NKF3.1 stabilizes p53, inhibits AKT activation, and blocks prostate cancer initiation caused by PTEN loss

Qunying Lei,1 Jing Jiao,1 Li Xin,2 Chun-Ju Chang,1 Shunyou Wang,1 Jing Gao,1 Martin E. Gleave,5 Owen N. Witte,1,2,3 Xin Liu,1,4 and Hong Wu1,*

1 Department of Molecular and Medical Pharmacology, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, California 90095
2 Department of Microbiology, Immunology, and Molecular Genetics, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, California 90095
3 Howard Hughes Medical Institute, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, California 90095
4 Department of Pathology and Laboratory Medicine, David Geffen School of Medicine at the University of California, Los Angeles, Los Angeles, California 90095
5 The Prostate Centre at Vancouver General Hospital, Vancouver, British Columbia V6H 3Z6, Canada
*Correspondence: hwu@mednet.ucla.edu

Summary

We demonstrate that PTEN loss causes reduced NKF3.1 expression in both murine and human prostate cancers. Restoration of Nkf3.1 expression in vivo in Pten null epithelium leads to decreased cell proliferation, increased cell death, and prevention of tumor initiation. Whereas androgen receptor (AR) positively regulates NKF3.1 expression, NKF3.1 negatively modulates AR transcription and consequently the AR-associated signaling events. Consistent with its tumor suppressor functions, NKF3.1 engages cell cycle and cell death machinery via association with HDAC1, leading to increased p53 acetylation and half-life through MDM2-dependent mechanisms. Importantly, overexpression of Nkf3.1 has little effect on Pten wild-type epithelium, suggesting that PTEN plays a predominant role in PTEN-NKF3.1 interplay. Manipulating NKF3.1 expression may serve as a therapeutic strategy for treating PTEN-deficient prostate cancers.

Introduction

Prostate cancer is the second leading cause of cancer-related death in males (Gregorakis et al., 1998; McDavid et al., 2004). Its development proceeds through a series of defined steps, including prostatic intraepithelial neoplasia (PIN), invasive cancer, and hormone-dependent or -independent metastasis. Although different stages of prostate cancer have been well defined histologically, relatively little is known about the molecular mechanisms contributing to the initiation and progression of prostate cancer.

The PTEN (phosphatase and tensin homolog deleted on chromosome 10) tumor suppressor gene is frequently mutated in human cancers (Dahia, 2000; Maehama et al., 2001; Parson et al., 1998). The major function of PTEN relies on its phosphatase activity toward PIP3 (phosphatidylinositol 3,4,5-triphosphate) and, consequently, antagonism of the PI3K (phosphatidylinositol 3-kinase) signaling pathway (Di Cristofano et al., 2001; Maehama et al., 2001). Loss of PTEN function results in accumulation of PIP3 and activation of its downstream effectors, such as AKT/PKB (Maehama et al., 2001). AKT, a serine/threonine protein kinase, phosphorylates key intermediate signaling molecules, leading to increased cell metabolism, growth, survival, and invasiveness, all hallmarks of cancer (Di Cristofano et al., 2001; Hanahan and Weinberg, 2000; Vivanco and Sawyers, 2002).

PTEN alteration is strongly implicated in prostate cancer development, as mutations of the PTEN gene are found in 30% of primary prostate cancers (Dahia, 2000; Sellers and Sawyers, 2002) and 63% of metastatic prostate tissue samples (Suzuki et al., 1998). Thus, PTEN mutations are among the most frequent genetic alterations in human prostate cancer. As PTEN-controlled signaling pathways are frequently altered in human prostate cancers, inhibiting the resultant signaling aberrations will likely serve as promising targets for therapeutic strategies.

Significance

Gene expression profiling of mouse tumor models or human cancers has identified many dysregulated genes that may contribute to tumor development. These wealth of data sets, upon functional validation, may help in elucidating the molecular mechanisms underlying tumorigenesis and providing potential novel targets for cancer therapies. Using a powerful prostate epithelial tissue reconstitution assay, we demonstrated the importance of NKF3.1 in prostate cancer initiation caused by PTEN loss. Our finding emphasizes the cooperative effects between ubiquitously expressed PTEN tumor suppressor genes and prostate-specific expressed NKF3.1 in prostate cancer development. Our study further indicates that validation of candidate genes using mouse models can yield valuable molecular insights that impact human cancer research.
We and others have developed murine models of prostate cancers by deleting the Pten tumor suppressor gene specifically in the prostatic epithelium (Chen et al., 2005; Ma et al., 2005; Trotman et al., 2003; Wang et al., 2003). The Pten prostate cancer model recapitulates many features of the disease progression seen in humans with defined kinetics: initiation of prostate cancer with PIN lesions, followed by progression to locally invasive adenocarcinoma, and subsequent metastasis (Wang et al., 2003). Similar to human cancer, Pten null murine prostate cancers regress in response to androgen ablation therapy but subsequently relapse and proliferate in the absence of androgens (Wang et al., 2003).

Global assessment of molecular changes caused by homozygous Pten deletion identified key genes known to be relevant to human prostate cancer, including those “signature” genes associated with human cancer metastasis (Wang et al., 2003). Among the genes that are downregulated in Pten null prostate cancer is Nkx3.1, a homeobox gene specifically expressed in the prostate epithelium. NXX3.1 is one of the earliest markers for prostate development and is continuously expressed at all stages during prostate development and in adulthood (Bhatia-Gaur et al., 1999). Human Nkx3.1 maps to chromosome 8p21, a region that frequently undergoes loss of heterozygosity (LOH) at early stages of prostate carcinogenesis (He et al., 1997; Voeller et al., 1997). Nkx3.1 mutant mice develop prostatic hyperplasia and dysplasia. However, these early lesions failed to progress to metastatic cancers (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999), consistent with a role for Nkx3.1 inactivation in prostate cancer initiation.

In this study, we employed a dissociated prostatic epithelial regeneration system to directly test the significance of Nkx3.1 loss in Pten null prostate cancer formation. Our data show that Nkx3.1 plays an important role in prostate cancer initiation caused by PTEN loss and that forced Nkx3.1 expression prevents Pten null prostate cancer initiation and progression. Thus, decreased Nkx3.1 expression contributes to prostate cancer development caused by PTEN loss.

**Results**

**PTEN loss leads to reduced NKKX3.1 expression in both murine and human prostate cancers**

Our previous gene expression profiling analysis revealed that Nkx3.1 mRNA level is downregulated in the Pten null prostate cancers (Wang et al., 2003). In this study, consecutive sections of ventral prostate lobe from 4-week-old (4W) Pten conditional knockout animals were probed with antibodies to either Nkx3.1 or phospho-AKT (P-AKT, Ser 473) (Figure 1A). In the acini where P-AKT levels are low, intense Nkx3.1 staining can be observed (Figure 1A, arrows). In contrast, areas with high P-AKT are either low or negative for Nkx3.1 staining (Figure 1A, arrowheads). Since increased AKT phosphorylation is a consequence of PTEN loss and that forced Nkx3.1 expression prevents Pten null prostate cancer initiation and progression. Thus, decreased Nkx3.1 expression contributes to prostate cancer development caused by PTEN loss.

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**Figure 1.** PTEN loss leads to decreased Nkx3.1 protein levels in both murine and human prostate epithelium

A: Consecutive sections of 4-week-old Pten mutant prostates were probed for Nkx3.1 (left) and phospho-AKT (Ser 473) (P-AKT, right) expression. Arrows and arrowheads point to the same duct. Note that Nkx3.1 expression inversely correlates with P-AKT staining.

B: High-magnification views of two representative samples from human prostate cancer tissue microarrays are shown here. Upper panels: H&E staining; middle and lower panels: double immunofluorescent staining using anti-PTEN (middle) and anti-NKX3.1 (lower) antibodies. Insert: high-power overlay of NKX3.1 and DAPI staining showing NKX3.1 nuclear localization.

C: Correlation of PTEN and Nkx3.1 protein levels in 153 human prostate samples. SPSS linear regression was used to analyze data, and the standardized coefficient value was 0.52 (p < 0.01 level; n = 153).

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prostate tissue microarrays (Rocchi et al., 2004). Among 153 samples surveyed (see Experimental Procedures), positive PTEN expression was significantly correlated with Nkx3.1 staining, whereas PTEN loss was associated with decreased Nkx3.1 staining (Figure 1C, p < 0.01). Photos from two representative samples are shown in Figure 1B. Therefore, PTEN loss leads to decreased Nkx3.1 expression in both human and murine prostate cancers, implying that Nkx3.1 may serve...
as an important regulator downstream of PTEN-controlled signaling pathway in prostate cancer development.

**Forced Nkx3.1 expression in Pten null epithelium using an exogenous promoter**

Several mechanisms have been proposed for loss of Nkx3.1 expression in human prostate cancers, including both transcriptional and posttranscriptional regulations (Asatiani et al., 2005; Bowen et al., 2000; He et al., 1997; Korkmaz et al., 2004; Ornstein et al., 2001; Voeller et al., 1997). To understand how PTEN controls Nkx3.1 expression and to evaluate the functional relevance of Nkx3.1 downregulation in Pten null prostate cancer initiation, we employed a dissociated prostate cell regeneration method (Xin et al., 2003, 2005) to test (1) whether we can restore Nkx3.1 expression in Pten null epithelium to a level comparable to the wild-type (wild-type) by using an exogenous promoter, and (2) the consequence of forced Nkx3.1 expression in Pten null prostate epithelium. In order to circumvent the low transfection efficiency of mouse prostate epithelium, we cloned Flag-tagged Nkx3.1 into a lentiviral vector (Xin et al., 2003, 2005) in which Nkx3.1 expression is driven by the ubiquitin promoter followed by an IRES-eGFP expression cassette (Figure 2A) (Lois et al., 2002). Nkx3.1 expression can be detected indirectly via eGFP expression (Figure 2B, left panels) and by Western blot analysis of transfected 293T cells using total protein lysate (Figure 2B, upper row in the right panel) or eGFP-sorted cells (Figure 2B, middle row in the right panel) with an anti-Flag antibody.

We then infected epithelium from 4W Pten null mice (Mut), corresponding to the hyperplastic stage, and the epithelium from their wild-type littermates with Nkx3.1 expressing (Nkx3.1 group) or control lentivirus (GFP group). Infected epithelium was then mixed with mesenchyme isolated from embryonic day 16 wild-type urogenital sinus mesenchyme (UGSM) and grafted under the renal capsule of CB17SCID/SCID mice and propagated for 6 weeks. Low but detectable levels of PTEN expression can be found in the mutant grafts by Western blot analysis (Figure 2C, insert), most likely attributable to the wild-type UGSM cells used for reconstitution. Importantly, lentivirus-mediated gene expression in Pten null grafts restored Nkx3.1 protein levels so that they were comparable to those of the WT graft (Figure 2C, insert). The fact that Nkx3.1 protein expression can be restored and maintained near the wild-type levels via an exogenous promoter suggests that PTEN modulates Nkx3.1 function largely through regulation of its transcription, e.g., by controlling its promoter activity.

**Introducing Nkx3.1 into Pten null prostatic epithelium leads to reduced graft growth**

To evaluate the effects of PTEN loss and forced Nkx3.1 expression, we quantified the graft weight (Figure 2C, upper panel) and DNA contents (Figure S2). To increase the confidence of our analysis, we considered only the epithelial compartment, instead of both the mesenchymal and epithelial ones, as a function of PTEN loss or forced Nkx3.1 expression (Figure 2C, lower panel). Compared to wild-type grafts (blue), Pten null grafts (in red) are significantly larger and include greater number of epithelium (Figure 2C, left, compare wild-type-GFP and Mut-GFP groups) while forced Nkx3.1 expression consistently reduced Pten null graft weight and epithelial cell numbers (Figure 2C, compare Mut-GFP and Mut-Nkx3.1 grafts; p < 0.05). To evaluate whether the effect of forced Nkx3.1 expression depends on the stage of cancer, epithelium from PIN lesions (6W) were harvested and yielded similar observations (Figure 2C, right). No significant difference was observed when mock-infected wild-type grafts were compared to Nkx3.1-infected wild-type grafts (Figure 2C, compare wild-type-GFP and wild-type-Nkx3.1 groups). These data show that forced Nkx3.1 expression can reverse the growth advantage of Pten null epithelium but has little effects on its wild-type counterpart.

**Nkx3.1 blocks Pten null prostate cancer initiation and progression**

Histological analysis indicated that wild-type and Pten null epithelium reconstituted prostate grafts recapitulate the
histological characterization of the donor epithelium (Figures 3A and 3C) (Wang et al., 2003). No significant difference was observed when comparing wild-type-GFP and wild-type-Nkx3.1 grafts (compare Figures 3A and 3B). In contrast, tissue recombinants from Pten null epithelium resulted in very differential phenotypes: those from the GFP group demonstrated hyperplasia and early mPIN lesions, whereas grafts from the Nkx3.1 expression group showed relative preservation of normal prostatic duct structure with protein secretion in the lumen (compare Figures 3C and 3D). Occasionally, isolated areas with hyperplastic phenotype were observed (Figure 3D, circled area). Further studies using consecutive sections suggest that these hyperplastic lesions are most likely due to incomplete viral infection, as evidenced by the low to undetectable NKX3.1 protein expression and high P-AKT staining in the same region (circled areas in Figures 3H and 3L, respectively). These results suggest that forced Nkx3.1 expression can rescue the hyperplastic phenotype caused by PTEN loss.

**Forced Nkx3.1 expression leads to decreased cell proliferation and increased apoptosis of Pten null grafts**

Previous studies showed that overexpression of NKX3.1 in human PC3 prostate cancer cells and rodent AT 6 cells leads to inhibition of cell growth (Kim et al., 2002a), whereas increased epithelial cell proliferation is observed in Nkx3.1 knockout mice (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Magee et al., 2003; Tanaka et al., 2000). We examined whether forced Nkx3.1 expression could inhibit cell proliferation in Pten null epithelium grafts. Detection of the Ki-67 epitope indicated that Nkx3.1 expression significantly decreased the proliferative index of 4W and 6W mutant grafts (compare Figures 3G and 3H; quantification shown in Figure 3M; p \( \leq 0.05 \)). Consistently, Pten null epithelium appears to be hypersensitive to NKX3.1’s suppression effect, since NKX3.1 at similar protein levels (Figure 2C, insert) has no significant effect on cell proliferation in the wild-type grafts (Figures 3E, 3F, and 3M).

Given that Pten null grafts infected with NKX3.1 were consistently smaller than those of the control GFP group (Figure 2C), the apoptotic index was evaluated by way of the TUNEL assay. In both 4W and 6W Pten null grafts, a significant increase in TUNEL-positive cells was observed in the presence of NKX3.1 (Figure 3N; p \( \leq 0.05 \)). When consecutive tissue sections were probed for the presence of NKX3.1, TUNEL-positive cells were consistently associated with areas of strong NKX3.1 expression (Figure S3). No increase in TUNEL-positive cells was observed in 4W or 6W wild-type grafts (Figure 3N). Taken together, our data demonstrate that the effects of NKX3.1 on cell proliferation and cell death depend on the PTEN status.
NKX3.1 negatively regulates AKT activity in an AR-dependent manner

To investigate possible changes in the known PTEN-controlled signaling pathways in the presence and absence of NKX3.1, we first examined AKT status on consecutive sections of the grafts. No difference in either PTEN or P-AKT levels can be detected in the wild-type grafts with or without Nkx3.1 overexpression (Figures 3I and 3J). Robust P-AKT staining can be detected in the mock-infected Pten null grafts (Figure 3K), whereas forced Nkx3.1 expression, at a level comparable to that of wild-type prostatic epithelium (compare Figures 3F and 3H, and Figure 2C), leads to a dramatic decrease in P-AKT levels (Figure 3L). In the uninfected area where NKX3.1 staining is weak or undetectable (Figure 3H, circled area), P-AKT level remains high, thus serving as an important internal control (Figure 3L, circled area).

To develop a system more amenable to in vitro biochemical analysis, we generated several prostate epithelial cell lines from the prostates of Pten conditional knockout mice. One such line, PTEN-CaP2, is characterized by positive AR expression but undetectable PTEN and NKX3.1 protein expression (our unpublished data). Nkx3.1 overexpression in PTEN-CaP2 cells suppressed AKT phosphorylation by 3-fold (Figure 4A, lower panel).

Given previous studies that androgen receptor (AR) can modulate AKT activation via a PI3K-dependent mechanism (Baron et al., 2004; Sun et al., 2003), we investigated whether AR serves as a mediator for NKX3.1-regulated AKT activity. For this, we used PC3 cells, a human prostate cancer cell line known to be null for AR, PTEN, and NKX3.1. When we compared P-AKT levels in the presence or absence of NKX3.1 without cotransfection of AR, we did not detect any significant difference (Figure 4B, first two lanes in the upper panel). While introduction of AR significantly increased the level of P-AKT (Figure 4B, compare first and third lanes in the upper panel), this effect was diminished by cotransfection of the Nkx3.1-expressing vector (Figure 4B, compare the third and fourth lanes). Previous studies also showed that AR modulates AKT phosphorylation via binding of the p85α subunit of PI3-kinase (Baron et al., 2004; Sun et al., 2003). We performed reciprocal coimmunoprecipitation experiments and showed that Nkx3.1 overexpression decreases the
amount of AR able to interact with p85α (Figure 4B, lower panels), further supporting the notion that NKX3.1 controls AKT phosphorylation via an AR/Pi3K-dependent mechanism.

**NKX3.1 negatively regulates the AR promoter**

Since AR serves as a mediator for NKX3.1-controlled AKT activation, we investigated the possible role of NKX3.1 on AR expression in the PTEN-CaP2 cells and found that NKX3.1 also inhibits AR expression at both mRNA (Figure 4A, upper panel) and protein levels (Figure 4A, lower panel). To determine if NKX3.1 negatively regulates AR in vivo, we examined AR protein levels in the prostate glands of Nkx3.1 knockout mice (Kim et al., 2002b) and found that AR levels are indeed increased (Figure 4C). The role of NKX3.1 in negatively regulating AR level was also confirmed in human primary prostatic PrEC cells (Figure 4D, upper panel; compare lanes 2 and 4 in the lower panel).

Similar to previous reports (Bieberich et al., 1996; He et al., 1997; Magee et al., 2003; Prescott et al., 1998), our study indicates that NKX3.1 expression can be positively modulated by androgen (Figure 4D, compare lanes 3 and 4; Figure 6B). That overexpression of NKX3.1 leads to AR downregulation (upper panels in Figures 4A and 4D) while NKX3.1 loss results in an increased level of AR (Figure 4C) suggests that AR and NKX3.1 may form an important feedback loop. Within this feedback loop, NKX3.1 may serve as an important negative regulator for AR expression as well as AR-controlled signaling pathway. To test this hypothesis, we searched sequences surrounding the murine AR promoter region and found a potential NKX3.1 consensus binding site, TAAGTA, within the 5' UTR. The functional significance of this consensus site was further investigated using a series of luciferase reporter constructs with truncated promoter regions (Figure 4E, upper panel). NKX3.1 significantly suppressed the wild-type reporter construct, while it had little effect on a Δ315 construct in which sequences surrounding the putative binding motif have been deleted (Figure 4E, lower panel). Changing “TAAGTA” to “TAAAAA” also decreased NKX3.1’s effect (Figure 4E, lower panel).

To assess whether NKX3.1 can associate with the endogenous AR promoter, we conducted chromosome immunoprecipitation (ChIP) analysis on the human prostate cancer cell line LNCaP, which expresses endogenous NKX3.1 and AR. Using a primer set that contains the human NKX3.1 “CAAG” motif (nt 1223–1386), we showed that the endogenous NKX3.1 can physically associate with the AR promoter and that this association can be further enhanced in the presence of androgen (Figure 4F, primer 2). We further demonstrate that this association is site specific: primer 1 set derived from nt 1113–1311 of the human AR promoter containing the “CAAG” motif, as well as control primers (nt 17–170) without the CAAG motif, can not detect NKX3.1 binding activity. Therefore, NKX3.1 inhibits AR transcriptional activity, at least in part, through its consensus binding site.

**NKX3.1 negatively regulates AR expression in both murine and human prostatic cancer samples**

To test whether the inverse relationship between NKX3.1 and AR is present in human prostate cancer samples, we conducted double immunofluorescent analysis of NKX3.1 and AR and found two basic scenarios: areas where NKX3.1 levels negatively correlate with AR levels (Figure 5A) and areas where NKX3.1 and AR are coexpressed (Figure 5B). Interestingly, even in the areas where both NKX3.1 and AR are expressed, mosaic patterns of NKX3.1 and AR expression can be easily detected (Figure 5B, high-powered image on the right), suggesting that the NKX3.1–AR feedback loop may function at a single cell level.

Importantly, despite upregulated AR levels (Figure 5C), NKX3.1 mRNA levels are downregulated in both PIN (Figure 5C) and Pten null prostate cancer (Wang et al., 2003), suggesting that PTEN either plays a predominant role over AR, the known positive regulator of Nkx3.1, or is essential for AR-mediated Nkx3.1 regulation. We then compared the relative expression levels of those NKX3.1 target genes identified by Magee et al. (2003) that are also present in our microarray data sets (Wang et al., 2003 and our unpublished data). Intriguingly, the trends of gene regulation in the Pten null prostate are very similar to what has been observed in the Nkx3.1 null prostate: Probasin, which is known to be positively regulated by NKX3.1, is downregulated in the Pten null PIN lesions, whereas Elafin-like II, which belongs to the repression group, is upregulated upon Pten deletion (Figure 5C). Furthermore, the mosaic expression patterns of NKX3.1 and AR shown in Figure 5B may provide an explanation for the stochastic expression patterns of NKX3.1-targeted genes within the same prostatic acini (Magee et al., 2003).

**NKX3.1 stabilizes p53 through MDM2-dependent and AKT-independent mechanisms**

PTEN regulates p53 protein levels and transcription activity via AKT-DM2-dependent and -independent mechanisms (Freeman et al., 2003; Mayo and Donner, 2001; Zhou et al., 2001). The fact that overexpression of NKX3.1 inhibits AKT phosphorylation in Pten null grafts prompts us to analyze whether NKX3.1 also alters p53 levels. Western blot analysis demonstrated that forced Nkx3.1 expression significantly increases p53 levels in Pten null grafts in vivo (Figure 6A, upper panels), whereas knockout of Nkx3.1 leads to reduced p53 levels (Figure 6A, lower panels).

To understand the molecular mechanisms involved in NKX3.1-regulated p53 activity, we first measured endogenous p53 levels in the LNCaP cells. The androgen analog R1881 can stimulate the endogenous NKX3.1 expression with a peak around 4 hr (Figure 6B, upper panel). Following this trend, p53 protein levels are also increased by 1.5-fold 4 hr after R1881 addition (Figure 6B, upper panel). Consistently, overexpression of Nkx3.1 leads to increased p53 protein levels without changing of its mRNA level (Figure S4, upper panel), suggesting that NKX3.1 modulates p53 at the posttranscriptional level. Furthermore, Nkx3.1 expression leads to an increase in p53 half-life from 21 min to 30 min in LNCaP cells (Figure 6B, lower panel). Similar results were also obtained when using the Pten null murine PTEN-CaP2 prostate cancer cell line (Figure S4, lower panel).

To determine whether NKX3.1 stabilizes p53 in a MDM2-dependent manner, we introduced Nkx3.1 into p53/Mdm2 double null (p53−/−;Mdm2−/−) mouse embryonic fibroblasts (Jones et al., 1995). Without MDM2, NKX3.1 has no significant influence on p53 expression levels (Figure 6C, compare lanes 3 and 4). When cotransfected with a vector containing Mdm2, NKX3.1 expression partially reverses the increased p53 degradation brought about by Mdm2 overexpression (Figure 6C, compare lanes 5 with 6), suggesting that NKX3.1 controls p53 half-life via a MDM2-dependent mechanism.

MDM2 nuclear translocation and stability are known to be controlled by AKT phosphorylation (Mayo and Donner, 2001;
To determine whether NKX3.1 antagonizes MDM2 function via an AKT-dependent mechanism, we treated PC3 cells with or without LY294002, a specific inhibitor for PI3K. As shown in Figure 6D, although P-AKT levels were significantly diminished, LY294002 treatment had no significant effect on either p53 (compare lanes 2 and 5) or NKX3.1-regulated p53 level (compare lanes 3 and 6). This result suggests that NKX3.1 regulates p53 half-life by modulating nuclear MDM2 activity, independent of AKT activation.

NKX3.1 can physically associate with HDAC1 and promotes p53 acetylation by recruiting HDAC1 from p53-MDM2-HDAC1 complex

Besides its E3 ubiquitin ligase activity, MDM2 can negatively regulate p53 half-life by recruiting a nuclear protein HDAC1, thereby promoting p53 deacetylation and degradation (Kobet et al., 2000; Ito et al., 2002; Brooks and Gu, 2003). To determine if enhanced p53 acetylation is one of the mechanisms involved in NKX3.1-mediated p53 half-life control, we examined acetylated p53 levels without or with Nkx3.1 overexpression and found that NKX3.1 indeed enhances p53 acetylation (Figure 6E, upper panel). In vivo, NKX3.1 can physically associate with either endogenous or exogenous HDAC1 (Figure 6E, middle and lower panels), suggesting that NKX3.1 can form a complex with HDAC1 and change p53 acetylation status. Furthermore, NKX3.1 cannot further increase p53 levels in the presence of TSA, an inhibitor for HDAC activity, suggesting that NKX3.1 indeed depends on HDAC activity to regulate p53 level (Figure S5).

We then tested whether NKX3.1 can recruit HDAC1 from p53-MDM2-HDAC1 complex. To do this, Mdm2 was introduced into p53−/−;Mdm2−/− cells, without or with Nkx3.1, followed by measurement of the relative amount of p53 associating with HDAC1. It was observed that Mdm2 expression leads to increased p53-HDAC1 association by 4-fold, resulting in decreased total p53 protein level (compare lanes 1 and 2 of Figure 6F). In contrast, coexpressing Nkx3.1 reduces p53-HDAC1 association, partially rescuing MDM2-mediated p53 degradation (Figure 6F, compare lanes 2 and 3). Thus, NKX3.1 regulates p53 half-life, as least in part, by recruiting HDAC1.
Discussion

The involvement of NKX3.1 in human prostate cancer development has been intensively studied since its cloning (for review see Abdulkadir, 2005). Despite strong correlation of loss of NKX3.1 expression in human prostate cancer initiation and progression, several outstanding questions remain to be answered; these include but are not limited to the following. (1) How and at what level is NKX3.1 expression controlled in the prostate epithelium? (2) What is the normal function of NKX3.1 in vivo? And (3) what are the targets of NKX3.1, and how do those targets, upon NKX3.1 loss, contribute to prostate cancer initiation and progression? Our current study suggests that PTEN and its controlled signal pathway regulate NKX3.1 expression in the prostatic epithelium. Within the prostate epithelium, AR and NKX3.1 form a signaling feedback loop in which NKX3.1 is the negative modulator that keeps AR level and AR-controlled pathways in check. NKX3.1 regulates prostate cell proliferation and survival not only via its transcription factor activity, i.e., by negatively modulating the AR promoter, but also through protein-protein interaction with HDAC1 and consequently regulating p53 half-life and activity (Figure 7). Our finding emphasizes the cooperative effects between ubiquitously expressed PTEN tumor suppressor genes and prostatic-specific expressed NKX3.1 in prostate cancer development.
Several mechanisms have been proposed for loss of NKX3.1 expression in human prostate cancers, including both posttranscriptional modification, such as protein degradation, as well as transcriptional and epigenetic regulation (Asatiani et al., 2005; Bowen et al., 2000; He et al., 1997; Korkmaz et al., 2004; Omstein et al., 2001; Voller et al., 1997). The facts that NKX3.1 mRNA (Figure 5C and Wang et al., 2003) and protein levels (Figure 1A and Wang et al., 2003) are concomitantly downregulated in the Pten null prostate, and we can successfully restore Nkx3.1 expression using an exogenous promoter and maintain near wild-type levels of NKX3.1 in different cell lines and in the renal capsule grafts (Figure 2C), suggest that PTEN modulates NKX3.1 function largely through regulation of its promoter activity. This conclusion is consistent with the strong correlation of NKX3.1 mRNA and protein levels in human prostate cancer specimens reported by a recent study (Korkmaz et al., 2004).

Nkx3.1 is positively regulated by androgen and its receptor AR at the transcriptional level (Bieberich et al., 1996; He et al., 1997; Magee et al., 2003; Prescott et al., 1998). Our results suggest that PTEN either predominantly controls NKX3.1 transcription or contributes significantly to AR-controlled mechanism. In the presence of PTEN, Nkx3.1 expression can be modulated by AR, while in its absence, Nkx3.1 expression is almost completely silenced despite increases in AR levels in Pten null prostate cancers (Wang et al., 2003; Figures 1A, 5C, and 7). Mechanisms involving enhancer/promoter and transcription activator/repressor interactions, epigenetic changes such as DNA methylation (Asatiani and Gelmann, 2005), and histone modification (Plass, 2002) are all potential possibilities by which NKX3.1 and AR interact and are regulated by PTEN and warrant a separate and more elaborate study.

Recently, Magee et al. identified Nkx3.1 target genes that are sensitive to Nkx3.1 dosage in a stochastic manner and, at the same time, are influenced by the androgen status in vivo (Magee et al., 2003). Among those target genes is a set regulated by androgens only in the absence of Nkx3.1, suggesting that Nkx3.1 functions as a selector for modulation of the expression of potential androgen target genes. We demonstrate that Nkx3.1 loss, in either human prostate cancer samples (Figure 5A) or murine prostates of Pten and Nkx3.1 knockout mice (Figures 5C and 4C), leads to increased AR levels. Conversely, overexpression of Nkx3.1 decreases AR mRNA and protein expression (Figures 4A and 4D). These results imply that Nkx3.1 is a negative regulator for AR expression, and AR and Nkx3.1 form a feedback loop important for both prostate development and cancer formation. With Nkx3.1 at steady-state levels, androgen/AR induces Nkx3.1 expression, which in turn inhibits AR expression. Interestingly, the mosaic expression pattern of Nkx3.1/AR target genes showed by Magee and colleagues (Magee et al., 2003) are similar to Nkx3.1 and AR expression patterns observed in human prostate samples (Figure 5B), suggesting that the balance of Nkx3.1-AR expression may ultimately determine the levels of target gene expression. The natural “set point” of this feedback loop and its range of action are currently unknown and require further investigation. Loss of Nkx3.1 in the prostatic epithelium will impair this feedback system (Figure 7), which in turn leads to AR overexpression and may contribute to PIN lesion in Nkx3.1 knockout mice (Abdulkadir et al., 2002; Kim et al., 2002a) and prostate cancer development in Pten null model (Wang et al., 2003), similar to what has been concluded from AR transgenic animals (Stanbrough et al., 2001) and human prostate cancer studies (Chen et al., 2004).

The growth-suppressive activities of Nkx3.1 have been demonstrated in vitro in cell culture system and in vivo in knockout mice (Bhatia-Gaur et al., 1999; Kim et al., 2002a; Schneider et al., 2000; Tanaka et al., 2000). Nkx3.1 haploinsufficiency or...
loss leads to significant delay in exiting from the cell cycle (Magee et al., 2003), but how NKX3.1 engages the cell cycle machinery to regulate prostatic epithelium growth is currently unknown. Here, we showed that restoration of Nkx3.1 expression to the wild-type level in Pten null prostatic epithelium leads to increased p53 protein levels in vivo and in vitro. NKX3.1 can physically associate with HDAC1 and recruit HDAC1 from the HDAC1-MDM2-p53 complex, protecting p53 from deacetylation and degradation. Furthermore, NKX3.1 stabilizes p53 through modulation of MDM2 activity in the nucleus, independent of AKT-mediated MDM2 phosphorylation and nuclear translocation. Whether the above-mentioned pathways involve or collaborate with Nkx3.1-regulated oxidative damage or p53-dependent senescence requires further investigation (Chen et al., 2005; Ouyang et al., 2005).

Although this study suggests that NKX3.1 plays an essential role in PTEN-controlled prostate cancer development, NKX3.1 loss alone only mimics a part of Pten deletion phenotype, suggesting that other PTEN-controlled pathways may synergistically interact with those NKX3.1-dependent events (Figure 7). AKT hyperphosphorylation has been observed in isolated clusters of cells within regions of high grade PIN lesions in Nkx3.1+/− and Nkx3.1−/− mice, and deletion of one allele of Pten in Nkx3.1 null mice leads to accelerated tumor initiation and increased incidence of high-grade PIN (Kim et al., 2002b). However, differing from the Pten null prostate cancer model (Wang et al., 2003), Pten+/−/Nkx3.1−/− mice do not progress to metastatic prostate cancer (Kim et al., 2002b). These results suggest that the dosage of PTEN plays a dominant role in the cooperative effect of NKX3.1 and PTEN.

We showed that forced Nkx3.1 expression in Pten null epithelium significantly induces cell apoptosis in the mutant grafts. This mechanism, together with NKX3.1’s growth suppression function, leads to reduced graft size. Interestingly, the effects of Nkx3.1 appear to depend on the PTEN status: Nkx3.1 overexpression leads to increased cell death and decreased cell proliferation in the Pten null grafts but has no significant impact on the wild-type grafts. One possible explanation is that Pten null cells have become “addicted” to high levels of PI3K/AKT activity and, consequently, are hypersensitive to inhibition of this pathway. We have previously shown that PTEN-deficient human cancer cell lines and murine Pten null tumors are sensitive to inhibitors specific for mTOR, a downstream effector of the PI3K/AKT pathway (Neshat et al., 2001). In the present study, we demonstrate that NKX3.1 inhibits AKT phosphorylation/activation via an AR-dependent mechanism and show that NKX3.1 expression in vivo can block the hyperproliferative and antia apoptotic effects brought on by PTEN loss. This mechanism elucidates a pathway that can potentially be targeted with specific therapies for human prostate cancer, given that 30% of primary prostate cancers and as many as 60% of metastatic cases exhibit PTEN LOH (Sellers and Sawyers, 2002).

**Experimental procedures**

**Immunohistochemical analysis**

Tissues were fixed in 10% buffered Formalin for 6 hr, followed by transfer to 70% alcohol. These paraffin-embedded tissues were sectioned (4 μm) and stained with hematoxylin and eosin. Antigen retrieval was performed by incubating the slides in 0.01 M citric acid buffer (pH 6.0) at 95°C for 30 min. The endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide (H₂O₂) in methanol. The following detection and visualization procedures were performed according to the manufacturer’s protocol. Negative control slides were performed without primary antibody. Control slides known to be positive for each antibody were incorporated. For fluorescence double staining, pretreated sections were first blocked with mouse IgG blocking reagent in the VECTOR M.O.M. Immunodetection Kit (Vector Laboratories) and then incubated with mouse antibody PTEN (2H6, Cell Signaling Technology), Ki67 (NCL-Ki67-MM1), or Nkx3.1 (a kind gift from Dr. Abate-Shen) at room temperature for 30 min, followed by signal amplification with TSA Plus Fluorescence Systems (PerkinElmer). After biotin blocking, the section was then stained with rabbit antibody P-AKT (9277, Cell Signaling Technology) or AR (PG21, Upstate), and signal was amplified with TSA system with different fluorescence. For human prostate tissue array, PTEN, Nkx3.1 (SC-816 and SC-15022, Santa Cruz), and AR (PG21, Upstate) were used.

**Tissue array analysis**

Double immunofluorescent staining was performed as above. PTEN and Nkx3.1 (Santa Cruz SC-816 and SC-15022, respectively) intensity was analyzed by Image Plus software, and only cores with epithelial structure were chosen for further analysis. Briefly, we chose four views for each core; measured the signal intensity using Image Plus software; and signed the signal intensity as 0–6, in which 0[0–50]; 1[50–75]; 2[75–100]; 3[100–125]; 4[125–150]; 5[150–175]; 6[>175]. According to the average intensity value, samples were categorized into grades. By using the intensity grade, a stacked line was generated with value of each sample displayed. Linear regression analysis was performed for the correlation between PTEN and Nkx3.1 expression by SPSS software and presented as Scattagram.

**Preparation of prostate epithelial cells, virus infection, and prostate regeneration**

Four- and six-week-old Pten wild-type and mutant mice were killed by carbon dioxide inhalation. Prostates were dissected, cut into small pieces with a steel blade, and digested with 0.8 mg/ml collagenase (GIBCO, 226 units/mg) in 10 ml of DMEM 10% FBS (GIBCO) at 37°C for 90 min. Cells were filtered through 100 μm nylon mesh (Becton Dickinson), washed twice with 10 ml of DMEM 10% FBS, resuspended in 1 ml of DMEM 10% FBS, and counted.

The lentivirus was prepared as described (Lois et al., 2002; Pfeifer et al., 2002). Infection was carried out according to Xin et al. (2003). Briefly, 1 × 10⁴ prostate cells were mixed with GFP or Nkx3.1. Lentivirus stock (titr 2 × 10⁴) in the presence of 8 μg/ml polybrene (Sigma), then centrifuged at 1500 rpm with a Beckman GS-6R centrifuge (Beckman Coulter) for 2 hr at room temperature and washed twice with 1 ml of DMEM 10% FBS. All procedures were performed under University of California Los Angeles safety regulations for lentivirus usage.

Mouse prostate regeneration was performed according to previous reports (Cunha and Donjacour, 1987; Thompson et al., 1989; Xin et al., 2003). Lentivirus-infected cells (1 × 10⁶) were combined with UGSM cells (1 × 10⁴) and 25 μl of type I collagen (Roche) and then grafted under the renal capsule. Each experiment contained grafts of UGSM alone to ensure that tissue growth did not result from contaminating urogenital sinus epithelial cells. Grafts were harvested and weighed after 6–9 weeks. All surgical procedures were performed under Division of Laboratory Animal Medicine regulations of the University of California, Los Angeles.

**Cell proliferation and apoptosis index**

Cell proliferation index was examined by Ki67 staining. Five different fields were chosen, and then 200 cells were counted in each field. Ki67+ cells were presented as the percentage of nucleated cells. Cell apoptosis was determined by TUNEL assay using the In Situ Cell Death Detection Kit from Roche according to the manufacturer’s instruction. Sections were dehydrated with xylene and rehydrated through graded alcohol. DNA fragmentation was labeled with fluorescein-conjugated DUTP and visualized with fluorescence. TUNEL-positive cells were counted and presented the percentage of nucleated cells.

**Western blot analysis**

Protein lysate was prepared by sonication graft tissues and prostate tissue from Nkx3.1 mice. R1881 treated or transfected LNCaP, PC3 and PTEN-GaP2 cells in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl,
0.1% SDS, 0.5% SD, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 25 mM NaF, and cocktail protease inhibitors (Roche). Tissue lysate (40 μg) was resolved to SDS-PAGE followed by Western blot analysis using anti-p53 (Ab-1, Oncogene; DO-1, Santa Cruz), P-AKT (9271, Cell Signaling), total AKT (Cell Signaling), Nkx3.1 (SC-15022, Santa Cruz), MDM2 (Ab-2, Oncogene), Flag (Stratagene), and Actin (#5060, Sigma) antibodies, respectively.

For immunoprecipitation experiments, 500 μg of cell lysate was incubated 16 hr at 4°C with 2 μg AR or p85x antibody (PG-21 and #06-496, Upstate), p53 antibody (DO-1, Santa Cruz), Nkx3.1 or HDAC1 (SC-10022, SC-07872, Santa Cruz), or Flag antibody (Sigma, F1804) plus 50 μl Protein A agarose beads (#16-125, Upstate). Beads were washed three times with lysis buffer and centrifuged for 5 min at 5000 g between each wash. Protein was eluted from beads with 50 μl Laemmli sample buffer (Bio-Rad). Lysates were resolved on a 10% SDS-PAGE gel and transferred onto nitrocellulose (Bio-Rad).

For endogenous Nkx3.1 induction, LNCaP cells were treated with 2 nM R1881 for different time periods after being plated in 10% charcoal serum for 2 days. For half-life experiments, Nkx3.1 was transfected into LNCaP cells 36 hr prior to the addition of 50 μg/ml cycloheximide (Cycloheximide, Calbiochem) in serum-free medium. Cells were then lysed at indicated time points and further analyzed by Western blot.

**Supplemental data**
The Supplemental Data include Supplemental Experimental Procedures and five supplemental figures and can be found with this article online at http://www.cancer.org/cgi/content/full/9/5/367/DC1/.

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