Emergence of Androgen Independence at Early Stages of Prostate Cancer Progression in *Nkx3.1; Pten* Mice

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Abstract

Although androgen deprivation therapy is a widely used treatment for patients with advanced prostate cancer, it ultimately results in the emergence of a hormone-refractory disease that is invariably fatal. To provide insights into the genesis of this disease, we have employed an *in vivo* model to investigate how and when prostate epithelial cells can acquire the ability to survive and proliferate in the absence of androgens. In particular, we have been studying the evolution of androgen independence in *Nkx3.1; Pten* mutant mice, which develop prostatic intraepithelial neoplasia and adenocarcinoma as a consequence of aging, as well as androgen-independent phenotypes following castration. We now find that the prostate epithelial cells from these *Nkx3.1; Pten* mutant mice are capable of surviving and proliferating in the absence of androgens and that they develop androgen-independent phenotypes well before they display overt prostatic intraepithelial neoplasia or cancer phenotypes. Our findings in this mouse model show that acquisition of androgen independence can be uncoupled from overt cancer progression and raise the possibility that hormone-refractory disease can arise at early stages of prostate carcinogenesis. (Cancer Res 2006; 66(16): 7929-33)

Introduction

All aspects of prostate development and prostate carcinogenesis are critically dependent on the activity of the androgen receptor and its primary ligands, testosterone and dihydrotestosterone (1–7). In fact, androgen deprivation therapy remains the most widely used treatment for patients with advanced prostate cancer. However, although androgen deprivation initially results in regression of prostate tumors, the tumors eventually reemerge and the resulting hormone-refractory (androgen independent) disease is invariably fatal. Therefore, understanding how and when prostate cancer cells acquire the ability to survive and proliferate in the absence of androgens is critical for the treatment of patients with prostate cancer.

We have been investigating the evolution of androgen-independent prostate cancer *in vivo* using a mouse model based on the loss-of-function of the *Nkx3.1* homeobox gene and the *Pten* tumor suppressor. *NKX3.1* maps within a critical region of human chromosome 8p21 that undergoes deletion in prostate cancer (8, 9). Furthermore, loss or reduction of *NKX3.1* expression is a hallmark of prostate cancer progression in humans as well as in mouse models of this disease, and inactivation of *Nkx3.1* in mutant mice leads to prostate intraepithelial neoplasia (PIN; refs. 10–16).

PTEN encodes a lipid phosphatase that is a key negative regulator of the PI-3 kinase signaling cascade, with one of its principal downstream targets being Akt/protein kinase B (17–19). *PTEN* maps to human chromosome region 10q23, which is deleted at high frequency in prostate cancer (8), and loss of PTEN protein expression is a frequent occurrence in advanced prostate cancer (20, 21). In mice, loss-of-function of *Pten* leads to high-grade PIN and/or carcinoma and can synergize with inactivation of other relevant genes, such as *Nkx3.1*, $p27^{kip1}$, or *p53*, in prostate cancer progression (15, 22–25).

Previously, we have found that loss-of-function of *Nkx3.1* and *Pten* leads to PIN and cancer with aging, as well as androgen independence following castration (22, 25). In the present study, we have investigated the evolution of androgen independence in *Nkx3.1; Pten* compound mutant mice. We find that these mice can acquire androgen-independent phenotypes before the formation of overt PIN or cancer. Our findings shed new light on the relationship between androgen independence and cancer progression, which may have implications for the treatment of patients with prostate cancer.

Materials and Methods

Measurement of androgen levels. The *Nkx3.1; Pten* mutant mice have previously been described (11, 22, 25). Castration was done by surgical removal of the testes and epididymus, and mice were analyzed 1 to 3 days or 3 to 10 months following surgery. Because the adrenal glands provide an alternative source of androgens that may be used in the absence of testosterone (26), we also did bilateral adrenalectomy along with castration to fully deplete all circulating androgens in several mice. Serum testosterone levels were determined using a competitive enzyme immunoassay [Cayman Chemical (Ann Arbor, MI) Testosterone EIA Kit]. Following castration, the levels of testosterone in the serum were reduced from the reference range of ~4 to 10 ng/mL to <0.001 ng/mL (n = 10).

Histologic and immunohistochemical analyses. The prostate and other organs of the male urogenital system were collected from euthanized mice and processed for histopathologic analyses as described (11, 22, 25). Histologic criteria for designation of PIN and/or cancer phenotypes have been described (27); analyses shown are for anterior prostate, with similar results observed in other prostatic lobes.[5] Procedures for immunohistochemical analyses were previously described (11, 13, 22, 25). Antibodies and dilutions were as follows: Ki67 (Novocastra, Newcastle, United Kingdom; 1:1,000), androgen receptor (Sigma, St. Louis, MO; 1:2,000), and p-Akt (Ser473; 1:200). Terminal deoxyribonucleotidyl transferase–mediated dUTP

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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nick end labeling (TUNEL) assays were done using an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany). Cell counting for determination of the relative numbers of proliferative and apoptotic prostate epithelial cells was done as described (11, 25).

Results

Survival and proliferation of *Nkx3.1; Pten* prostate epithelial cells following castration. In previous studies, we have shown that compound mutant mice lacking one or both alleles of *Nkx3.1* as well as one allele of *Pten* (*Nkx3.1*$^{+/-\ or\ -/-}$; *Pten*$^{+/-}$, hereafter referred to as *Nkx3.1; Pten* mutant mice) develop low-grade PIN by 3 to 6 months of age, high-grade PIN by 6 to 9 months of age, invasive adenocarcinoma by 12 months, and androgen-independent phenotypes following androgen ablation (refs. 22, 25; Fig. 1*A*). We have now used these *Nkx3.1; Pten* mutant mice to investigate the emergence of androgen independence *in vivo*.

Specifically, we have examined the consequences of surgical castration of these mice as a function of animal age and prostate cancer progression using four experimental regimens: group I, 0.75 months (3 weeks) of age, before complete maturation of the prostate; group II, 2 months, subsequent to prostate maturation but before the onset of PIN; group III, 6 months, subsequent to the onset of PIN, but before the onset of cancer; or group IV, 9 months or older, subsequent to the onset of cancer (Fig. 1*A*; Table 1). These mice were analyzed immediately following castration (1-2 days) for analyses of cell survival and proliferation, or were examined 3 to 6 months later for analyses of androgen-independent phenotypes. In this study, we define "androgen independence" as the ability of prostate epithelial cells to survive and display a robust proliferation index following removal of testicular androgens and for the mice to display PIN and/or cancer phenotypes several months after castration.

In striking contrast to wild-type control mice (n = 4), castration of *Nkx3.1; Pten* mutants (n = 6) did not induce massive apoptosis of the prostate at 2 days following castration, as shown by TUNEL analysis (Fig. 1*B-G* and Supplementary Fig. S1*A-D*). In particular, the high-grade PIN lesions of mutants castrated at 8 months and analyzed 2 days later (group III) displayed significantly less apoptosis (4%; n = 1,727 epithelial cells) than the prostate epithelium of wild-type littermates (20%; n = 976; Fig. 1*B-G*). Furthermore, the epithelial cells within these high-grade PIN lesions were highly proliferative, as determined by Ki67 immunoreactivity (16%; n = 1,188), compared with wild-type control epithelium (1%; n = 928; Fig. 1*H-M*). These findings suggest that at the time of castration, the high-grade PIN lesions of *Nkx3.1; Pten* mutant mice contain a significant percentage of cells that can survive and proliferate in the absence of androgens.

Interestingly, the *Nkx3.1; Pten* mutant mice castrated at 2 months of age and analyzed 2 days later (group II) also did not display massive apoptosis of the prostate, as shown by TUNEL analysis (Supplementary Fig. S1*A-D*) and instead showed a high proliferation index (Supplementary Fig. S1*E-H*). Together, these observations indicate that prostate epithelial cells from *Nkx3.1; Pten* mutant mice are able to survive and proliferate when testicular androgens are initially removed, even before when they display a PIN phenotype.

Acquisition of androgen-independent phenotypes before PIN or cancer formation in *Nkx3.1; Pten* mutant mice. When analyzed several months following androgen deprivation, a majority of the castrated *Nkx3.1; Pten* mutant mice displayed androgen-independent high-grade PIN and/or adenocarcinoma (Fig. 2*D-G*; Table 1). Such androgen-independent lesions were also observed in castrated *Pten* heterozygous single mutants (Fig. 3*C* and *F*) but were never found in the wild-type mice or in the *Nkx3.1* single mutant mice (Fig. 2*A* and *B*; Fig. 3*A, B, D*, and *E*; Table 1). These findings indicate that loss-of-function of *Pten* is sufficient for emergence of androgen independence in *Nkx3.1; Pten* mutant mice.

Because adrenal hormones can potentially activate androgen receptor in the absence of testosterone (28), we investigated whether high-grade PIN lesions also arise in *Nkx3.1; Pten* mutant mice that have undergone both castration and adrenalectomy, which would deplete all circulating androgens. We found that *Nkx3.1; Pten* mutant mice, but not the wild-type mice, displayed high-grade PIN lesions following castration and adrenalectomy

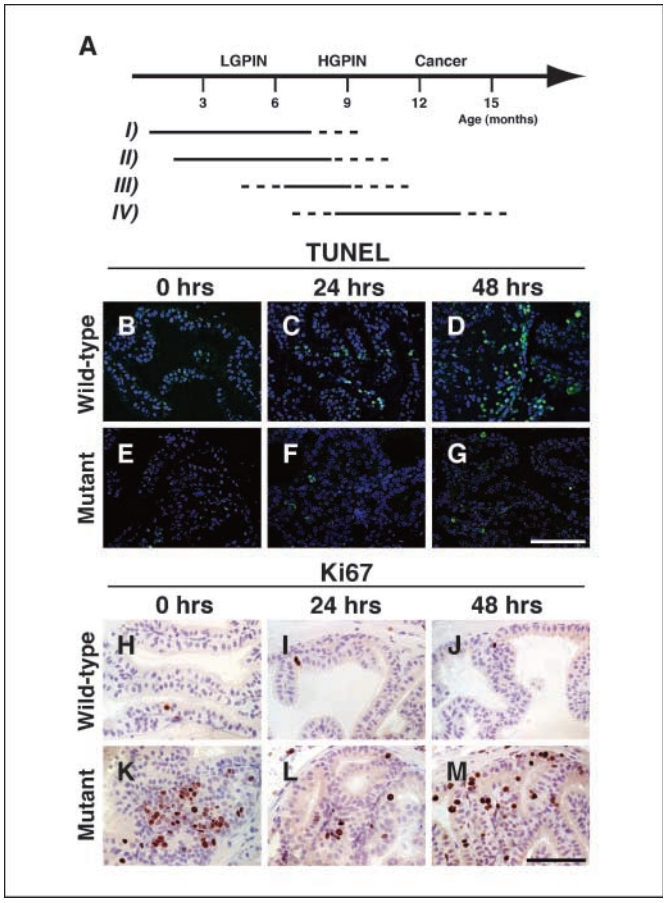

Figure 1. Analyses of androgen independence in *Nkx3.1; Pten* mutant mice. *A,* diagram of the experimental paradigm. On the top is shown the characteristic progression of prostate cancer in *Nkx3.1; Pten* mutant mice from low-grade PIN (*LGPIN*) to high-grade PIN (*HGPIN*) to cancer as previously reported (22, 25). Below are the experimental paradigms used in this study to investigate androgen independence in *Nkx3.1; Pten* mutants: *I,* castrated at 0.75 months and analyzed 1 to 2 days or 7 to 9 months later; *II,* castrated at 2 months and analyzed 1 to 2 days or 6 to 8 months later; *III,* castrated at 5 to 8 months and analyzed 1 to 2 days or 2 to 5 months later; *IV,* castrated at 9 months and analyzed 1 to 2 days or 3 to 6 months later. *B* to *M,* analyses of apoptosis and cellular proliferation in castrated *Nkx3.1; Pten* mutant mice. Wild-type (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$) or mutant (*Nkx3.1*$^{+/-}$; *Pten*$^{+/-}$) mice at 8 months (group III) were analyzed immediately (0 hours) or at 24 or 48 hours after castration for apoptosis by TUNEL assay (*B-G*), or for proliferation by Ki67 immunostaining (*H-M*). Note that 48 hours following castration, the *Nkx3.1; Pten* mutant mice do not display significant apoptosis (*green staining, B-G*) and are highly proliferative (*brown staining, H-M*). Similar data for group II mice are provided in Supplementary Fig. S1.

Table 1. Summary of androgen-independent phenotypes

Genotype	*N*	Group	Description of phenotype
Nkx3.1$^{+/+}$; *Pten*$^{+/+}$	30	[I-IV]	0 of 30 PIN*
Nkx3.1$^{-/-\ or\ +/-}$; *Pten*$^{+/+}$	30	[I-IV]	0 of 30 PIN*
Nkx3.1$^{+/+}$; *Pten*$^{+/-}$	8	[I-II]	6 of 8 focal PIN†
	9	[III-IV]	6 of 9 focal HGPIN‡
Nkx3.1$^{+/-\ or\ -/-}$; *Pten*$^{+/-}$	12	I	7 of 12 focal PIN
	8	II	7 of 8 focal HGPIN
	8	III	8 of 8 extensive HGPIN§
	13	IV	13 of 13 extensive HGPIN with invasive adenocarcinoma‖

NOTE: Summary of mice analyzed in each group and the phenotypes.
*The prostates of these mice were fully regressed without any evidence of PIN.
†The prostates of these mice displayed isolated regions of PIN.
‡The prostates of these mice displayed isolated regions of high-grade PIN.
§The prostates of these mice displayed high-grade PIN throughout most of the tissue.
‖The prostates of these mice displayed high-grade PIN throughout most of the tissue with areas of adenocarcinoma.

(Fig. 4). Therefore, we conclude that these lesions are truly independent of all androgen sources.

The histologic features of the androgen-independent high-grade PIN and/or cancer lesions that occur in the castrated *Nkx3.1; Pten* mice were comparable to lesions that occur in age-matched intact (noncastrated) *Nkx3.1; Pten* mutant mice (refs. 22, 25, 27; Fig. 2, compare *C* with *D-G*). These androgen-independent lesions displayed cytoplasmic as well as nuclear localization of androgen receptor, consistent with depletion of endogenous androgens (Fig. 2*K-N*). Notably, they also displayed robust activation of membrane-localized Akt (Figs. 2*Q* and *R-U*), indicative of complete *Pten* loss-of-function. Activation of Akt was never observed in the wild-type mice either before or after castration (Fig. 2*O* and *P*).

To determine the earliest time point at which *Nkx3.1; Pten* mutant mice can acquire androgen-independent phenotypes, we compared the phenotype of mice castrated at 0.75 or 2 months (groups I and II) with those castrated at 6 months or older (groups III and IV). Surprisingly, we found that most of the *Nkx3.1; Pten* mutants castrated at 2 months (group II; $n = 7$ of 8) and half of those castrated at 0.75 months (group I; $n = 7$ of 12) displayed androgen-independent lesions when examined at 8 to 9 months of age (Table 1). Whereas the group I castrates displayed small, focal PIN lesions, the group II castrates displayed high-grade PIN lesions that were histologically indistinguishable from those of the group III mice, which were castrated at 6 months or older (Fig. 2*D* and *E*). Furthermore, regardless of their age when castrated, many *Nkx3.1; Pten* mutant mice analyzed at 8 months of age displayed

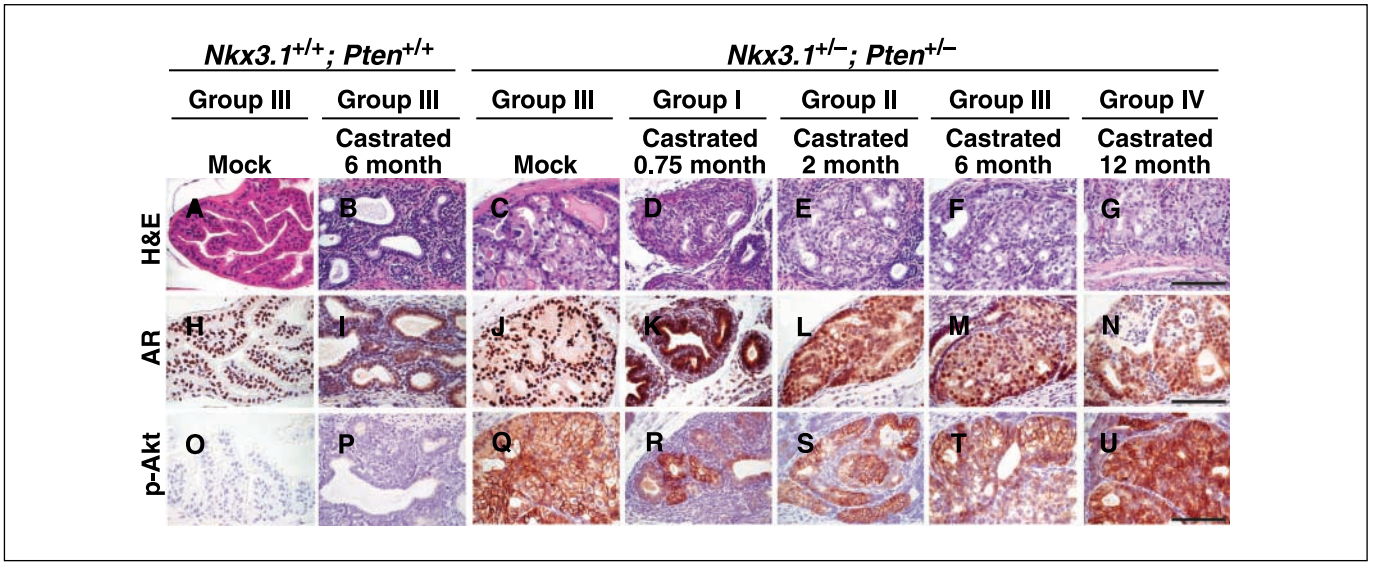

Figure 2. Androgen-independent PIN and cancer lesions in *Nkx3.1; Pten* mutant mice. Wild-type (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$) or mutant (*Nkx3.1*$^{+/-}$; *Pten*$^{+/-}$) mice were mock-castrated (*Mock*) or were surgically castrated at 0.75 months (*Group I*), 2 months (*Group II*); or 6 months (*Group III*) and analyzed at 8 months of age, or were castrated at 10 months and analyzed at 15 months (*Group IV*). Sections from the anterior prostate were analyzed by H&E staining or by immunostaining for androgen receptor or activated Akt (p-Akt). Representative data from group III for the wild-type mice (mock and castrated) and the noncastrated (mock) mutant mice. Note that in each experimental group, the castrated mutant mice, but not the wild-type mice, display PIN lesions that express androgen receptor and robust levels of p-Akt, although the PIN lesions in the group I mutants are small and focal.

high-grade PIN, whereas those analyzed at 12 months or greater displayed high-grade PIN with invasion (Table 1), which resembles the time course of disease progression in their noncastrated counterparts (22, 25). These results indicate that acquisition of androgen independence in *Nkx3.1; Pten* mice can occur before the onset of PIN or cancer, and therefore suggest that androgen independence can emerge in parallel with disease progression, rather than as an end-point.

Discussion

In this study, we have investigated the acquisition of androgen independence *in vivo* using a mouse model that is based on loss-of-function of *Nkx3.1* and *Pten*. The major findings of our study are that *Nkx3.1; Pten* mutant mice can develop androgen-independent phenotypes before the occurrence of overt PIN or cancer and that *Pten* loss-of-function is sufficient for these androgen-independent phenotypes. Notably, our findings in these mutant mice complement a growing body of evidence supporting the idea that *Pten* loss-of-function is critical for the development of hormone-refractory cancer in humans. Indeed, recent studies have shown that *Pten* expression is reduced in hormone-refractory cancer (29) whereas forced expression of *Pten* in human prostate cancer cell lines promotes their androgen responsivity (30).

In contrast to *Pten*, we find that loss-of-function of *Nkx3.1* is not sufficient to promote androgen independence. However, it is likely that *Nkx3.1* loss promotes androgen independence in the *Nkx3.1; Pten* mutant mice by potentiating the effects of *Pten* inactivation (25). Indeed, our findings in the *Nkx3.1; Pten* mutant mice differ somewhat from a previous study showing that conditional deletion of *Pten* in the prostatic epithelium leads to androgen independence because, in this previous study, the prostate epithelium underwent apoptosis immediately following castration (15). Whereas this difference may be due to conditional deletion of *Pten* in the prostatic epithelium (previous study) versus germ-line deletion of *Pten* (our study), it may also reflect the collaborative activities of *Nkx3.1* and *Pten* in our mouse model. The role of *Nkx3.1* in this capacity will be interesting to explore in future studies.

Notably, an important distinction between the emergence of androgen independence in *Nkx3.1; Pten* mice versus the onset of hormone-refractory prostate cancer in humans is the sizeable proportion of prostate epithelial cells that can survive and proliferate immediately following androgen deprivation. This contrasts with the proportion of "androgen-independent" cells in human prostate cancer, which has been estimated to represent ~1 in every 10^5 to 10^6 cells before androgen ablation (31). We believe that this difference reflects the preexisting uniform loss of one *Pten* allele in all prostate epithelial cells in the *Nkx3.1; Pten* mutant mice, greatly increasing the likelihood of complete *Pten* inactivation, whereas in humans, loss of both *Pten* alleles (or other possible molecular alterations that promote androgen independence) would presumably be stochastic and occur in a limited cell population.

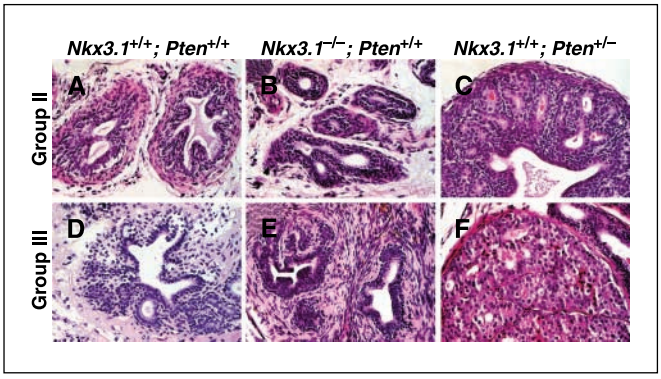

Figure 3. Loss-of-function of *Pten* is sufficient for androgen independence. H&E staining of sections from the anterior prostates of wild-type mice (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$), *Nkx3.1* single mutant mice (*Nkx3.1*$^{-/-}$; *Pten*$^{+/+}$), or *Pten* single mutant mice (*Nkx3.1*$^{+/+}$; *Pten*$^{+/-}$) that were castrated at 2 months (*Group II*) or 6 months (*Group III*) and analyzed at 8 months of age, as indicated. Note that the prostate epithelia from the wild-type and *Nkx3.1* single mutants are atrophic whereas the *Pten* single mutant displays PIN lesions.

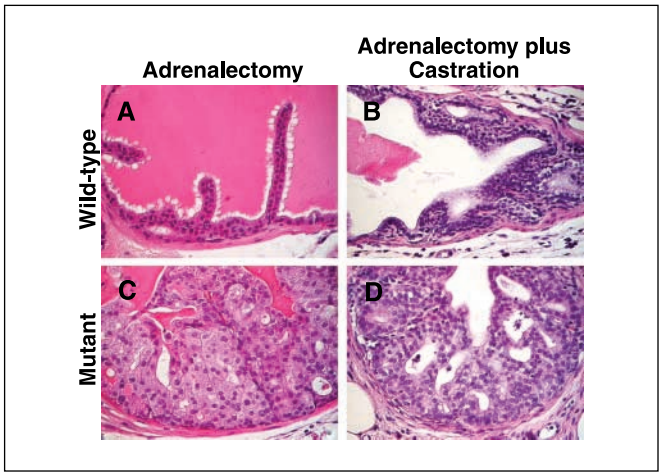

Figure 4. Androgen-independent lesions following adrenalectomy and castration. H&E staining of sections from the anterior prostates of wild-type mice (*Nkx3.1*$^{+/+}$; *Pten*$^{+/+}$) or *Nkx3.1; Pten* mutant mice (*Nkx3.1*$^{+/-}$; *Pten*$^{+/-}$) that had undergone adrenalectomy or adrenalectomy as well as castration as indicated; surgeries were done on mice at 6 months and the mice were then analyzed at 8 months. Note that the mutant mice display high-grade PIN lesions following adrenalectomy and castration, which depletes all circulating androgens.

Consequently, our findings favor a clonal selection mechanism for the emergence of hormone-refractory disease, which predicts that androgen-independent cells are present even in the absence of overt cancer progression, as opposed to an adaptation model in which androgen-dependent cells acquire hormone independence through genetic and/or epigenetic alterations (32). In particular, Craft et al. (31) proposed that androgen independence arises in two distinct stages, with an initial selection for preexisting cells that can survive in the absence of androgens and their subsequent clonal expansion.

In conclusion, our analysis of *Nkx3.1; Pten* mutant mice has enabled investigation of the relationship between cancer progression and the emergence of androgen-independent disease. Notably, our finding that androgen independence can emerge before overt cancer phenotypes in *Nkx3.1; Pten* mice has implications for interpreting the outcome of the recently concluded Prostate Cancer Prevention study, which investigated the long-term consequences finasteride, a 5(α)-reductase inhibitor (33). Somewhat paradoxically, although the finasteride-treated population displayed a significant reduction in prostate cancer incidence, those individuals who developed cancer had higher-grade disease (33). Whereas several possible explanations have been discussed (34, 35), our current results support the interpretation that finasteride promotes an "androgen-independent" state in some prostate cancers, perhaps those with preexisting mutations of

Pten or other genes that enable prostatic cells to survive under conditions of androgen deprivation. This interesting possibility can be tested in the context of the *Nkx3.1; Pten* mutant mice.

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