 days (C) and 4 weeks (D) after castration (scale bars, 75 μm).

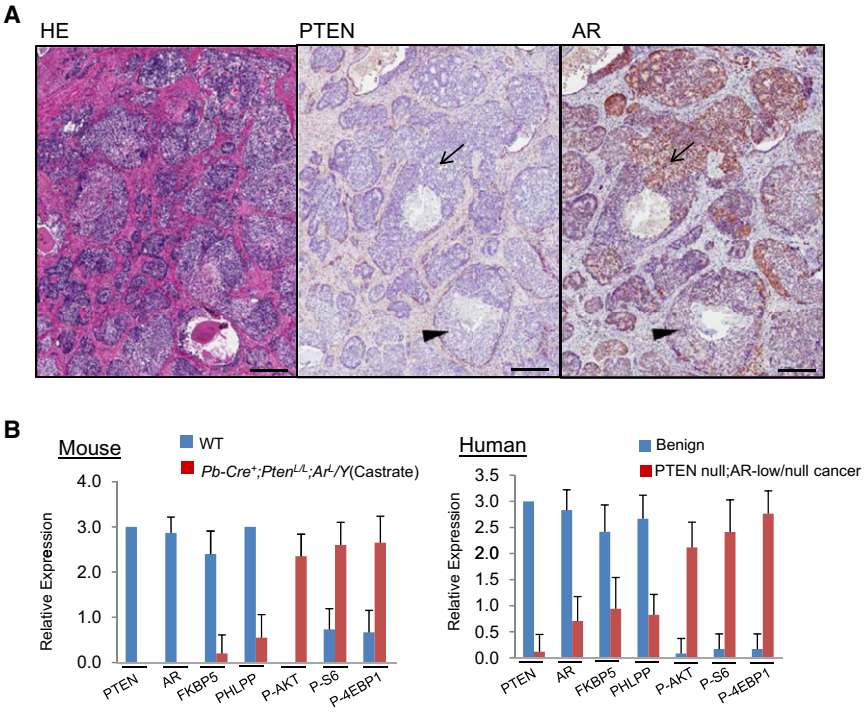
See also Figure S5.

or even 2 days post-castration (Figures 5A, 5C, and 5D; Figure S5A).

In searching for androgen-responsive genes that are decreased during castration, we found FKBP5, a member of the *cis-trans* prolyl isomerase family (Ratajczak et al., 2003). *FKBP5* is an established androgen-regulated gene in humans (Magee et al., 2006; Mostaghel et al., 2007) whose expression is immediately suppressed after castration but upregulated upon hormone replacement in the WT mouse prostate (Figure S5B) and downregulated in the *Pten* null intact and castrated prostates (Figure S5C), similar to *Mme* and *Msmb* (Figure S5D). We found that FKBP5 expression in *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* prostate correlates with AR expression (Figure 5B). A recent study showed that FKBP5, by serving as a scaffolding protein for AKT and PHLPP, promotes PHLPP dephosphorylation of AKT at amino acid S473 (Brognard et al., 2007; Gao et al., 2005) and thereby suppresses AKT activity (Pei et al., 2009). In castrated *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* prostates, we found that regions with *Pten* and *AR* deletion have hyperphosphorylated AKT (S473) and reduced PHLPP, especially membrane-associated PHLPP (Figures 5C and 5D, arrows), whereas Pten$^-$;AR$^+$ regions showed the opposite effect (Figures 5C and 5D, arrowheads). These results suggest that downregulation of FKBP5 either by castration or AR loss could release FKBP5-PHLPP-mediated suppression of AKT activity and promote AKT-dependent but androgen- and AR-independent cell proliferation.

Correlations among PTEN, AR, FKBP5, and PHLPP in Human CaPs

To assess the relevance of our finding to human CaPs, we took consecutive sections from a whole-mounted, fresh, surgically resected human CaP sample and performed IHC analyses for PTEN, AR, FKBP5, PHLPP, P-AKT, P-S6, and Ki67 levels. We observed considerable heterogeneity of AR expression in PTEN null cancerous regions (Figure 6A, arrow) (PTEN$^-$;AR$^+$ staining, arrow, PTEN$^-$;AR$^-$ staining, arrowhead), whereas adjacent noncancer regions showed uniformed PTEN$^+$ and AR$^+$ staining (Figure S6A). Importantly, high AR expression regions are associated with higher FKBP5 and PHLPP but lower P-AKT and P-S6, similar to our observations in the murine model (Figure S6B). We then took 40 high-power images from benign and PTEN-negative;AR-low/negative cancer regions and scored the expression of PTEN, AR, FKBP5, PHLPP, P-AKT, P-S6, and P-4EBP1 in a range of 0–3, with 3 being the highest expression level. Similar to the *Pb-Cre*$^+$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mouse model (Figure 6B, left panel), we observed moderate to high levels of PI3K pathway effectors in PTEN-negative;AR-low/negative human cancer regions (Figure 6B, right panel). Moreover, PTEN-negative;AR-low/negative regions maintained elevated cell proliferation in comparison to benign regions (19.2% versus 1.4%; $p < 0.01$) (Figure S6C). We then surveyed CaP tissue microarrays (TMAs) generated by the UCLA Prostate SPORE program. Again, consecutive sections were used for IHC analyses, and only cores showing >30% epithelia were scored. Among 91 cores we analyzed, we found extensive heterogeneous AR expression levels, ranging from nearly 100% AR$^+$ to complete AR$^-$ (Figure S6D). Using



p-value*	PTEN	PHLPP	AR
PHLPP	1.73E-07		
AR	4.11E-02	6.33E-03	
FKBP5	1.22E-01	1.74E-02	1.67E-03

Figure 6. Heterogeneous AR Expression in Human CaPs Correlating with PI3K/AKT Signaling and FKBP5 and PHLPP Levels

(A) PTEN and AR expression in human CaP (scale bars, 500 μm).

(B) PI3K pathway components (P-AKT, P-S6, P-4EBP1) in Pten^{-};AR^{-} regions of *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mice and in PTEN-negative;AR-low/negative regions of human CaPs. Error bars, mean ± SD.

(C) Unsupervised clustering analysis of PTEN, PHLPP, AR, and FKBP5 in human TMA samples (n = 91) (left). Chi-square test p values (n = 91) were used to quantitate the strength of association between each pair (right, table). Protein level was categorized to high (IHC >1) and low (≤1) levels). See also Figure S6.

unsupervised cluster analysis, we affirmed that PTEN is significantly correlated to PHLPP expression (p = 1.73e-7, χ^2 test), whereas AR and FKBP5 are closely clustered together (p = 1.67e-3, χ^2 test) (Figure 6C; Figure S6E). These data suggest that the AR-FKBP5-PHLPP feedback loop may also function in human PTEN-negative;AR-low/negative CaP.

Combined AR/Androgen Ablation and mTOR Inhibition Results in Enhanced Therapeutic Efficacy in *Pten* null CaP

Because ablation of the AR/androgen-signaling axis further activates AKT, we hypothesized that cancers with low levels of AR and PTEN loss would have greater dependency on the PI3K/AKT/mTOR-signaling axis. To test this in vitro, we used CaP8 cells, a Pten null;AR+ cell line derived from the *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ CaP model (Jiao et al., 2007), infected with either a scramble or a sh-AR RNAi lentivirus containing a RFP tag for FACS. Analysis of cell viability at 0, 2, 4, and 6 days indicated a cooperative reduction in cell viability using AR knockdown combined with the mTOR inhibitor rapamycin (1 nM) (Figure 7A, left graph) (n = 6). We also treated the PTEN null;AR^{+} LNCaP human CaP cell line with rapamycin (1 nM), the second-generation anti-AR inhibitor MDV3100 (10 μM) (Scher et al., 2010; Tran et al., 2009), or both. Again, we observed a cooperative inhibition effect when both AR and PI3K/mTOR pathways were targeted (Figure 7A, right graph) (n = 6).

To test our hypothesis in vivo, we treated intact and castrated *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ and *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mice at cancer stage with rapamycin and evaluated the effects by Ki67 index (Figure 7B) and histopathology (Figure 7C; Figure S7; n = 4). Rapamycin treatment (4 mg/kg daily for 4 weeks) led to a reduction of Ki67 index in *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ intact mutants from 17% to 11%; castration and castration plus MDV3100 (10 mg/kg/day) further reduced the rates of cell proliferation to 8% and 3%, respectively (Figure 7B). Similarly, when *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants were castrated and treated with or without rapamycin for 4 weeks, we found a significant reduction of prostate volume in comparison to the placebo cohort (Figure 7C). At the histological level, double mutants with rapamycin treatment showed significant atrophy in most glandular structures, similar to that of WT castrated and *Pten* null castrated mice treated with combination of rapamycin and MDV3100 (Figure S7). A reduction in P-S6 (Ser240) staining and reduced cell proliferation (Figures 7B and 7D) (33.7% versus 4%; **p < 0.01) were also observed. Interestingly, many residual epithelia in rapamycin-treated castrated mutants appeared to be AR^{+} (high-power inset). Collectively, these data suggest that CaPs with AR loss have greater reliance upon the PI3K/AKT/mTOR-signaling pathways and that combined AR/androgen blockage in conjunction with PI3K/AKT/mTOR inhibition is more effective for CaPs initiated by PTEN loss or PI3K/AKT activation.

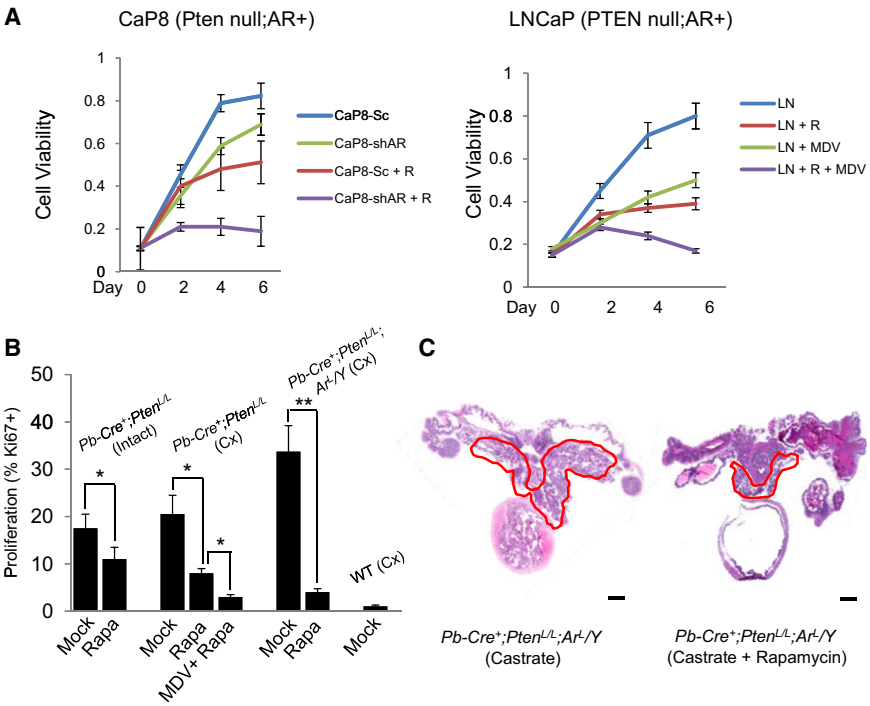


Figure 7. Cooperative Effects of AR and mTOR Inhibition In Vitro and In Vivo

(A) In vitro response of *Pten* null;Ar^{+} murine (CaP8) and human (LNCaP) prostate cancer cells to AR knockdown (sh-AR) or pharmacological inhibition of AR (MDV3100, 10 μM) with and without rapamycin (R: 1 nM) treatment (Sc, control sh oligo). (B and D) In vivo response to treatments with castration, MDV3100, rapamycin, or their combinations as measured by cell proliferation ($Ki67^{+}$ cells) and (C and D) tumor burden in *Pb-Cre*$^{+}$*;Pten*$^{L/L}$ and *Pb-Cre*$^{+}$*;Pten*$^{L/L}$*:Ar*^{L}*/Y* mutants. Scale bars represent 2 mm (C), 200 μm (D), and 75 μm (D, inset). Error bars represent mean ± SD.
See also Figure S7.

DISCUSSION

PTEN loss or PI3K pathway activation represents one of the most frequent genetic alterations found in human CaPs (Taylor et al., 2010). Dysregulation of the PTEN/PI3K pathway has also been associated with resistance to conventional antiandrogen therapies (Ham et al., 2009). Despite these clinical observations, the consequence of PTEN loss and how its loss influences the androgen/AR-signaling axis and CRPC development is unclear. By genetically deleting *Pten* and *Pten*/*Ar* in the prostatic epithelium and analyzing human CaP samples, our studies support the hypothesis that PTEN loss or PI3K pathway activation may function in a cell autonomous manner to promote androgen/AR-independent CaP progression and CRPC development. Although *Pten* null epithelial cells remain sensitive to androgen withdrawal or AR ablation, the resulting activated PI3K/AKT pathway is sufficient to compensate for androgen/AR-signaling blockage, mobilize basal and transient-amplifying stem/progenitor cells, and promote cell proliferation. Therefore, in CaPs initiated by PTEN loss or PI3K activation, the overall outcome of cancer development, especially CRPC development, depends on the balance of androgen-dependent cell survival/differentiation and androgen-independent cell proliferation. PTEN loss enhances the expressions of EGR1, c-JUN, and EZH2, which in turn suppresses AR TFA and output. Inhibiting FKBP5/PHLPP-mediated negative feedback to AKT activation, as a result of castration or AR inhibition, may further enhance the strength of the PI3K/AKT pathway and tilt the balance toward androgen-independent growth (Figure 8). Importantly, results derived from our genetically engineered mouse models, including PTEN-controlled AR transcription output and AR-FKBP5-PHLPP regulatory loop, can also be observed in human CaP samples. Given the heterogeneous *PTEN* deletion/mutation and AR expression patterns within individual human CaPs, shown in our study and by others (Attard et al., 2009b; Taylor et al., 2010), we would expect to observe a broad range of phenotypes correlating between PTEN loss, AR expression, and CRPC development in human patients.

Using genetically engineered animal models, our findings may provide potential insight into the clinical settings. Although hormone therapy immediately after radical prostatectomy improves survival and reduces recurrence (Messing et al., 1999), it is not clear whether early androgen-deprivation therapy (ADT) will prevent cancer progression and CRPC development. That early castration of the *Pten* null CaP model does not significantly impede carcinoma development and castration-resistant growth suggests that patients with PTEN loss or PI3K pathway activation may not benefit from aggressive early hormone treatment. Although neoadjuvant ADT may partially reduce tumor load and PSA levels, it may select for cells with activated compensatory cell survival and proliferating signaling pathways, such as those *PTEN* null or PI3K/AKT

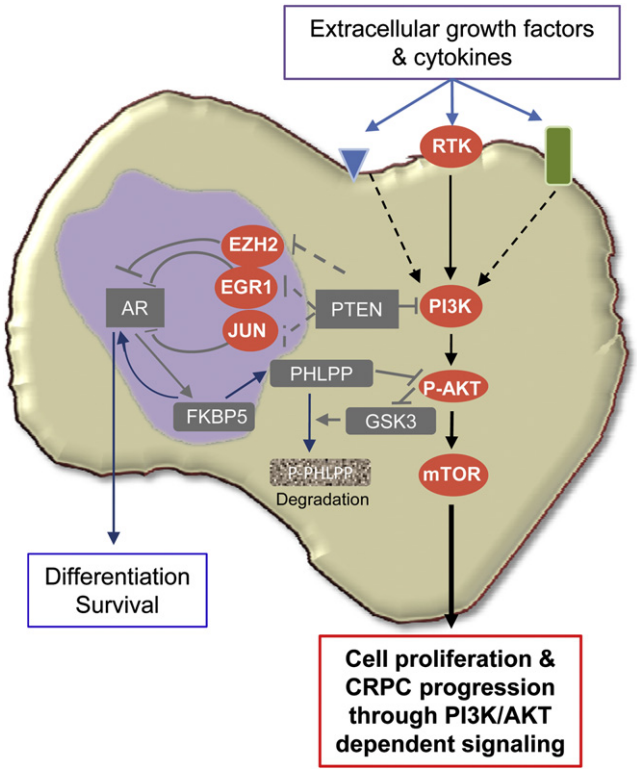


Figure 8. PTEN Loss Promotes CRPC Development by Two Collaborative Mechanisms

By regulating EGR1, c-JUN, and EZH2 expression and activities, PTEN loss suppresses AR TFA and output, leading to reduced prostate epithelial differentiation and survival. Collaboratively, PTEN loss activates the PI3K/AKT-signaling pathway and reduces the AR-regulated FKBP5-PHLPP negative feedback loop, further enhancing AKT activation, leading to androgen/AR-independent prostate epithelial proliferation.

activated, and ultimately facilitate resistance to antiandrogen therapy.

An important finding from our study is that PTEN loss suppresses AR TFA and androgen-responsive gene expression in both murine models and human prostate samples. Interestingly, AR itself is not downregulated in *Pten* null CaPs, even after castration (data not shown), similar to human cancers after short-term castration or after 9 months of neoadjuvant ADT (Mostaghel et al., 2007). Previous studies suggest that the PI3K pathway can either activate or suppress AR activity (Kaarbo et al., 2010; Lei et al., 2006; Lin et al., 2003, 2004). Our analyses demonstrate that PTEN, through regulating the expressions and activities of EGR1 and c-JUN transcription factors and EZH2 levels, controls AR transcription output. This results in *PTEN* null CaP cells being less or completely independent of signaling provided by androgens and epithelial AR, hence promoting castration-resistant growth. However, how the PTEN/PI3K pathway controls EGR1, c-JUN, and EZH2 expression and activities requires further study.

The mosaic pattern of AR deletion observed in our in vivo model also mimics the heterogeneous AR expression observed in our human CaP studies and those reported by others (Attard et al., 2009b). After castration of *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants, we observed significant outgrowth of Pten^{-};AR^{-} cancerous regions, accompanied by elevated P-PAKT and cell proliferation. Adjacent to these outgrowth are AR^{+} regions. Therefore, it is possible that within heterogeneous malignant CaPs, those with low or negative AR expression fail to respond to conventional antiandrogen treatment and continue to proliferate and survive as a consequence of PTEN loss and PI3K/AKT activation. Although such cells would not constitute PSA-producing cancer cells, they would contribute toward overall tumor load.

An intriguing finding of our study is the elevated P-AKT (S473) levels, despite genetic deletion of *Ar* and surgical castration. The recently defined relationship between FKBP5 and the AKT phosphatase, PHLPP (Gao et al., 2005; Brognard et al., 2007; Pei et al., 2010), provides a potential mechanism as to how PI3K/AKT signaling can be activated upon inhibiting the AR/androgen-signaling axis. Specifically, in normal cells PHLPP levels are high, which keeps P-AKT levels low, downregulation of FKBP5 in Pten^{-} cells that have undergone AR/androgen ablation would lead to a reduction of PHLPP's association with P-AKT and, consequently, enhance AKT activity. Although it is difficult to assess P-AKT levels in all human CaP samples due to various sample harvesting and preparation procedures used, we did observe statistically significant correlation between the levels of PTEN and PHLPP as well as AR and FKBP5. Importantly, when a fresh resected CaP sample was analyzed, we found that regions with low or no AR expression had lower levels of FKBP5 and PHLPP and enhanced P-AKT and P-S6 staining. Besides the AR-FKBP5-PHLPP feedback loop, we also observed changes in other scaffold proteins and mediators of the PI3K pathway (Table S1). It will be interesting for future studies to ascertain whether other AR/androgen-regulated genes, such as FKBP11 and IGFBP3/6, also have the capacity to modulate PI3K/AKT signaling in a manner similar to FKBP5.

Increasing evidence suggests that different cellular compartments may contribute differently toward cancer initiation. For example the prostate epithelial AR may confer some suppressor function, whereas the stromal AR has been postulated to be mitogenic (Cunha et al., 2002; Simanainen et al., 2009), thus raising the possibility that stromal AR plays a dominant role in Pten null cancer initiation or during the development of CRPC. In fact, using the TRAMP CaP model, a previous report has shown that reduction of stromal AR content may lead to reduced tumor progression (Niu et al., 2008b). However, extrapolating these data to human disease is challenging because the neuroendocrine carcinoma found in the TRAMP model is a phenotype not commonly observed in human disease (Abbas et al., 1995; Tetu et al., 1987). It is also possible that while AR loss or degradation in differentiated luminal epithelium results in cell death, AR loss in basal or transient-amplifying initiating cells is well tolerated. In fact recent reports have shown that the basal "LSC" stem/progenitor cells either from the Pten null CaP model (Mulholland et al., 2009) or from primary human tissue that has been transformed (Goldstein et al., 2010) are sufficient for tumor reconstitution. Thus, it would be interesting and potentially clinically relevant to ascertain if AR null stem progenitor cells would be capable of cancer initiation. Interestingly, cells with high N-cadherin expression, a marker associated with epithelial-mesenchymal transition and cancer metastasis at late-stage

disease, are also low or null for AR. Moreover, forced expression of N-cadherin results in reduced AR expression and elevated P-AKT in human CaPs (Tanaka et al., 2010).

Previous studies have shown that mTOR inhibition alone is relatively ineffective at reducing overall tumor load in *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ mutants (Kinkade et al., 2008; Zhang et al., 2009). To test the hypothesis that combined AR and mTOR targeting may be more effective where single-agent use is not, we evaluated the impact of total AR/androgen ablation in conjunction with mTOR inhibition. When comparing *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* mutants under castration alone to those with rapamycin, we observed marked inhibition of cell proliferation and reduction of tumor load. Thus, these preclinical data suggest that complete abolishment of the AR/androgen-signaling axis combined with mTOR inhibition is superior to single-agent use, most likely by inhibiting the crosstalk between the two pathways. Thus, more effective blockade of the androgen/AR axis with new generation inhibitors such as abiraterone and MDV3100 in combination with mTOR or PI3K/mTOR dual inhibitors may prove to be further advantageous in treating CRPC cases initiated by alterations of PTEN/PI3K pathway.

EXPERIMENTAL PROCEDURES

Generation of *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* and *Pten*$^{L/L}$;*Tfm* Mutant Mice

Mice with conditional deletion of *Pten* and AR in the murine prostate were developed by crossing male *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ mice with *Cre*$^{-}$;*Ar*^{L}/*Ar*^{L} female mice on a mixed background. The AR conditional line (AR$^{loxP\text{-}exon2\text{-}loxP}$) was obtained from and previously described by the laboratory of Dr. Guido Verhoeven (De Gendt et al., 2004), whereas *Pb-Cre*$^{+}$;*Pten*$^{L/L}$ mutants were described by our laboratory (Wang et al., 2003). *Pten*$^{L/L}$;*Tfm* mutant mice were obtained by crossing *Pten*$^{L/L}$ mutants with Tfm mice (JAX) (Cunha and Chung, 1981). All animal experiments were approved by the UCLA Animal Research Committee and conducted according to relevant regulatory standards.

Cell Isolation and Flow Cytometry

The isolation of murine prostate epithelium was carried out by mechanical dissociation (5 min mincing) and enzymatic digestion (collagenase type I digestion at 37°C for 2 hr) of all prostate lobes. Single-cell suspensions were generated and stained in DMEM 10% FBS for 20 min at 4°C with anti-CD49f (BioLegend), and with a Lin cocktail comprised of anti-CD45, anti-CD31, and anti-Ter119 (eBioscience). Cell sorting was performed on the BD FACS Vantage or BD FACS Aria II (BD Biosciences). Lin^{-}CD49f$^{hi/mid}$ cells were collected and subsequently used for FACS analysis and/or tissue regeneration assays (Mulholland et al., 2009).

Prostate Regenerations

Donor (Lin^{-}CD49f$^{hi/mid}$) epithelia isolated from Cre^{-}, *Cre*$^{-}$;*Pten*$^{L/L}$, or *Cre*$^{-}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y*, or *Pten*$^{L/L}$;*Tfm* mutant mice were infected with high-titer (>1 × 10^{9}) Cre-lentivirus (Cre-FUCRW) by low-speed centrifugation over 90 min. Infected epithelia (1–2 × 10^{5}) were recombined with UGSM (2 × 10^{5}) in 15 μl collagen pellets and incubated for 6–10 hr in DMEM (high glucose, 10% FBS; insulin, 0.005 mg/ml; bovine pituitary extract, 10.7 μg/ml; EGF/FGF, 3.0 ng/ml). Tissue recombinants were then surgically implanted below the kidney capsule of NOD;SCID;IL2rγ null mice for 6–10 weeks (Mulholland et al., 2009; Lawson et al., 2010; Goldstein et al., 2010).

Plasmids

The murine *Ar*-specific short hairpin RNAs (shRNA) (Jiao et al., 2007) were cloned into the BamHI-EcoRI site the lentivector FUCRW under regulation with a U6 promoter. The open reading frame for the human *AR* gene with Flag tag was cloned at the XbaI site under the human ubiquitin promoter.

Orthotopic Lentivirus Injection

The LSL-Akt (loxP-RFP-loxP-myr-Akt-Ires-EGP) lentivector and virus were generated as previously described (Marumoto et al., 2009). To carry out orthotopic injections, 2–3 μl of high titer lentivirus (>1 × 10^{8}) was injected to the base of the anterior prostate lobe (proximal to urethra) of *Pb-Cre*$^{+}$;*Ar*^{L}/*Y* mice aged 2–3 weeks using a cemented, 10 μl microsyringe (Hamilton). Mice were then aged for 6–8 weeks and subsequently evaluated at the histological level.

Collection of Patient Samples

Clinical samples were obtained with informed consent under approval from the University of California at Los Angeles (UCLA) Institutional Review Board. All samples were obtained in a deidentified manner to maintain patient confidentiality.

Human Whole-Mount and TMA

Pathologic specimens (whole-mount specimens) were acquired from the UCLA Tissue Procurement Core Laboratory in a deidentified manner. IHC analysis and scoring were carried out on cores containing >30% epithelial content (Thomas et al., 2004).

Drug Treatment

Pb-Cre^{+};*Pten*$^{L/L}$ and *Pb-Cre*$^{+}$;*Pten*$^{L/L}$;*Ar*^{L}/*Y* were castrated and immediately treated with 4 mg/kg/day (i.p.) rapamycin (Selleck Chemicals), 10 mg/kg/day MDV3100, or vehicle control (p.o.). Mice were treated for 4 weeks and then evaluated at the histological level.

ACCESSION NUMBERS

Gene expression microarray data have been deposited in the GEO database with the accession number GSE29010.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at doi:10.1016/j.ccr.2011.05.006.

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