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Award Number: W81XWH-04-1-0824

TITLE: Molecular Mechanism of Nkx3.1 Deregulation and its Function in Murine Pten Prostate Cancer Model

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REPORT DATE: September 2005

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-09-2005	Annual Summary	1 Sep 04 - 31 Aug 05

4. TITLE AND SUBTITLE
Molecular Mechanism of Nkx3.1 Deregulation and its Function in Murine Pten Prostate Cancer Model

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0824

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Los Angeles, CA 90095

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Both PTEN and Nkx3.1 play important role in prostate cancer control. Preliminary results from *Pten* prostate model indicated that reduced Nkx3.1 expression is correlated with Cre-mediated *Pten* deletion. The goal of this study is to investigate how these two proteins interplay in prostate cancer development. We generated and characterize the primary cell lines from Pten prostate cancer model, which will facilitate us to study the molecular mechanism of Nkx3.1 deregulation by Pten loss. We demonstrate that restoration of Nkx3.1 into Pten null prostate epithelium can lead to increases cell apoptosis, decreased cell proliferation and prevention of tumor initiation. The anti-tumor effect of Nkx3.1 is correlated with stabilization p53, inhibition Akt activation and AR function.

15. SUBJECT TERMS
PTEN, Nkx3.1, Animal model, molecular mechanism

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	48	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The *PTEN* tumor suppressor gene is one of the most frequently mutated/deleted genes in human prostate cancer, especially in advanced or metastatic forms[1]. Our murine *Pten* prostate cancer model mimics human prostate cancer development and provides an ideal experimental tool for characterization of the molecular events in disease initiation and progression[2]. Preliminary results from *Pten* prostate model indicated that reduced Nkx3.1 expression is correlated with Cre-mediated *Pten* deletion. Since loss of NKX3.1, which correlates with human prostate cancer initiation and progression, is regulated by unknown mechanism, the goal of this study is to investigate the mechanism by which PTEN regulate Nkx3.1 and to evaluate the role of Nkx3.1 loss in PTEN controlled prostate cancer development.

Two specific tasks should be accomplished in this study:

Task 1: To investigate the molecular mechanism by which PTEN regulates NKX3.1;
Task 2: To investigate the role of Nkx3. 1 loss in PTEN controlled prostate cancer development by tissue reconstitution assay.

BODY: STUDIES AND RESULTS

I. To investigate the molecular mechanism by which PTEN regulates NKX3.1

I-1. To establish and characterize epithelial cell lines corresponding to different stages of tumor development (Partially finish)

We generated and characterized two set of primary cells ($Pten^{lox/-}$P2, $Pten^{-/-}$CaP2 and $Pten^{lox/-}$P8, $Pten^{-/-}$CaP8) from late stage of Pten null prostate cancer. They have AR expression, demonstrated androgen sensitivity and can grow in androgen-depleted serum medium. Our study further emphasize the important dosage effect of PTEN on tumor suppress. Tumor formation study demonstrated subcutaneous injection only Pten-CaP2 and Pten-CaP8, not $Pten^{+/-}$P2 and $Pten^{+/-}$P8, can form tumor in both female and male SCID mice. Therefore, these primary cells are useful tools for in vitro study the molecular mechanism of prostate cancer progression, especially the role of PTEN on prostate cancer control. This part of study "***Establishment and characterization of primary epithelial cell lines from Murine Pten prostate cancer model***" was presented in **2005 AACR annual meeting**. This part of study is on the manuscript preparation.

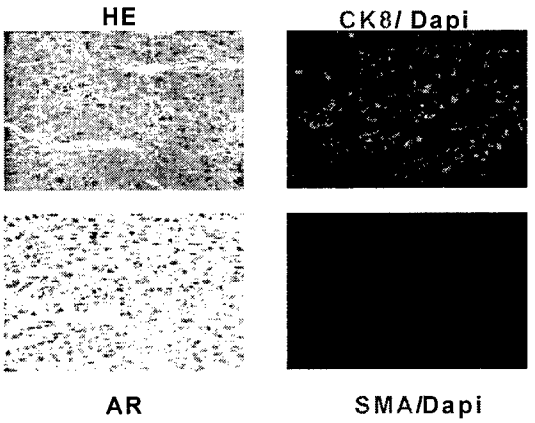

Figure 1: Tumor formation by subcutaneous injection of Pten-CaP8 in SCID mice - HE and immunostaining of tumor section.

To quickly establish and characterize epithelial cell lines corresponding to different stages of tumor development, we tried to use the Cre-activable enhanced green fluorescent protein (eGFP) reporter mice to facilitate the isolation of *Pten* null prostate epithelial cells from stromal cells. By crossing strategy as proposed in this fellowship, we got $Pten^{loxp/loxp}$; $ARR2PB\text{-}Cre^{+}$, *e-GFP*+ male mice. However, the FACS analysis demonstrated there was only very low amount of e-GFP positive prostate cells. Therefore, the unexpected low expression of e-GFP makes this approach unpractical. Another approach we proposed in the fellowship is to use Cre-Lox recombination system to generate the isogenic $Pten^{loxp/loxp}$ and $Pten^{?\,loxp/?\,loxp}$ epithelial cells. During establishement lines $Pten^{loxp/loxp}$(functional *Pten* WT) isolated from the prostate of $Pten^{lox/lox}$ mice, cells can't stand for trypsnize and became dead.

Since the purpose of this I-1 was to generate the cell lines for in vitro study, the two primary cell line and their corresponding Pten null cells will provide us the unique tool for future study.

I-2. To characterize Nkx3.1 expression, localization, and protein level in the established murine epithelial cells (partially finish)

Western analysis of primary cell lines generated from I-1 showed no Nkx3.1 protein expression. RT-PCR study from our murine Pten prostate cancer model showed that in 10 weeks, mutant prostate don't have Nkx3.1 transcription expression, which suggest that PTEN may regulate Nkx3.1 at the transcriptional level.

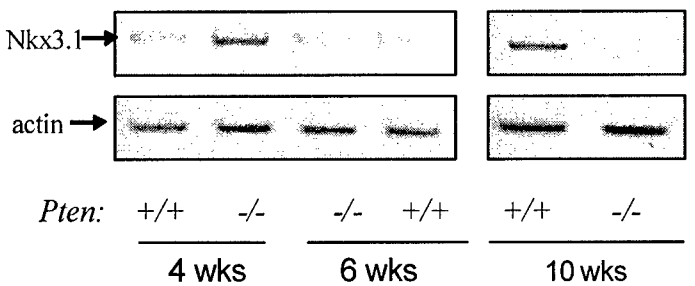

Figure 2: Characterize Nkx3.1 mRNA level from different stage of Pten prostate mouse tissue.

I-3. To study the molecular mechanism controlling NKX3.1 degradation (partially finish)

Previous study suggested that Nkx3.1 undergo a proteosome-dependent degradation passway and has a very short half life. To exam the mechanism by which PTEN regulates Nkx3.1, I generated the myc-tag and flag-taged human Nkx3.1 as well as mouse Nkx3.1 construct. Though hNkx3.1 and p27 share the similar homolog sequence related Skp2 recognition, we didn't find dominant negative of Skp2 can block the endogenous Nkx3.1 degradation, which suggest that PTEN may regulate Nkx3.1 undergo Skp2-independent passway.

Ongoing study for task 1 part is to continue exam the role of PTEN in regulating Nkx3.1 transcription and protein stability: 1) I will generate lucififase-Nkx3.1 promoter and exam PTEN'S role in transcriptional regulation. To increase the transcription efficiency, I will use the inducible Pten lenti-construct; 2) I will use the cell line generate in this study as well as human cell lines to exam the role of PTEN on Nkx3.1 post-transcriptional expression.

II. To investigate the role of Nkx3.1 loss in PTEN controlled prostate cancer development

II-1. Reintroduce Nkx3.1 into *Pten* null prostate epithelium and test its effect on prostate cancer development **(Accomplished in year 1, for detail, see attached manuscript)**

Using renal capsule prostate reconstitution assay[3] with the collaboration with another postdoc(Lei qunyin) in our lab, we demonstrated that restoration of *Nkx3.1* expression to the WT level in the *Pten* null epithelium leads to decreased cell proliferation, increased cell death and prevention of tumor initiation.

Significantly, by reintroducing Nkx3.1 into the Pten-Cap2 cells, we observed the increased p53 expression and decreased AR expression. The positive regulation of p53 by Nkx3.1 was further confirmed in human Lncap cell line as well as Nkx3.1 knock out prostate tissue. RT-PCR analysis and p53 half-life study demonstrated that Nkx3.1 regulated p53 at the post-transcriptional level. I further demonstrated that NKX3.1 stabilizes p53 through MDM2-dependent and AKT-independent mechanisms, regulates androgen receptor (AR) transcription and inhibits AKT activation in AR-dependent manner.

II-2. Test the molecular mechanism by which Pten modulates Nkx3.1 in the tissue reconstitution assay

Since we can successfully reintroduce *Nkx3.1* expression using an exogenous promoter, and maintain near WT levels of NKX3.1 in different PTEN null cell lines and in the renal capsule grafts, it suggest that PTEN may modulate NKX3.1 expression largely through regulation of its transcription. Therefore, it also gave us the new focus in Aim I-3.

KEY RESEARCH ACCOMPLISHMENTS

- Establish and characterize of primary cell lines from Pten-null prostate tissue
- Finish the investigation of Nkx3.1's role in PTEN controlled cancer initiation and progression (task 2)
- demonstrate p53, Akt, AR as downstream Nkx3.1 anti-tumor effectors

REPORTABLE OUTCOMES

- Publication

Jing Jiao, Qunyin Lei, Shunyou Wang, Hong Wu (2003) Establishment and characterization of primary epithelial cell lines from Murine *Pten* prostate cancer model. In: Ninty-six Annual Meeting of the American Association for Cancer Research, Anaham, April 2005

Qunying Lei, **Jing Jiao**, Li Xin, Chun-Ju Chang, Shunyou Wang, Jing Gao, Martin E Gleave, Owen N Witte, Xin Liu, Hong Wu (2005, manuscript, Cancer Cell) NKX3.1 Stabilizes p53, Inhibits AKT Activation and Blocks Prostate Cancer Initiation Caused by PTEN Loss

CONCLUSIONS

During the first funding period of this project, we have accomplished the task 2: we demonstrated that restoration of Nkx3.1 can reverse the pathological phenotype associated with PTEN loss in the prostatic epithelium. Notably, restoration of NKX3.1 to WT level in Pten null prostate epithelium results in decreased cell proliferation and increased cell apoptosis, which can be correlated to the enhancement of p53 expression and reduction of AKT activation. NKX3.1 stabilizes p53 in a MDM2-dependent but AKT-independent manner and inhibits AKT activation through an AR-dependent mechanism. This part of study highlights the important role of Nkx3.1 in preventing tumor initiation and the collaboration of the two tumor suppressor in prostate disease control.

We generated the primary epithelial cells from Pten null prostate for task 1. We found only the cell lines having completely Pten deletion can form tumor in SCID mice. These cell lines have androgen response and can grow in the androgen-depleted serum medium. These cell lines therefore provide unique tool for study the role of PTEN in prostate cancer control and progression. To investigate the molecular mechanism by which PTEN regulates NKX3.1, we will focus our work more on the transcriptional regulation of Nkx3.1 by PTEN.

REFERENCES

1. Porkka, K.P. and T. Visakorpi, *Molecular mechanisms of prostate cancer.* Eur Urol, 2004. **45**(6): p. 683-91.
2. Wang, S., et al., *Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer.* Cancer Cell, 2003. **4**(3): p. 209-21.
3. Xin, L., et al., *In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme.* Proc Natl Acad Sci U S A, 2003. **100 Suppl 1**: p. 11896-903.

APPENDICES

Manuscript

Qunying Lei, **Jing Jiao**, Li Xin, Chun-Ju Chang, Shunyou Wang, Jing Gao, Martin E Gleave, Owen N Witte, Xin Liu, Hong Wu (2005, manuscript, Cancer Cell) NKX3.1 Stabilizes

p53, Inhibits AKT Activation and Blocks Prostate Cancer Initiation Caused by PTEN Loss

Copy of an abstract

Jing Jiao, Qunyin Lei, Shunyou Wang, Hong Wu (2003) Establishment and characterization of primary epithelial cell lines from Murine *Pten* prostate cancer model. In: Ninty-six Annual Meeting of the American Association for Cancer Research, Anaham, April 2005

NKX3.1 Stabilizes p53, Inhibits AKT Activation and Blocks Prostate Cancer Initiation Caused by PTEN Loss

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Summary

Loss of *NKX3.1*, a homeobox gene specifically expressed in the prostate epithelium, has been implicated in human prostate cancers. In this study, we show that *Nkx3.1* expression is decreased immediately following Cre-mediated excision of the *Pten* tumor suppressor gene in the murine prostate. Restoration of *Nkx3.1* expression to the WT level in the *Pten* null epithelium leads to decreased cell proliferation, increased cell death and prevention of tumor initiation. NKX3.1 stabilizes p53 through MDM2-dependent but AKT-independent mechanisms, regulates androgen receptor (AR) transcription and inhibits AKT activation in AR-dependent manner. The suppressive effects that NKX3.1 confers depend on the PTEN status: when PTEN remains intact, NKX3.1 has little effect on either cell proliferation or cell survival. Our study suggests that manipulating NKX3.1 expression may serve as a potential therapeutic target for treating prostate cancers that have undergone loss of PTEN function.

SIGNIFICANCE

Gene expression profiling of mouse tumor models or human cancers has identified many dysregulated genes that may contribute to tumor development. These wealthy data-sets, upon functional validation, may help in elucidating the molecular mechanisms underlying tumorigenesis and provide potential new targets for cancer therapies. Using a powerful prostate epithelial tissue reconstitution assay, we demonstrated the importance of NKX3.1 in prostate cancer initiation caused by PTEN loss. Our findings suggest that validation of candidate genes using mouse models can yield valuable molecular insights that impact human cancer research.

Introduction

Prostate cancer is the second leading cause of cancer-related death in males (Gregorakis et al., 1998). 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, relative little is known about the molecular mechanisms contributing to the initiation and progression of prostate cancer.

The *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) tumor suppressor gene is the first phosphatase identified to be frequently mutated/deleted in human cancers (Dahia, 2000; Maehama et al., 2001; Parson et al., 1998). The major function of PTEN relies on its phosphatase activity and consequent 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 (phosphatidyl inositol 3,4,5-triphosphate) 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, cell growth, cell survival, and cell 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 deletion or mutation of the *PTEN* gene (10q23.3) are found in approximately 30% of primary prostate cancers (Dahia, 2000; Sellers and Sawyers, 2002) and 63% of metastatic prostate tissue samples (Suzuki et al., 1998). This places *PTEN* mutation among the most frequent genetic alterations

in human prostate cancer. PTEN-controlled signaling pathways are frequently altered in human prostate cancer, making them promising targets for therapeutic strategies (DeMarzo et al., 2003; Sellers and Sawyers, 2002; Vivanco and Sawyers, 2002).

We have developed a murine model of prostate cancer by deleting the *Pten* tumor suppressor gene specifically in the prostatic epithelium. 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 down-regulated in *Pten* null prostate cancer is *Nkx3.1*, a homeobox gene specifically expressed in the prostate epithelium. NKX3.1 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 LOH (loss of heterozygosity) at early stages of prostate carcinogenesis (He et al., 1997; Voeller et al., 1997). *Nkx3.1* mutant mice develop prostatic epithelial hyperplasia and dysplasia. However, these early lesions failed to progress before 2 years (Bhatia-Gaur et al., 1999), an observation that is 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 shows 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 *NKX3.1* down regulation in both murine and human prostate cancers.

Our previous gene expression profiling analysis revealed that *Nkx3.1* mRNA level was down regulated by more than 20-fold in the *Pten* null prostate cancers. This down regulation was confirmed using Northern and Western blot analyses (Wang et al., 2003). In this study, consecutive sections of ventral prostate lobe from 4-week-old *Pten* conditional knock-out 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, arrow heads). Since increased AKT phosphorylation is a consequence of PTEN loss (Supplement Figure 1), these staining patterns suggest that *Nkx3.1* down regulation is an early event linked to *Pten* deletion and prostate cancer initiation.

To test whether PTEN regulated NKX3.1 expression can be observed in human prostate cancers, we conducted double immunofluorescent analysis of PTEN and NKX3.1, using human 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 were shown in Figure 1B. Therefore, down regulation of NKX3.1 expression is associated with PTEN loss in both human and murine prostate cancers.

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

To evaluate the functional relevance of *Nkx3.1* down regulation in *Pten* null prostate cancer initiation, we employed a dissociated prostate cell regeneration method, in which UGSM (urogential sinus mescenchyme) cells support epithelial growth from stem and progenitor elements in young adult murine prostate (Xin et al., 2003, 2005). The consequence of forced *Nkx3.1* expression in *Pten* null prostate epithelium was tested using a viral infection system. 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 epithelial cells from 4W old *Pten* null mice (Mut), corresponding to the hyperplastic stage of cancer development, and their wild type (WT) littermate with *Nkx3.1* expressing (NKX3.1 group) or control lentivirus (GFP group). The infected epithelial cells were

then mixed with the mescenchymal cells isolated from E16 WT UGSM region and grafted under the renal capsule of $CB17^{SCID/SCID}$ mice for six 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 WT UGSM cells used for reconstitution. Importantly, lentivirus-mediated gene expression in *Pten* null grafts restored NKX3.1 expression to a level comparable to that of the WT graft (Figure 2C, insert). Comparing to the WT grafts (in blue), *Pten* null grafts (in red) are significantly bigger in size and by weight (Figure 2C left, comparing two GFP groups) while forced *Nkx3.1* expression consistently reduced *Pten* null graft size and weight (Figure 2C, comparing GFP and NKX3.1 *Pten* null grafts; $P<0.05$). To determine whether the effect of *Nkx3.1* overexpression depends on cancer stage, we also harvested epithelial cells from PIN lesion stage (6W) and observed similar effects (Figure 2C, right). No significant difference was observed when mock infected WT grafts were compared to *Nkx3.1* infected WT grafts (Figure 2C, WT grafts in blue). This data shows that forced expression of *Nkx3.1* is able to reverse the growth advantage of *Pten* null epithelium

***Nkx3.1* blocks *Pten* null prostate cancer initiation and progression**. Histological analysis indicated that WT and *Pten* null epithelium reconstituted prostate grafts recapitulate the histological characterization of the donor epithelium (Figure 3A and 3C), similar to our previous reports (Xin et al., 2003, 2005). No significant difference was observed when comparing GFP- and NKX3.1-virus infected WT epithelial grafts (comparing Figure 3A and 3B). In contrast, tissue recombinants from *Pten* null epithelium resulted in very different phenotypes: grafts from the GFP group demonstrated clear hyperplasia and early mPIN lesions, while grafts from the

Nkx3.1 expression group showed relative preservation of normal prostatic duct structure with protein secretion in the lumen (comparing Figure 3C and 3D). Occasionally, isolated areas were observed that retained a hyperplastic phenotype (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 (Figure 3, circled areas in H and L). These results suggest that forced *Nkx3.1* re-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 have shown 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* knock-out mice (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Tanaka et al., 2000). We examined whether forced *Nkx3.1* expression could inhibit cell proliferation in *Pten* null epithelium in vivo using our renal capsule reconstitution system. Using an anti-Ki67 antibody, we demonstrated that *Nkx3.1* re-expression significantly decreases the proliferation index of 4W and 6W mutant grafts (comparing Figure 3G and H; quantification shown in Figure 4A; $p \leq 0.05$) consistent with the growth-suppressive function of NKX3.1 in previous studies. Interestingly, *Pten* null epithelium appears to be hyper-sensitive to NKX3.1's suppression effect since at similar protein levels (Figure 2C, insert), NKX3.1 has no significant effect on the WT grafts (Figure 3E and F; Figure 4A).

We then assessed the role of NKX3.1 in controlling apoptosis using TUNEL assay, given the observation that *Pten* null grafts of the NKX3.1 group were consistently smaller than those of the

control GFP group (Figure 2C). A significant increase in TUNEL positive cells was observed in both 4W and 6W *Pten* null grafts transduced with NKX3.1 (Figure 4B, upper left panel; quantification shown in Figure 4B, lower panel, $P \leq 0.05$). When consecutive sections were stained with an anti-NKX3.1 antibody, we found that TUNEL positive cells were consistently associated with areas of strong NKX3.1 expression (Figure 4B, upper right panel). No increase in TUNEL positive cells was observed in 4W or 6W WT grafts (Figure 4B, lower panel). Taken together, our data demonstrate that the effects of NKX3.1 on suppressing cell proliferation and promoting cell death depend upon PTEN status: when PTEN function is disrupted, restoration of NKX3.1 expression attenuates both the proliferative and anti-apoptotic properties of *Pten* null epithelium.

NKX3.1 negatively regulates AKT activity in vivo

PTEN is well known for its function as an antagonist of the PI3K/AKT pathway (Dahia, 2000; Sellers and Sawyers, 2002; Stiles et al., 2004). We have shown that restoration of NKX3.1 expression in *Pten* null prostatic epithelial cells leads to near complete reversal of the phenotype associated with PTEN loss. The next step was to investigate possible changes in PTEN controlled signaling pathways in the presence and absence of NKX3.1. Using PTEN and P-AKT double immunofluorescent staining, we examined AKT status on consecutive sections of Figure 3. No difference in either PTEN or P-AKT levels can be detected in the WT grafts with or without *Nkx3.1* expression (Figure 3I and J). Robust P-AKT staining can be detected in mock infected *Pten* null grafts (Figure 3K). In contrast, forced *Nkx3.1* expression, at a level comparable to that of WT prostate gland (comparing Figure 3F and H, and Figure 2C), leads to

dramatic down-regulation of P-AKT levels (Figure 3L). In 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). Therefore, reintroducing NKX3.1 reverts the phenotype associated with *Pten* deletion in the prostatic epithelium, at least in part, by down regulating AKT phosphorylation and activation.

NKX3.1 regulates androgen receptor (AR) expression and modulates AKT activation through an AR-dependent mechanism.

To develop a system more amenable to *in vitro* biochemical analysis, we generated several *Pten* null mouse prostate epithelial cell lines from the prostate cancer model described above. One such line, PTEN-CaP-2, is characterized by positive AR expression and undetectable PTEN and NKX3.1 protein expressions (Jiao et al., manuscript in preparation). We first examined the effect of NKX3.1 on AKT activation in PTEN-CaP-2. *Nkx3.1* overexpression suppressed AKT phosphorylation by 2-fold (Figure 5A, lower panel). Interestingly, *Nkx3.1* overexpression also inhibited AR expression at both the mRNA (Figure 5A, upper panel) and protein levels (Figure 5A, lower panel). To determine if NKX3.1 negatively regulates AR in vivo, we examined AR protein levels in the prostate glands of *Nkx3.1* knock-out mice (Kim et al., 2002b). As shown in Figure 5B, AR levels are increased by more than 2-fold in *Nkx3.1* null prostate, suggesting that NKX3.1 serves as an important negative regulator of AR expression in vivo. Since overexpression of AR leads to PIN lesion in transgenic animals (Stanbrough et al., 2001), increased AR levels observed in this study may, therefore, contribute to PIN lesion development in *Nkx3.1* knock-out mice (Abdulkadir et al., 2002; Kim et al., 2002a) and prostate cancer

development in *Pten* null model (Wang et al., 2003).

It is intriguing that NKX3.1 negatively regulates AR expression, given previous studies that AR can modulate AKT activation via a PI3K-dependent mechanism (Baron et al., 2004; Sun et al., 2003). To further investigate whether AR serves as a mediator for NKX3.1 regulated AKT activity, we tested the effect of NKX3.1 on AKT activity in 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 co-transfection of AR, we did not detect any significant difference (Figure 5C, first two lanes in the upper panel). However, reintroducing AR significantly increased P-AKT level in PC3 cells (Figure 5C, comparing 1st and 3rd lanes in the upper panel), and this effect was diminished by co-transfection of the *Nkx3.1* expressing vector (Figure 5C, comparing the 3rd and 4th lanes). Consistent with previous publication (Baron et al., 2004; Sun et al., 2003), these results suggest that NKX3.1 regulates AKT activity via an AR-dependent mechanism. Previous studies have shown that AR modulation of AKT phosphorylation occurs via interaction between AR and the p85α subunit of PI3-kinase (Baron et al., 2004; Sun et al., 2003). We performed reciprocal co-immunoprecipitation experiments and showed that *Nkx3.1* re-expression decreases the amount of AR able to interact with p85α (Figure 5E, lower panels). Taken together, our data suggest that NKX3.1 modulates AR activity at the transcriptional level and controls AKT phosphorylation via an AR/PI3K-dependent mechanism.

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

We showed previously that *Pten* deletion leads to impaired p53 expression and transcription

activity via both MDM2-AKT-dependent and –independent mechanisms (Freeman et al., 2003). Having observed that NKX3.1 re-expression leads to reduced levels of P-AKT in *Pten* null grafts, we tested whether NKX3.1 also alters p53 levels. Western blot analysis demonstrated that *Nkx3.1* re-expression significantly increased p53 protein levels in *Pten* null grafts in vivo (Figure 6A) as well as in murine and human *Pten* null prostate cancer cells lines in vitro (Supplement Figure 2). Additionally, p53 protein level is moderately but significantly decreased in *Nkx3.1* knock-out mice, as compared to their WT littermates (Figure 6B).

p53 is a short-lived protein whose activity is tightly controlled at low levels in normal cells (Levine, 1997). MDM2 controls the level of p53 by acting as an E3 ubiquitin ligase, initiating p53 proteasomal degradation (Corcoran et al., 2004). To test whether NKX3.1 affects p53 protein stability, we expressed *Nkx3.1* in either LnCaP (Figure 6C) or PTEN-CaP-2 cells (Supplement Figure 3). *Nkx3.1* expression leads to an increase in p53 half-life from 21 min to 30 min (Figure 6C), while does not affecting p53 transcriptional (Supplement Figure 4). 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). Expression of NKX3.1 was able to partially reverse the increased p53 degradation brought about by MDM2 over expression but not in the absence of MDM2 (Figure 6D), suggesting that NKX3.1 controls p53 half-life via a MDM2-dependent mechanisms.

AKT phosphorylates MDM2 and controls its nuclear translocation and stability, key steps in p53 regulation (Mayo and Donner, 2001; Zhou et al., 2001). To determine whether NKX3.1 stabilizes p53 via an AKT-dependent mechanism, we treated cells with or without LY294002, a specific inhibitor for PI3K. As shown in Figure 6E, LY294002 treatment had no significant affect

on either p53 (comparing lanes 4 and 5) or NKX3.1 regulated p53 level (comparing lanes 5 and 6), although P-AKT levels are significantly diminished. Therefore, NKX3.1 appears to regulate p53 protein level through MDM2-dependent but AKT-independent mechanisms. NKX3.1-mediated p53 upregulation may lead to decreased cell proliferation, increased cell death and the reversal of phenotype seen upon restoration of NKX3.1 expression in *Pten* null prostatic epithelium. Conversely, NKX3.1 loss after *Pten* deletion may further impair p53 function, promoting tumor progression.

Discussion

In this study, we demonstrate that dysregulated *Nkx3.1* expression and function play important roles in prostate cancer initiation caused by PTEN loss. Re-introducing *Nkx3.1* into *Pten* null prostate epithelium, driven by a constitutive promoter, can efficiently increase cell death, decrease cell proliferation, and block prostate cancer initiation. Mechanistically, NKX3.1 stabilizes p53 in a MDM2-dependent but AKT-independent manner and represses AKT activation through an AR/PI3K-dependent pathway (Fig 7).

The growth-suppressive activities of NKX3.1 have been demonstrated *in vitro* in cell culture system and *in vivo* in either transgenic and knockout mice (Bhatia-Gaur et al., 1999; Kim et al., 2002a). In the Myc-induced prostate cancer mouse model, *Nkx3.1* expression is decreased along tumor progression (Ellwood-Yen et al., 2003). *Nkx3.1* null mice display abnormal prostatic growth, differentiation, as well as epithelial hyperplasia and dysplasia in aged animals (Abdulkadir et al., 2002; Bhatia-Gaur et al., 1999; Tanaka et al., 2000). Deletion of one allele

of *Pten* in *Nkx3.1* null mice leads to accelerated tumor initiation, increased incidence of high-grade PIN, and localized AKT hyperphosphorylation. However similar to *Nkx3.1* null mice, there is not progression to metastatic prostate cancer (Kim et al., 2002b). In our model, *Nkx3.1* expression is significantly down-regulated to near undetectable levels immediately after *Pten* deletion, and prostate cancer develops from PIN to invasive carcinoma followed by metastasis. These results suggest that concurrent PTEN and NKX3.1 down regulation act synergistically to promote prostate cancer initiation, but that the dosage of PTEN plays a critical role in this cooperative effect.

Several mechanisms have been proposed for NKX3.1 downregulation in human prostate cancers, including both posttranscriptional modification, such as protein degradation, as well as transcriptional and epigenetic regulation (Bowen et al., 2000; He et al., 1997; Ornstein et al., 2001; Voeller et al., 1997). The fact that we can successfully reintroduce *Nkx3.1* expression using an exogenous promoter, and maintain near WT levels of NKX3.1 in different cell lines and in the renal capsule grafts, suggest that PTEN may modulate NKX3.1 function largely through regulation of its transcription. 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 is regulated by PTEN in our mouse model and merit further study.

We showed that *Nkx3.1* re-expression significantly induces cell apoptosis in the mutant grafts. This novel 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: although we observed strikingly increased cell death and decreased cell proliferation with *Nkx3.1*

re-expression in the *Pten* null grafts, we failed to detect any significant alterations in WT grafts. One possible explanation is that *Pten* null cells have became "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 anti-apoptotic effects brought on by PTEN loss. This novel mechanism provides a new pathway which can potentially be targeted with specific therapies for human prostate cancer, given that 30% of primary prostate cancers display LOH at the PTEN locus and as many as 60% of metastatic cases exhibit LOH (Sansal and Sellers 2004).

Nkx3.1 is an androgen-regulated homeobox gene. Androgens can induce *NKX3.1* expression at a transcriptional level (He et al., 1997). In the present study we demonstrate that overexpression of *Nkx3.1* decreases AR mRNA expression, suggesting that AR and NKX3.1 form an important self-feedback loop. With *Nkx3.1* at steady-state endogenous levels, androgen binding to the AR, induces NKX3.1 expression, in turn inhibiting AR expression. Loss of NKX3.1 in the prostatic epithelium likely impairs this feedback system, which may contribute to AR overexpression and, in combination with impaired p53 stability, result in tumor initiation. The natural "set point" of this feedback loop and its range of action are currently unknown and require further investigation.

Dysregulation of AKT activation plays a critical role in prostate cancer initiation and progression (Majumder et al., 2003; Paweletz et al., 2001). We observed dramatic inhibition of

AKT activation with re-expression of *Nkx3.1* in *Pten* null prostate epithelium. Notably, we also found that NKX3.1 inhibits AKT activation only in the presence of AR, which is consistent with previous reports (Baron et al., 2004). Our results suggest AR functions as a mediator in NKX3.1 regulated AKT activity.

Restoration of *Nkx3.1* expression to WT level in *Pten* null prostatic epithelium leads to increased p53 protein levels in vivo and in vitro. Control of p53 function is mediated via several mechanisms, including the regulation of p53 protein stability (Brooks and Gu, 2003). Indeed, the half-life of p53 was increased in *Nkx3.1* transfected LnCaP and PTEN-CaP-2 cells. Interestingly, NKX3.1 stabilizes p53 through a MDM2-dependent but AKT-independent mechanism. How NKX3.1 stabilizes p53 is currently unknown. One possibility might be through modulation of MDM2 activity in the nucleus, independent of AKT-mediated MDM2 phosphorylation and nuclear translocation. Alternatively, NKX3.1 may affect p53 stability through its interaction with co-repressors such as HIPK2 (homeodomain-interacting protein kinase 2). HIPK2 is a member of a family of co-repressors that interact with homeo-domain containing transcription factors, such as *Drosophila* NK-1 and NK-3, and mouse Nkx-1.2 and Nkx-2.5 (Kim et al., 1998). Another study has since shown that HIPK2 co-localizes and interacts with p53 in PML (promyelocytic leukemia) nuclear bodies (Hofmann et al., 2000).

In conclusion, forced *Nkx3.1* expression can reverse the pathological phenotype associated with PTEN loss in the prostatic epithelium. Restoration of NKX3.1 to WT level results in decreased cell proliferation and increased cell apoptosis, which can be correlated to the enhancement of p53 expression and reduction of AKT activation. NKX3.1 stabilizes p53 in a MDM2-dependent but AKT-independent manner and inhibits AKT activation through an

AR-dependent mechanism. Our findings further emphasize the significance of cooperative effects between various tumor suppressor genes and oncogenes in prostate cancer development and progression.

Experimental Procedures

Preparation of prostate epithelial cells from *Pten* mice.

Four week and 6W old *Pten* WT 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 100μm nylon mesh (Becton Dickinson), washed twice with 10 ml of DMEM 10% FBS, resuspended in 1 ml of DMEM 10%FBS, and counted.

Preparation of Lentivirus and infection of prostate cells.

The lentivirus transfer vector FUGW expressing enhanced GFP and packaging vectors VSVG and Δ8.9 were kindly provided by Drs. David Baltimore (California Institute of Technology, Pasadena) and Inder Verma (Salk Institute for Biological Sciences, La Jolla, CA). The lentivirus was prepared as described (Lois et al., 2002; Pfeifer et al., 2002). Prostate cells were infected as described (Xin et al., 2003). Briefly, 1 $x10^5$ prostate cells made as above were mixed with GFP or NKX3.1 Lent virus stock (titer $2x10^7$); 0.8 mg/ml polybrene (Sigma) was added to the mixture to a final concentration of 8 μg/ml. Cells were infected by centrifuging at 1,500 rpm with a Beckman GS-6R centrifuge (Beckman Coulter) for 2 h 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.

Prostate regeneration.

Mouse prostate regeneration followed the protocol from previous reports (Cunha and Donjacour, 1987; Thompson et al., 1989; Xin et al., 2003). Lentivirus infected Cells ($1x10^5$) from *Pten* mice prostatic tissue were combined with UGSM cells ($1x\ 10^5$) and resuspended in 25 µl of type I collagen (Roche). The collagen grafts were grafted under the renal capsule (Cunha and Donjacour, 1987). 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. UGSM was isolated from the urogenital sinus of embryos 16 days old from C57/BL mice (Cunha and Donjacour, 1987; Xin et al., 2003). All surgical procedures were performed under Division of Laboratory Animal Medicine regulations of the University of California, Los Angeles.

Immunohistochemical analysis.

Tissues were fixed in 10% buffered Formalin for 6 hr, followed by transfer to 70% alcohol. These paraffin-embedded tissues were sectioned (4µm) and stained with hematoxylin & eosin. Antigen retrieval was performed by incubating the slides in 0.01 M citric acid buffer (pH 6.0) at 95^0C for 30 min. The endogenous peroxidase activity was inactivated in a solution containing 3% hydrogen peroxide (H_2O_2) in methanol. The following detection and visualization procedures were performed according to 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 Ig blocking reagent in the VECTOR M.O.M. Immunodetection Kit (Vector Laboratories) and then incubated with mouse antibody PTEN (26H9, Cell Signaling Technology) or Ki67

(NCL-Ki67-MM1) 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 NKX3.1 (a kind gift from Dr. Aben-Shen), and signal was amplified with TSA system with different fluorescence.

Cell proliferation and apoptosis index.

Cell proliferation index was examined by Ki67 staining and four or five different fields were chosen, then 200 cells were counted each field. Ki67 positive cells were presented as numbers per 100 nucleated cells. Cell apoptosis were determined by TUNEL assay using the In Situ Cell Death Detection Kit from Roche according to manufacturer's instruction. Sections were de-waxed 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 as above.

Cell culture and transfection.

Mouse *Pten*$^{-/-}$ prostate epithelial PTEN-CaP-2 cells, mouse *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEF cells and human prostate cancer LnCaP and PC3 cells were cultured in ISOVE medium, DMEM medium and RPMI 1640 medium supplemented with 10% fetal calf serum (FCS Hyclone), 100U/ml penicillin and streptomycin (Gibco), respectively. Cell transfection was performed using lipofectAmine (Invitrogen). Cells were harvested 36 or 48hr post transfection for RNA and protein analysis. For checking *NKX3.1* overexpression's effect on P-AKT level in PC3 cells, 30 hr post transfection, starved overnight (18hr), transferred to full medium for 30 min and

lysated. For AR promoter luciferase activity assay, 24 hr post transfection, the cells were lysated and luciferase activity was measured by luminometer TD-20/20. For comparison, luciferase activity from each experiment was normalized to the mock transfection and presented as fold changes. Transfection efficiency was normalized to thymidine kinase-driven Renilla luciferase activity.

Western blot analysis.

Protein lysate was prepared by sonicating graft tissues and prostate tissue from *Nkx3.1* mice or transfected LnCaP and clone2 cells in buffer containing 50mM Tris-HCl (pH 8.0), 150mM NaCl, 0.1% SDS, 0.5%SD, 1% NP-40, 1mM EDTA, 1mM PMSF, 25mM NaF and cocktail protease inhibitors (Roche). 40μg tissue lysate were 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, GFP (#A-11122, Molecular probe), respectively.

For immunoprecipitation experiments, 500μg of cell lysate were incubated 12 hr at 4^0C with 2ug AR antibody (AR, PG-21, Upstate) or P85α antibody (#06-496, Upstate) plus 50μl Protein A agarose beads(#16-125, Upstate). Beads were washed three times with lysis buffer and centrifuged for 5 min at 5000g between each wash. Protein was eluted from beads with 50μl laemmli sample buffer (Bio-Rad). Lysates were resolved on a 10% SDS-PAGE gel and transferred onto nitrocellulose (Bio-Rad).

For half-life experiments, *NKX3.1* was transfected into LNCaP cells 36 hr prior to the

addition of 50μg/ml cyclohexamide (Cyclohexamide, Calbiochem) in serum free medium. Cells were then lysated at indicated time points and further analyzed by Western blot.

Tissue array analysis

PTEN and NKX3.1 intensity was analysis by Image Plus software and only cores with epithelial structure were chosen for intensity analysis. Briefly, we chose 4 views for each core, measured the signal intensity using Image Plus software, and signed the signal intensity as 0, 1 or 2, in which 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. Liner regression analysis was performed for the correlation between PTEN and NKX3.1 expression by SPSS software.

ACKNOWLEDGEMENT

We thank Drs. Sawyers, Mulholland, Lasky, Thomas and colleagues in Wu and Witte laboratories for helpful comments and suggestion; Dr. Mary Perry for Mdm2 plasmid; Dr. Edward P. Gelmann for human NKX3.1 plasmid; and Dr. Cory Abete-Shen for NKX3.1 antibody and depositing NKX3.1 knockout mice to MMHCC/NCI mouse cancer model repository and John Wongvipat from Dr. Charles Sawyers' laboratory for providing *Nkx3.1* knock-out mice. QL is supported by the seed grant from the UCLA'S Jonsson Cancer Center Foundation. JJ is supported by the DOD postdoctoral fellowship. This work is partly supported by funds from DOD PC031130 and NCI UO1 CA84128-06 and RO1 CA107166 (for HW) and SPORE Mouse models (OW HHMI investigator).

References

Abdulkadir, S. A., Magee, J. A., Peters, T. J., Kaleem, Z., Naughton, C. K., Humphrey, P. A., and Milbrandt, J. (2002). Conditional loss of Nkx3.1 in adult mice induces prostatic intraepithelial neoplasia. Mol. Cell Biol. *22*, 1495-1503.

Asatiani, E., and Gelmann, E. P. (2005). Targeted therapies for prostate cancer. Expert. Opin. Ther. Targets *9*, 283-298.

Baron, S., Manin, M., Beaudoin, C., Leotoing, L., Communal, Y., Veyssiere, G., and Morel, L. (2004). Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells. J. Biol. chem. *279*, 14579-14586.

Bhatia-Gaur, R., Donjacour, A. A., Sciavolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., *et al.* (1999). Roles for Nkx3.1 in prostate development and cancer. Genes Dev. *13*, 966-977.

Bowen, C., Bubendorf, L., Voeller, H. J., Slack, R., Willi, N., Sauter, G., Gasser, T. C., Koivisto, P., Lack, E. E., Kononen, J., *et al.* (2000). Loss of NKX3.1 expression in human prostate cancers correlates with tumor progression. Cancer Res. *60*, 6111-6115.

Brooks, C. L., and Gu, W. (2003). Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. Curr. Opin. Cell. Biol. *15*, 164-171.

Corcoran, C. A., Huang, Y., and Sheikh, M. S. (2004). The p53 paddy wagon: COP1, Pirh2 and MDM2 are found resisting apoptosis and growth arrest. Cancer.Biol.Ther. *3*, 721-725.

Cunha, G. R., and Donjacour, A. (1987). Mesenchymal-epithelial interactions: technical considerations. Prog. Clin. Biol. Res. *239*, 273-282.

Dahia, P. L. (2000). PTEN, a unique tumor suppressor gene. Endocr. Relat. Cancer *7*, 115-129.

DeMarzo, A. M., Nelson, W. G., Isaacs, W. B., and Epstein, J. I. (2003). Pathological and molecular aspects of prostate cancer. Lancet *361*, 955-964.

Di Cristofano, A., De Acetis, M., Koff, A., Cordon-Cardo, C., and Pandolfi, P. P. (2001). Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse. Nat. Genet. *27*, 222-224.

Ellwood-Yen, K., Graeber, T. G., Wongvipat, J., Iruela-Arispe, M. L., Zhang, J., Matusik, R., Thomas, G. V., and Sawyers, C. L. (2003). Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer cell *4*, 223-238.

Freeman, D. J., Li, A. G., Wei, G., Li, H. H., Kertesz, N., Lesche, R., Whale, A. D., Martinez-Diaz, H., Rozengurt, N., Cardiff, R. D., *et al.* (2003). PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. Cancer cell *3*, 117-130.

Gregorakis, A. K., Holmes, E. H., and Murphy, G. P. (1998). Prostate-specific membrane antigen: current and future utility. Semin Urol Oncol *16*, 2-12.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

He, W. W., Sciavolino, P. J., Wing, J., Augustus, M., Hudson, P., Meissner, P. S., Curtis, R. T., Shell, B. K., Bostwick, D. G., Tindall, D. J., *et al.* (1997). A novel human prostate-specific, androgen-regulated homeobox gene (NKX3.1) that maps to 8p21, a region frequently deleted in prostate cancer. Genomics *43*, 69-77.

Hofmann, T. G., Mincheva, A., Lichter, P., Droge, W., and Schmitz, M. L. (2000). Human homeodomain-interacting protein kinase-2 (HIPK2) is a member of the DYRK family of protein kinases and maps to chromosome 7q32-q34. Biochimie. *82*, 1123-1127.

Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Kim, M. J., Bhatia-Gaur, R., Banach-Petrosky, W. A., Desai, N., Wang, Y., Hayward, S. W., Cunha, G. R., Cardiff, R. D., Shen, M. M., and Abate-Shen, C. (2002a). Nkx3.1 mutant mice recapitulate early stages of prostate carcinogenesis. Cancer Res. *62*, 2999-3004.

Kim, M. J., Cardiff, R. D., Desai, N., Banach-Petrosky, W. A., Parsons, R., Shen, M. M., and Abate-Shen, C. (2002b). Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis. Proc. Natl. Acad. Sci. USA *99*, 2884-2889.

Kim, Y. H., Choi, C. Y., Lee, S. J., Conti, M. A., and Kim, Y. (1998). Homeodomain-interacting protein kinases, a novel family of co-repressors for homeodomain transcription factors. J. Biol. chem. *273*, 25875-25879.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Maehama, T., Taylor, G. S., and Dixon, J. E. (2001). PTEN and myotubularin: novel phosphoinositide phosphatases. Annu. Rev. Biochem. *70*, 247-279.

Majumder, P. K., Yeh, J. J., George, D. J., Febbo, P. G., Kum, J., Xue, Q., Bikoff, R., Ma, H., Kantoff, P. W., Golub, T. R., *et al.* (2003). Prostate intraepithelial neoplasia induced by prostate restricted Akt activation: the MPAKT model. Proc. Natl. Acad. Sci. USA *100*, 7841-7846.

Mayo, L. D., and Donner, D. B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A *98*, 11598-11603.

Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H., and Sawyers, C. L. (2001). Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc. Natl. Acad. Sci. USA *98*, 10314-10319.

Ornstein, D. K., Cinquanta, M., Weiler, S., Duray, P. H., Emmert-Buck, M. R., Vocke, C. D., Linehan, W. M., and Ferretti, J. A. (2001). Expression studies and mutational analysis of the androgen regulated homeobox gene NKX3.1 in benign and malignant prostate epithelium. J Urol *165*, 1329-1334.

Parson, W., Parsons, T. J., Scheithauer, R., and Holland, M. M. (1998). Population data for 101 Austrian Caucasian mitochondrial DNA d-loop sequences: application of mtDNA sequence analysis to a forensic case. Int. J. Legal. Med. *111*, 124-132.

Paweletz, C. P., Charboneau, L., Bichsel, V. E., Simone, N. L., Chen, T., Gillespie, J. W., Emmert-Buck, M. R., Roth, M. J., Petricoin, I. E., and Liotta, L. A. (2001). Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene *20*, 1981-1989.

Pfeifer, A., Ikawa, M., Dayn, Y., and Verma, I. M. (2002). Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. Proc. Natl. Acad. Sci. USA *99*, 2140-2145.

Plass, C. (2002). Cancer epigenomics. Hum. Mol. Genet. *11*, 2479-2488.

Rocchi, P., So, A., Kojima, S., Signaevsky, M., Beraldi, E., Fazli, L., Hurtado-Coll, A., Yamanaka, K., and Gleave, M. (2004). Heat shock protein 27 increases after androgen ablation and plays a cytoprotective role in hormone-refractory prostate cancer. Cancer Res. *64*, 6595-6602.

Sellers, W., and Sawyers, C. (2002). Somatic Genetics of Prostate cnacer: Oncogene and Tumor Suppressors. (Philadelphia, Lippincott Williams & Wilkins).

Stiles, B., Groszer, M., Wang, S., Jiao, J., and Wu, H. (2004). PTENless means more. Dev. Biol. *273*, 175-184.

Sun, M., Yang, L., Feldman, R. I., Sun, X. M., Bhalla, K. N., Jove, R., Nicosia, S. V., and Cheng, J. Q. (2003). Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src. J. Biol. chem. *278*, 42992-43000.

Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W. B., and Bova, G. S. (1998). Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. Cancer Res. *58*, 204-209.

Tanaka, M., Komuro, I., Inagaki, H., Jenkins, N. A., Copeland, N. G., and Izumo, S. (2000). Nkx3.1, a murine homolog of Ddrosophila bagpipe, regulates epithelial ductal branching and proliferation of the prostate and palatine glands. Dev. Dyn. *219*, 248-260.

Thompson, T. C., Southgate, J., Kitchener, G., and Land, H. (1989). Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. Cell *56*, 917-930.

Vivanco, I., and Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat. Rev. Cancer *2*, 489-501.

Voeller, H. J., Augustus, M., Madike, V., Bova, G. S., Carter, K. C., and Gelmann, E. P. (1997). Coding region of NKX3.1, a prostate-specific homeobox gene on 8p21, is not mutated in human prostate cancers. Cancer Res. *57*, 4455-4459.

Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G. V., Li, G., Roy-Burman, P., Nelson, P. S., *et al.* (2003). Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. Cancer cell *4*, 209-221.

Xin, L., Ide, H., Kim, Y., Dubey, P., and Witte, O. N. (2003). In vivo regeneration of murine prostate from dissociated cell populations of postnatal epithelia and urogenital sinus mesenchyme. Proc. Natl. Acad. Sci. USA *100 Suppl 1*, 11896-11903.

Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. (2001). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol *3*, 973-982.

Figure legends

Figure 1. *Pten* deletion leads to reduced NKX3.1 protein levels in prostate epithelium.

A. Consecutive sections of 4 weeks old *Pten* mutant prostate were probed for NKX3.1 (left) and phospho-AKT expression (P-AKT, right). Arrows and arrowheads point to the same duct. Note that NKX3.1 expression reversely correlates with P-AKT staining.

B. Decreased PTEN expression in human prostate cancer correlates with reduced NKX3.1 protein levels. High power views of two representative samples from human prostate cancer tissue microarray are shown here. Upper panels: H&E staining; middle and lower panels: double immunofluorescent staining using anti-PTEN (middle) and anti–NKX3.1 antibodies.

C. Correlation of PTEN and NKX3.1 protein levels in 153 human prostate cancer samples. Relative intensity grades of PTEN and NKX3.1 from each sample are illustrated. SPSS liner regression was used to analyze data and the standardized coefficients value was 0.52 ($p<0.01$ level; n=153).

Figure 2. Overexpression of Nkx3.1 reduces the growth of reconstituted *Pten* null epithelium graft.

A. The lentivirus FUGW-IRES-eGFP vector used for expressing Nkx3.1.

B. Flag-tagged NKX3.1 protein expression in 293T cells. NKX3.1 expression can be indirectly detected by the expression of GFP from the same vector (left panels). Right panels show the expression of Flag-tagged NKX3.1 protein in 293T cells (upper row) and infected, eGFP-sorted cells (middle row) by Western blot analysis.

C. A comparison of the wet weight of regenerated tissue without or with forced Nkx3.1

expression. Single cell suspension was prepared from the prostate epithelium of 4W or 6W-old *Pten* WT (in blue) and Mut mice (in red), infected with either lenti-eGFP or lenti-eGFP-NKX3.1 virus, mixed with UGSM and grafted under the renal capsule. Quantization of the wet weight is shown as mean±SD. Similar results were obtained in three independent experiments ($P \leq 0.05$). Insert, Western blot shows the levels of PTEN and NKX3.1 in mock infected (GFP group) and NKX3.1 viral infected (Nkx3.1 group) grafts.

Figure 3. *Nkx3.1* re-expression in *Pten* null epithelium leads to decreased cell proliferation and P-AKT level, and reverse of hyperplasia phenotype.

Upper panel **A-D**: histopathology of regenerated grafts. H&E stained paraffin sections of grafts generated from 4-week-old WT (A and B) and *Pten* null epithelium (C and D). Without *Nkx3.1* overexpression (GFP group), the regenerated tissues recapitulate the phenotypes of the donor epithelium (A and C). Forced *Nkx3.1* expression in WT epithelium does not lead any significant changes (comparing A and B). Forced *Nkx3.1* expression in *Pten* null epithelium, however, reverses hyperplasia phenotype (comparing C and D). Circled area in (D) remains small hyperplasia lesion in otherwise normal duct.

Middle panel **E-H** and lower panel **I-L**: Consecutive sections from upper panels were double immunofluorescent stained with anti-Ki67 and -NKX3.1 antibodies (middle panels), as well as anti-PTEN and -P-AKT antibodies and then counter stained with DAPI. NKX3.1 re-expression significantly decreases cell proliferation of mutant graft but has no influence of the WT graft (comparing E and F, G and H). Lower panels show NKX3.1 expression significantly reduces P-AKT staining in the *Pten* null graft without influence PTEN protein levels. Circled area shows

negative correlation of P-AKT staining (L) with NKX3.1 expression (H).

Figure 4. *Nkx3.1* re-expression in *Pten* null epithelium leads to quantitatively decreased cell proliferation increased cell death.

A. Quantitation of cell proliferation. Similar results were obtained in three independent experiments ($P \leq 0.05$).

B. Forced *Nkx3.1* expression induces cell death in *Pten* null graft. Consecutive sections show TUNEL positive cells (left) overlap with NKX3.1 expressing cells (right). Lower panel, quantitation of cell apoptosis was represented as mean±SD from three independent experiments ($P \leq 0.05$).

Figure 5. NKX3.1 regulates AKT phosphorylation through an AR-dependent mechanism.

A. NKX3.1 regulated AR at transcriptional level and down regulated AKT phosphorylation in vitro. Total RNA and protein were harvest from PTEN-CaP-2 cells transfected with *Nkx3.1*. RT-PCR (upper panel) and Western blot analysis (lower panel) were performed as indicated.

B. Loss of NKX3.1 leads to AR up regulation in vivo. Western blot analysis of WT and *Nkx3.1* null prostate tissues.

C. NKX3.1 regulated AKT phosphorylation depends on AR expression. PC3 cells were transfected with *Nkx3.1* without (left two lanes) and with (right tow lanes) AR. Cell lysates were immunopreciptated with indicated antibodies and analyzed by western blot.

Figure 6. NKX3.1 regulates p53 expression in a MDM2-dependent but AKT-independent

manner.

A. Re-introducing *Nkx3.1* into *Pten* null epithelium increases p53 protein level in regenerated grafts. Protein lysates from grafts regenerated from 6W WT and *Pten* null epithelium were resolved by SDS/PAGE and blotted with indicated antibodies.

B. NKX3.1 modulates p53 protein levels in vivo. p53 protein levels in *Nkx3.1* WT and null prostate tissues were analyzed using Western blot.

C. NKX3.1 regulated p53 at post-translational level. *Nkx3.1* was transfected into LnCaP as indicated. Thirty-six hours after transfection, cells were incubated with cycloheximide for the indicated time and total proteins were analyzed by Western blot. P53 half-life was calculated using Quantity One (Bio-Rad).

D. NKX3.1 stabilizes p53 via Mdm2 dependent mechanism. Western blot analysis of cell lysates from p53-/-Mdm2-/- MEFs after transfection of indicated plasmids.

E. NKX3.1 stabilizes p53 in an AKT-independent manner. PC3 cells were transfected with indicated plasmids with or without LY treatment before harvesting. Cell lysates were analyzed by Western blot.

Figure 7. Signaling pathways involved in NKX3.1 modulated p53 protein level and AKT activity. NKX3.1 suppresses AR transcription, leading to down regulated AR/PI3K(p85α) association, down regulating AKT activation. NKX3.1 also stabilizes p53 through a MDM2–dependent but AKT –independent manner.

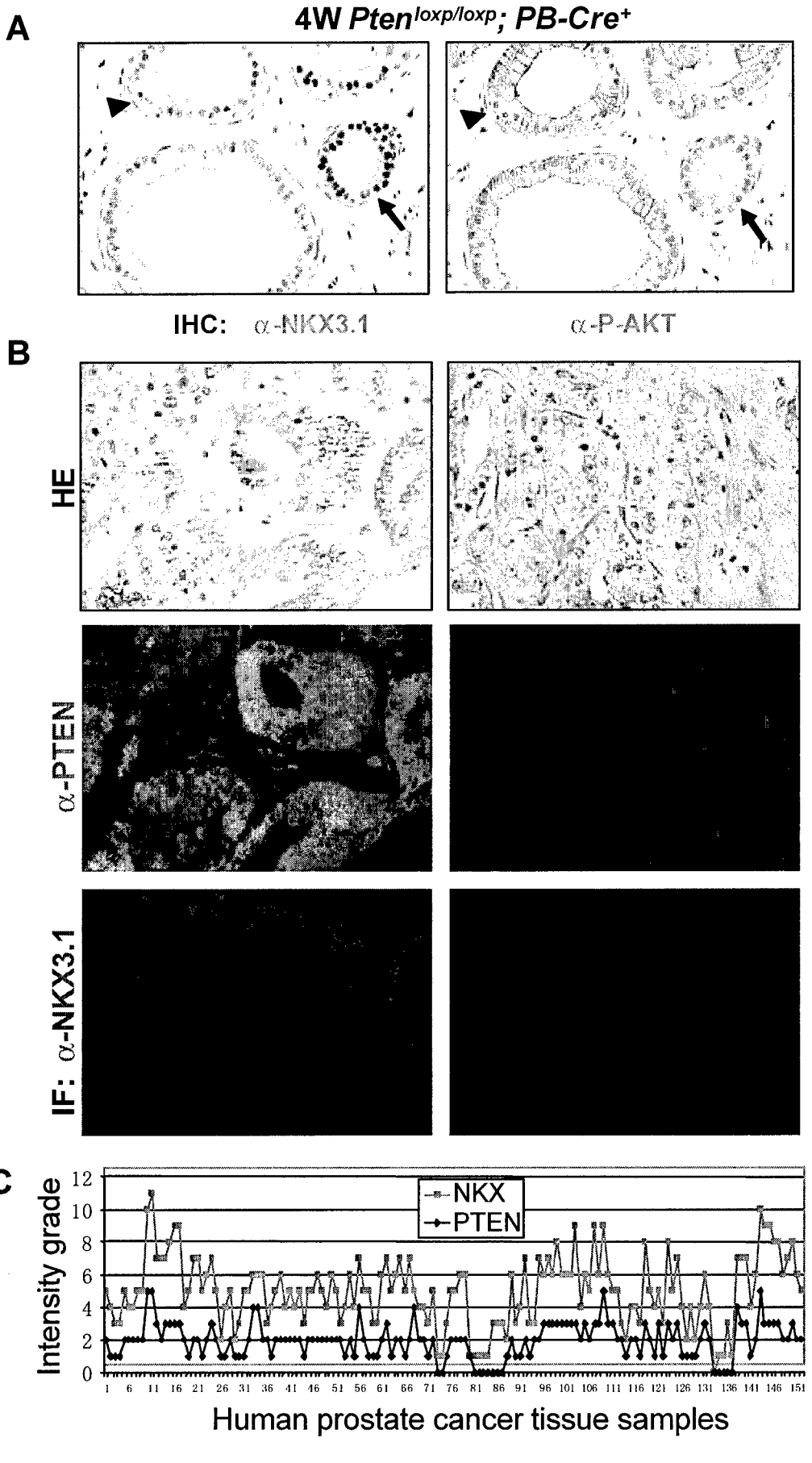

A
4W Pten loxp/loxp; PB-Cre+
IHC: α-NKX3.1
α-P-AKT
B
HE
α-PTEN
IF: α-NKX3.1
C
Intensity grade
NKX
PTEN
Human prostate cancer tissue samples

Figure 1

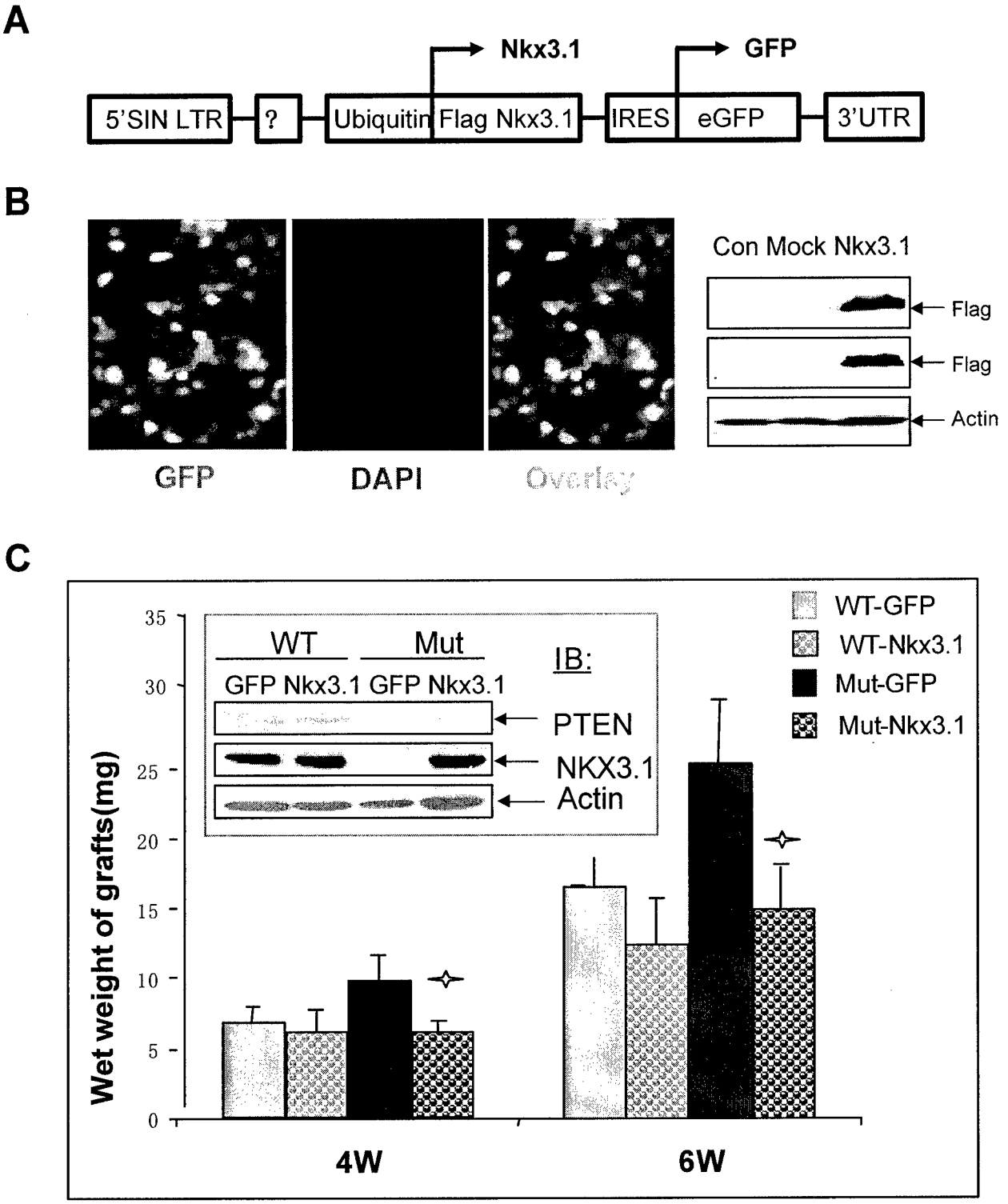

A
Nkx3.1
GFP
5'SIN LTR
?
Ubiquitin
Flag Nkx3.1
IRES
eGFP
3'UTR
B
Con Mock Nkx3.1
Flag
Flag
Actin
GFP
DAPI
Overlay
C
WT-GFP
WT-Nkx3.1
Mut-GFP
Mut-Nkx3.1
WT
Mut
IB:
GFP Nkx3.1 GFP Nkx3.1
PTEN
NKX3.1
Actin
Wet weight of grafts(mg)
35
30
25
20
15
10
5
0
4W
6W

Figure 2

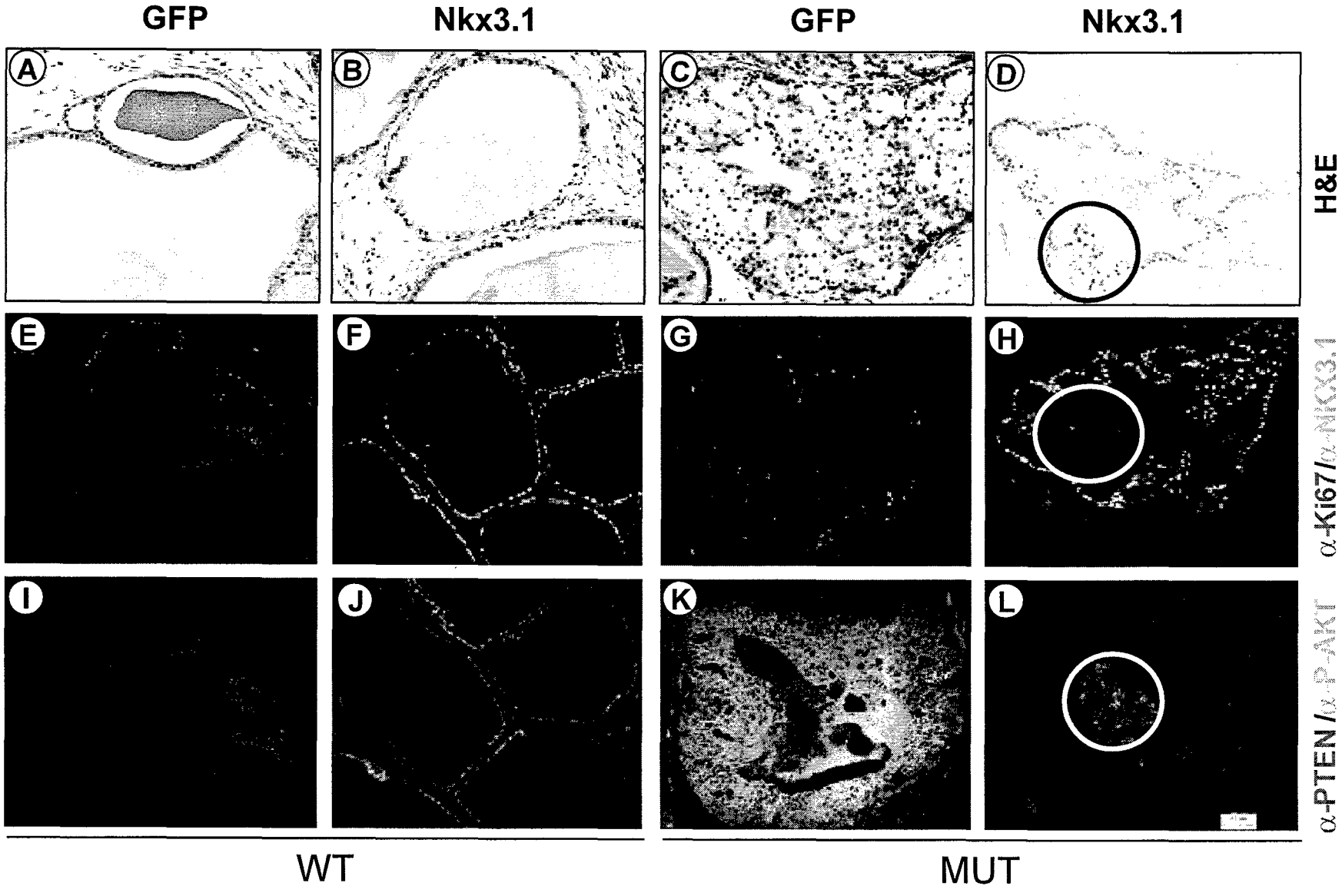
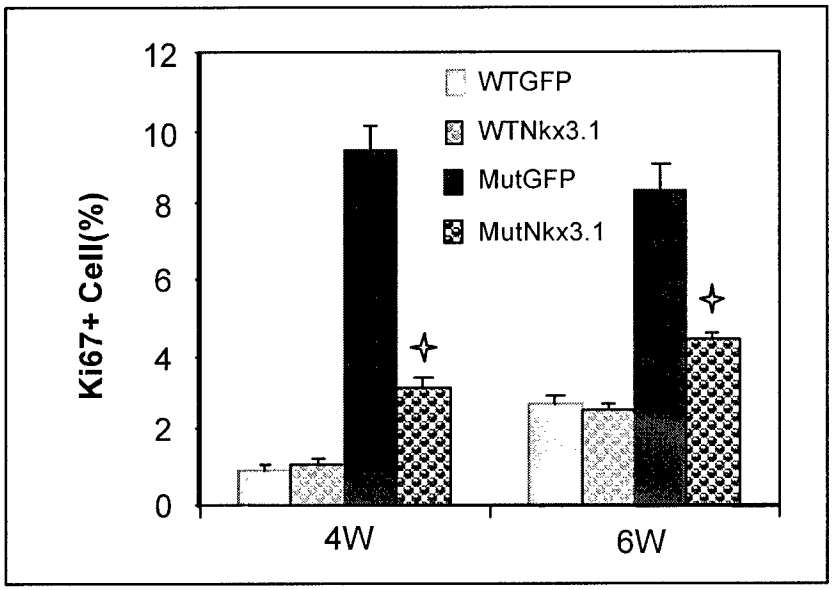
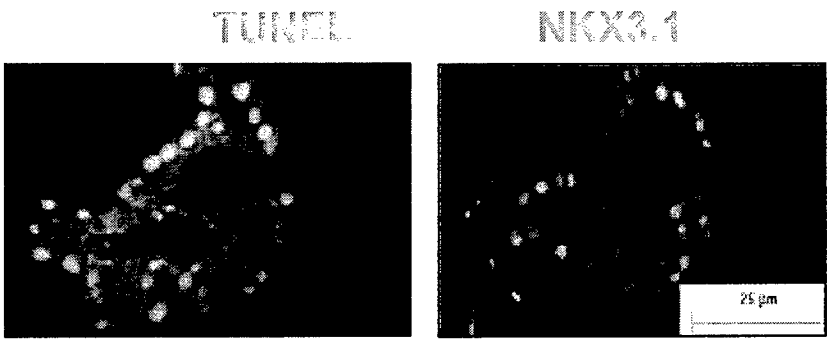

GFP
Nkx3.1
GFP
Nkx3.1
A
B
C
D
H&E
E
F
G
H
α-Ki67/α-NKX3.1
I
J
K
L
α-PTEN/α-P-AKT
WT
MUT

Figure 3

A


12
10
8
6
4
2
0
Ki67+ Cell(%)
WTGFP
WTNkx3.1
MutGFP
MutNkx3.1
4W
6W

B


TUNEL
NKX3.1
25 μm

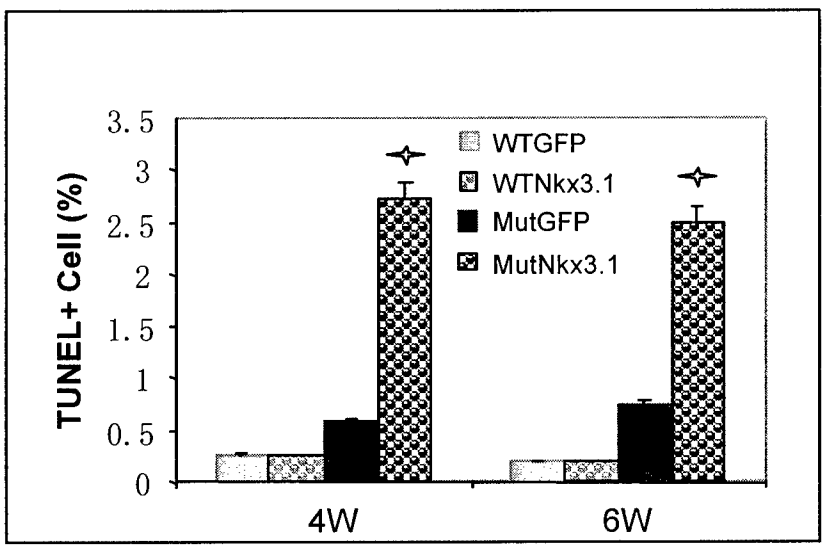

3.5
3
2.5
2
1.5
1
0.5
0
TUNEL+ Cell (%)
WTGFP
WTNkx3.1
MutGFP
MutNkx3.1
4W
6W

Figure 4

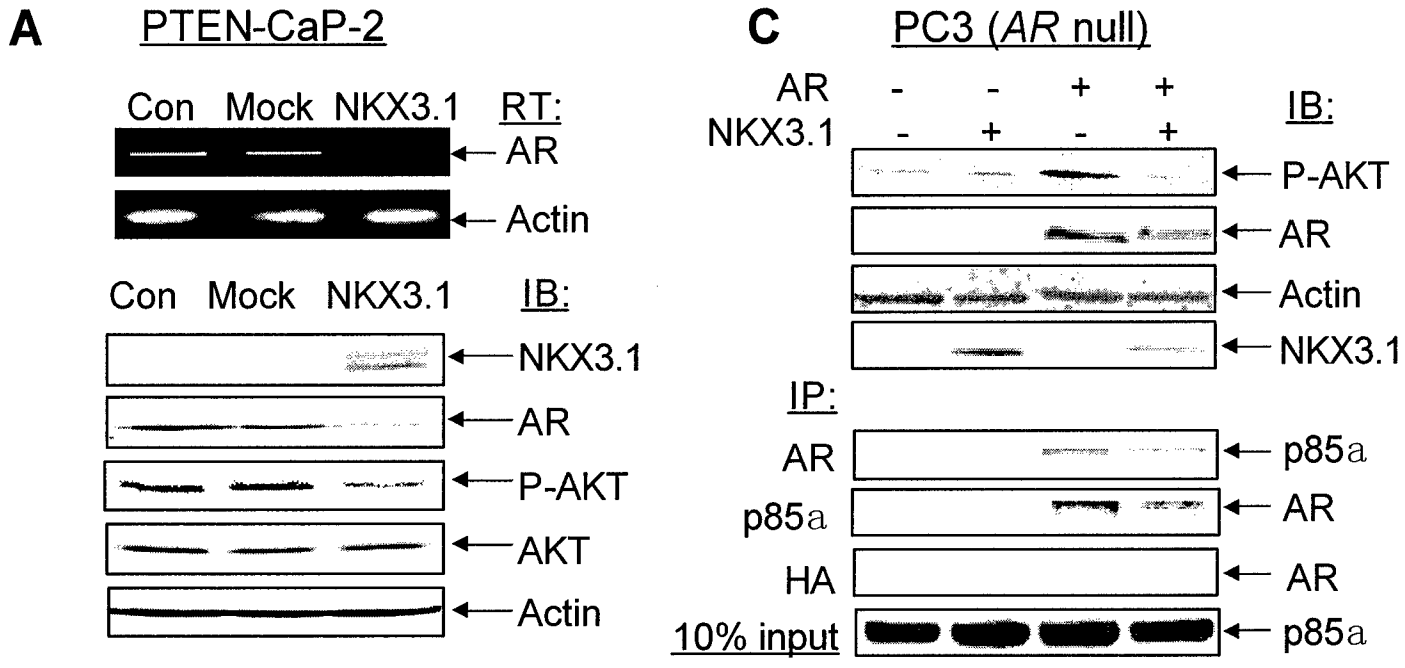

A
PTEN-CaP-2
Con Mock NKX3.1 RT:
AR
Actin
Con Mock NKX3.1 IB:
NKX3.1
AR
P-AKT
AKT
Actin
B
Nkx3.1 null prostate tissue
WT Mut IB:
NKX3.1
AR
Actin
C
PC3 (AR null)
AR - - + +
NKX3.1 - + - +
IB:
P-AKT
AR
Actin
NKX3.1
IP:
AR
p85a
p85a
AR
HA
AR
10% input
p85a

Figure 5

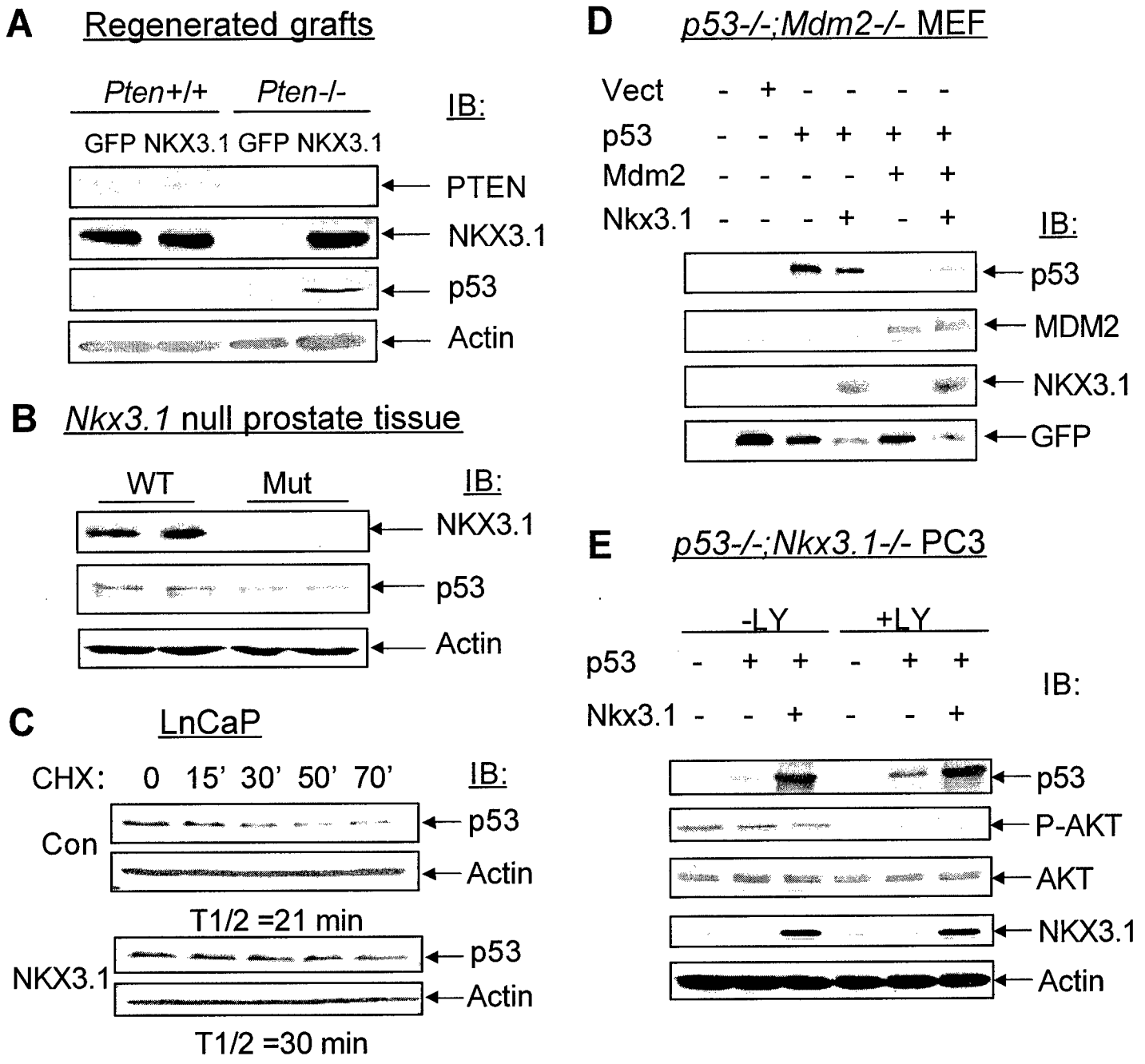

A Regenerated grafts
Pten+/+
Pten-/-
GFP NKX3.1 GFP NKX3.1
IB:
PTEN
NKX3.1
p53
Actin
B Nkx3.1 null prostate tissue
WT
Mut
IB:
NKX3.1
p53
Actin
C LnCaP
CHX: 0 15' 30' 50' 70'
IB:
Con
p53
Actin
T1/2 =21 min
NKX3.1
p53
Actin
T1/2 =30 min
D p53-/-;Mdm2-/- MEF
Vect - + - - - -
p53 - - + + + +
Mdm2 - - - - + +
Nkx3.1 - - - + - +
IB:
p53
MDM2
NKX3.1
GFP
E p53-/-;Nkx3.1-/- PC3
-LY
+LY
p53 - + + - + +
Nkx3.1 - - + - - +
IB:
p53
P-AKT
AKT
NKX3.1
Actin

Figure 6

NKX3.1 modulates p53 protein level and AKT activity

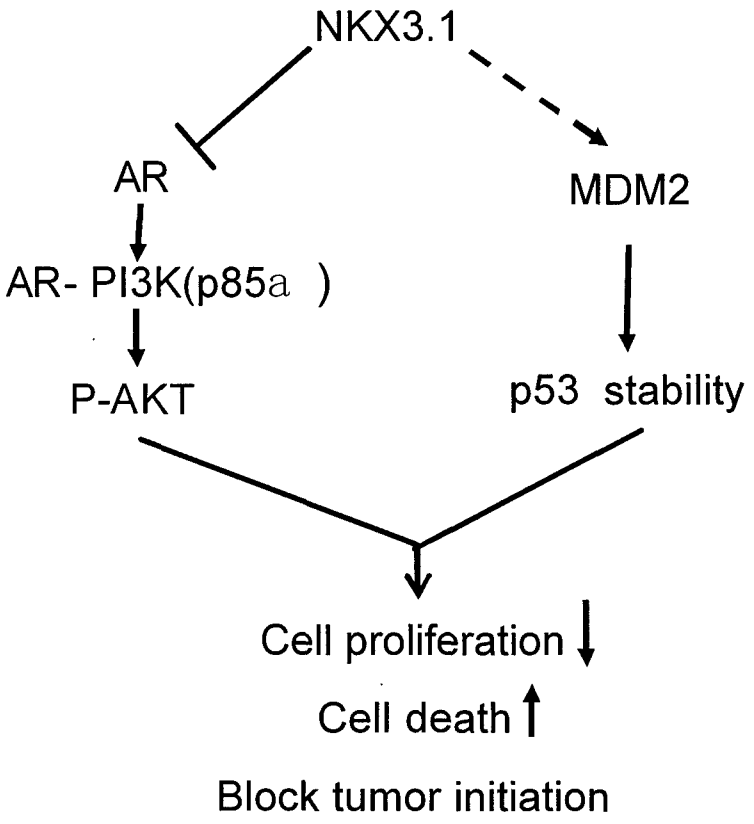


Figure 7

Establishment and characterization of primary epithelial cell lines from Murine *Pten* prostate cancer model

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Abstract

Among genetic alterations frequently found in human prostate cancers, *PTEN* (phosphatase and tensin homologue deleted on chromosome 10) loss of function is strongly implicated in prostate cancer development. We have created a murine model of prostate cancer by specifically deleting the *Pten* tumor suppressor gene using the Cre-Loxp system. The *Pten* prostate cancer mouse model mimics the disease progression seen in humans: initiation of prostate cancer with PIN lesions, followed by progression to local invasive adenocarcinoma, and subsequent metastasis with defined kinetics. Gene expression analysis also reveals similarities in the molecular mechanisms underlying *Pten* murine cancers and human prostate cancers. Importantly, we have identified several candidate genes which may be involved in prostate cancer initiation and progression.

To test the functional relevance of these candidate genes, we need a more amenable system for biochemical analysis. Since current available prostate cell lines are often derived from late stage or metastasis tumors which are not ideal for investigating the events associated with tumor initiation and progression, we established several epithelial cell clones from *Pten* null prostate cancer samples. These cell lines were further characterized for their androgen-dependent growth, their expressions of specific cellular markers, as well as alterations in PTEN controlled signaling pathways. Our preliminary analysis indicate that the cell lines we generated from the murine *Pten* prostate cancer model are very useful tool for studying the molecular mechanism of prostate cancer development caused by PTEN loss.