Synergy of p53 and Rb Deficiency in a Conditional Mouse Model for Metastatic Prostate Cancer

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Abstract

Pathways mediated by p53 and Rb are frequently altered in aggressive human cancers, including prostate carcinoma. To test directly the roles of p53 and Rb in prostate carcinogenesis, we have conditionally inactivated these genes in the prostate epithelium of the mouse. Inactivation of either p53 or Rb leads to prostatic intraepithelial neoplasia developing from the luminal epithelium by 600 days of age. In contrast, inactivation of both genes results in rapidly developing (median survival, 226 days) carcinomas showing both luminal epithelial and neuroendocrine differentiation. The resulting neoplasms are highly metastatic, resistant to androgen depletion from the early stage of development, and marked with multiple gene expression signatures commonly found in human prostate cancers. Interestingly, gains at 4qC3 and 4qD2.2 and loss at 14qA2-qD2 have been consistently found by comparative genomic hybridization. These loci contain such human cancer–related genes as Nfib, I-myc, and Nkx3.1, respectively. Our studies show a critical role for p53 and Rb deficiency in prostate carcinogenesis and identify likely secondary genetic alterations. The new genetically defined model should be particularly valuable for providing new molecular insights into the pathogenesis of human prostate cancer.

Introduction

Prostate cancer is the most common noncutaneous cancer in U.S. men. In 2006, more than 234,460 men will likely to be diagnosed with prostate cancer and 27,350 may die from the disease (1). Androgen depletion is the most widely used therapy for advanced metastatic prostate cancer. Although the majority of patients initially respond well to androgen ablation, they invariably relapse and die from androgen-independent prostate cancer and metastatic disease (2). There are no therapeutic options for the treatment of androgen-independent prostate cancer because the etiology and molecular mechanisms of the disease remain poorly understood.

p53 and Rb are classic tumor suppressors. Mutations of the p53 gene may occur early during prostate carcinogenesis (3) and are frequently associated with metastasis and an androgen depletion–independent phenotype (4, 5). Loss of heterozygosity at the Rb locus occurs in at least one third of human prostate cancer cases (6) and reconstitution of Rb in prostate cancer cell lines suppresses tumorigenicity (7).

Experiments examining individual mutations in p53 or Rb in the prostatic epithelium have resulted in mixed conclusions. Conditional inactivation of p53 does not lead to any neoplastic phenotype (8) whereas expression of the human mutant p53 results in prostatic intraepithelial neoplasia (PIN; ref. 9). Rb loss of function results in hyperplasia after Rb inactivation in situ (10) or carcinoma after transplantation of Rb-deficient cells as a prostatic graft (11).

Alterations of p53 and Rb or their respective pathways frequently coincide in neoplasia, including prostate carcinogenesis (3–6). Such coincidence is usually explained by cooperation between critical and interrelated mechanisms by which p53 and Rb execute their control on cell proliferation, survival, and differentiation, as well as genomic stability (reviewed in ref. 12). However, in some cell lineages such synergy can be very minor if present at all (13).

Previous experiments with expression of the transforming region of SV40 large T antigen (SV40 Tag) in the prostate epithelium have resulted in development of metastatic prostate cancers (reviewed in refs. 14, 15). Expression of the SV40 large T antigen, which binds both p53 and Rb, in the prostate epithelium is sufficient for fully metastatic prostate cancer (16), but expression of a SV40 large T antigen mutant (SV40 Tag T121) that only binds the Rb family proteins (Rb/p107/p130) results in PIN lesions followed by focally invasive well-differentiated adenocarcinomas (17). These observations suggest that inactivation of both p53 and Rb might be important for complete neoplastic progression in this organ. However, this conclusion is complicated by the fact that the SV40 early region produces several viral proteins by means of alternative splicing, including the large T, small t, and 17 kT antigens. All three proteins share NH2-terminal J domain that directly contacts Hsc70. Large T and 17 kT antigens also share a binding motif (LXQXE) that binds to all known members of the Rb-protein family (Rb, p107, and p130). Additionally, large T antigen contains a nuclear localization signal, a specific DNA binding domain, a zinc finger motif, ATPase domain, a bipartite p53 binding domain that is also essential for mediating interaction with the transcriptional adapter protein p300, and an HR specificity region. Small t and 17 kT antigens contain COOH-terminal domain that binds to the multimeric protein phosphatase 2A and four unique amino acids, respectively (reviewed in ref. 18). Notably, small t has been implicated in cell transformation (19).

To directly test the role of p53 and Rb deficiency in prostate cancer, we have conditionally inactivated these genes in the prostate epithelium using B6.D2-Tg(Phsn-Cre)4Hprb (PB-Cre4) mice which express Cre recombinase under the control of a composite
rat prostate-specific probasin ARRP2B promoter in all prostate lobes (20). We show that combined p53 and Rb alterations lead to fast-progressing metastatic carcinomas that share many functional and molecular properties of advanced human prostate cancer. Using a comparative genomic hybridization (CGH), we also identify three genetic loci consistently affected in this cancer model.

Materials and Methods

Generation of prostate-specific p53 and Rb gene deletion mice. To generate PB-Cre, p53loxPloxP, PB-Cre, RbloxPloxP, and PB-Cre, p53loxPloxP, RbloxPloxP mice, ARRP2B-Cre transgenic line, PB-Cre (20) male mice on C57BL/6xdB2A2 background were crossed with p53loxPloxP, RbloxPloxP, or p53loxPloxP, RbloxPloxP (21, 22) female mice on FVB/NJ29 background. The F1 male offspring with PB-Cre, p53loxPloxP, PB-Cre, RbloxPloxP, and PB-Cre, p53loxPloxP, RbloxPloxP genotype were then crossed to p53loxPloxP, RbloxPloxP, females, respectively. The F2 male offspring with PB-Cre, p53loxPloxP, PB-Cre, RbloxPloxP, and PB-Cre, p53loxPloxP, RbloxPloxP genotype were then crossed to p53loxPloxP, RbloxPloxP, females, respectively. To avoid potential genetic variations, only F3 generation of male offspring with PB-Cre, p53loxPloxP, PB-Cre, RbloxPloxP, or PB-Cre, p53loxPloxP, RbloxPloxP genotype were used in this study. They were designated as p53loxPloxP, RbloxPloxP, and p53loxPloxP, RbloxPloxP, respectively. Nonrecombinant littersmates were used as a control. All mice were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee.

Genotyping. p53loxPloxP and RbloxPloxP were identified essentially as previously described (23). PB-Cre transgenic mice have been identified by primers Cre5′-GGACATGTTCAGGATGCCAGCAGG-3′ and Cre2′-GGCATACCGTGAACAGCATGGTCG-3′. PCR amplification of CRE sequences resulted in 296-bp DNA fragment. The PCR profile yielded 94°C for 30 seconds, 60°C for 1 minute, and 72°C for 2 minutes with extension of the last cycle for 10 minutes at 72°C (24).

Pathologic assessment. Moribund mice, as well as those sacrificed according to schedule, were anesthetized with avertin and, if necessary, subjected to cardiac perfusion at 90 mm Hg with PBS followed by phosphate-buffered 4% paraformaldehyde. After macroscopic evaluation during necropsy, tissues were embedded in paraffin and 4-μm-thick sections were stained with Mayer’s HE. Pathology studies were done according to the classification endorsed by the Mouse Models of Human Cancers Consortium NIH/National Cancer Institute (NCI; ref. 25). Given the complexity and controversial nature of the interpretation of stromal microinvasion, only neoplasms infiltrating muscles, other organs, blood, and lymphatic vessels were considered to be invasive.

Immunohistochemical analyses. Immunohistochemical analysis of paraffin sections of paraformaldehyde-fixed tissue was done by a modified avidin-biotin-peroxidase (ABC) technique (26). Antigen retrieval was done by boiling the slides in 10 mmol/L citric buffer (pH 6.0) for 15 minutes. The antibodies to Ki67 (Novocasta Laboratories, Newcastle upon Tyne, United Kingdom, #NCL-Ki67p; 1:1,000 dilution), caspase-3 (Cell Signaling, Danvers, MA, #9661; dilution 1:200), and Ki67 antibody staining (Novocastra Laboratories, #NCL-Ki67p; 1:1,000 dilution). Antigen retrieval was done by boiling the slides in 10 mmol/L citric buffer (pH 6) for 15 minutes. For estimation of apoptosis and proliferation indexes, images were captured with a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) using 40× objective under a Zeiss Axioskop 2 Plus microscope. Five digital images were captured for each slide and transferred to Photoshop 6.0 for manual counting of all the cells (at least 2,000 cells) and proliferating or apoptotic cells after overlaying a grid.

Gene expression microarray analysis. Six metastatic primary prostate tumors (diameters = 0.8 cm) from p53RbPE/C0; RbloxPloxP mice and three normal prostate tissues from normal nonrecombinant littersmates were removed at necropsy. Half of each prostate tumor was fast frozen for RNA and DNA isolation and the other half was fixed for pathologic evaluation. All of these prostate tumors contained at least 85% prostate cancer cells. Total RNA was prepared from normal prostate or prostate tumor tissues using RNeasy lipid tissue mini kit (Qiagen, Valencia, CA, #74804). The generation of biotin-labeled cRNA (using 10 μg of total RNA per sample) fragmentation, hybridization to the Affymetrix murine U74Av2 arrays (Santa Clara, CA), washing, and scanning were done according to Affymetrix protocols.

The gene expression signals from Affymetrix GCOS software were normalized by scaling each GeneChip to a target signal of 500. An offset of 32 was further added to the scaled signals to stabilize the high variance for genes of very low signal values. The log 2-transformed signals of each gene were centered across the nine samples by subtracting the gene-wise median value. Gene filtering was applied to focus on genes having significant variation across all samples, and 4,962 genes with ≥2-fold variation in at least one sample were retained. Genes differentially expressed between normal prostates and prostate tumors were selected by applying two-sample t tests on the log 2-transformed signals of each gene. Under the stringent Bonferroni correction for multiple comparison testing, 475 of the 4,962 genes were significant (P < 0.00001). Hierarchical clustering analysis was done using cosine correlation as distance and complete linkage algorithm with GeneMaths-2.0 software (Applied Maths, Austin, TX).

Comparative genomic hybridization assay. Genomic DNA from five primary prostate tumors used for microarray analysis was assayed by comparative genomic hybridization. Genomic DNA from C57BL/6J male mice was used as a control. Because p53RbPE/C0; RbloxPloxP mice have mixed background, comparative genomic hybridization assay was also done on DNA from the pathologically normal brain of p53RbPE/C0; RbloxPloxP mice to control for differences in regional DNA content reported for mice with different genetic background. Genomic DNA was extracted using DNeasy Tissue Kit (Qiagen, #69504). Mouse BAC genomic arrays, each composed of 6500 RPCI-23 or PRCI-24 clones, were prepared in the Roswell Park Cancer Institute Microarray Core Facility (Buffalo, NY). Genomic DNA from tumors, normal brain, and C57BL/6J control were fluorescently labeled by random priming and hybridized as previously described (27). The hybridized slides were scanned using an Affymetrix model 428 scanner to generate high-resolution images for both Cy3 and Cy5 channels.

The raw fluorescent intensities from both channels were background adjusted and values below 1 were raised to 1. Lowess normalization was
applied on the log 2–transformed intensities after adding an offset of 64. The normalized log-ratio values from replicate spots for the same BAC clone were averaged and clones with consistently weak signals were not included in further analysis.

Tumor/normal brain DNA and control C57BL/6J DNA were sex mismatched; thus, high positive log-ratios were observed for most of clones on Y chromosome and negative log-ratios for those on X chromosomes. The log-ratios of normal-normal hybridization were centered around 0, mostly between −0.3 and 0.3. Gains or losses were scored based on tumor/control fluorescence log-ratios that were >0.5 or <−0.4, respectively.

Real-time PCR. Quantitative PCR analysis was carried out on an ABI 7900 HT Real-time PCR System (Applied Biosystems, Foster City, CA). The primers for quantitative PCR are as follows: nuclear factor I/B (Nfib) gene, Nfib5′ (5′-AGGGTAAGATTAGGAGGATCGACT-3′) and Nfib3′ (5′-ACGTAGTATGCCAAAAACAAAGTCA-3′); L-myc gene, L-myc 5′ (5′-CAG-TCTGGAGTATGCCAAAAACAAAGTCA-3′) and L-myc 3′ (5′-TCAGGAGTCTAGATGCCAAAAACAAAGTCA-3′). The reactions were done in a 25-μL volume in 96-well plate using the fluorescent dye SYBR Green (Invitrogen, Carlsbad, CA). Quantitative PCR parameters were 94°C for 3 minutes, followed by 40 cycles at temperature profile 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. Increase in real-time fluorescence was measured and relative fold changes were calculated using the 2−ΔΔCt method (28).

Statistical analyses. All statistical analyses in this study were done with InStat 3.05 and Prism 4.03 software (GraphPad, Inc., San Diego, CA). Survival fractions were calculated using the Kaplan-Meier method and survival curves were compared by log-rank Mantel-Haenszel tests.

Results

Inactivation of either p53 or Rb gene alone leads to PIN with no progression to invasive carcinoma. To determine the consequences of p53 or Rb deletion, the prostates of p53PE−/− and RbPE−/− mice, as well as those of their nonrecombinant littermates, were collected at different time points between 60 and 600 days after birth and evaluated histologically. In p53PE−/− mice, dysplastic lesions were located in all lobes and detected from postnatal day 170 onwards (Fig. 1A, p53PE−/−). These lesions represented multifocal accumulation of tufting mitotic atypical cells confined within the boundaries of the gland space and were diagnosed as PIN. The degree of cytologic and structural atypia of PIN increased with age, although no invasive carcinoma was detected in 20 serially sectioned prostates of 600-day-old mice (Supplementary Table S1).

Consistent with the results reported earlier (10), all mice (n = 5) with prostate-specific inactivation of the Rb gene developed only hyperplasia by postnatal day 500. The areas of hyperplastic epithelium could be easily identified by Ki67-positive nuclear

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Figure 1. Prostate epithelium–specific inactivation of either p53 or Rb alone leads to PIN. A, WT, regular structure of the dorsolateral lobe of the prostate of adult wild-type mouse. PIN (arrow) in the prostate of 550-day-old p53PE−/− and RbPE−/− mice. B, increased number of Ki67-positive cells (arrow) in PIN and hyperplasia induced by inactivation of p53 or Rb, respectively. PIN cells contain androgen receptor (AR; arrow) but no synaptophysin (SYN). Note the presence of synaptophysin in the nerve terminals (arrow). A, H&E; B, ABC Elite method, hematoxylin counterstaining. Bar, 40 μm (A); 50 μm (B).
staining and were detected in all lobes. By postnatal day 600, all RbPE−/− mice (n = 10) developed PIN (Fig. 1A, RbPE−/−), similar to those associated with p53 inactivation.

It was noted that cells of all proliferative lesions, initiated by either p53 or Rb loss of function, expressed nuclear androgen receptor and luminal epithelium-specific cytokeratin 8 (CK8) but lacked neuroendocrine marker synaptophysin and basal cell marker CK5 (Fig. 1B and data not shown).

Taken together, these results indicate that inactivation of either p53 or Rb alone is insufficient for formation of invasive carcinoma of the prostate.

Deletion of both p53 and Rb genes in prostate epithelial cells leads to metastatic carcinoma. To test the synergistic effects of p53 and Rb inactivation, compound p53PE−/−; RbPE−/− mice were produced and evaluated. All mice succumbed to rapidly growing invasive and metastatic neoplasms of the prostate between postnatal days 168 and 351 (median, 226 days). Histologic analysis of these tumors in moribund mice showed that neoplasms replaced most of the prostate, sometimes contained multifocal areas of necrosis, and invaded surrounding adipose and muscular tissues, vessels, the bladder, and the urethra. Neoplastic cells formed trabecular, solid, and, sometimes, acinar patterns, separated by moderate desmoplastic stroma, and exhibited numerous, frequently atypical, mitotic figures and apoptosis (Fig. 2A). Immunostaining revealed a phenotypic diversity of these neoplasms, with cells containing androgen receptor (20-80% of cells) (T1 and T3), CK8-negative tumor cells (T2 and T5), synaptophysin-positive tumor cells (T4 and T7), and synaptophysin-negative tumor cells (T6 and T7). 295-, 266-, and 247-bp fragments are diagnostic for fixed, excised, and wild-type alleles of the Rb gene, respectively. 316-, 198-, and 163-bp fragments are diagnostic for fixed, excised, and wild-type alleles of the p53 gene, respectively. D, survival of p53PE−/−; RbPE−/− (n = 30), p53PE−/−; Rb+/− (n = 6), and p53PE−/−; Rb+/− (n = 6) mice. Median survival for p53PE−/−; RbPE−/− mice was 226 days.
generated. By 510 days, PINs similar to those in p53<sup>−/−</sup> and Rb<sup>−/−</sup> mice were observed in both p53<sup>−/+;</sup> Rb<sup>−/−</sup> and p53<sup>+/−;</sup> Rb<sup>−/−</sup> mice. By the same time, none of p53<sup>−/+;</sup> Rb<sup>−/−</sup> developed malignant neoplasms whereas half of p53<sup>−/−;</sup> Rb<sup>+/−</sup> mice developed p53- and Rb-deficient metastatic carcinomas similar to those observed in p53<sup>−/−;</sup> Rb<sup>−/−</sup> mice (Fig. 2D).

Metastases were detected in p53<sup>−/+;</sup> Rb<sup>−/−</sup> mice from 200 days of age. Prostate carcinomas metastasized to the lung (67%, 16 of 24 mice), liver (85%, 23 of 27 mice), adrenal gland (11%, 2 of 18 mice), and regional lymph nodes (68%, 13 of 19 mice). Liver and lymph node metastases were usually large and could be routinely identified with macroscopic evaluation during necropsy. The metastatic cells retained expression of androgen receptor, CK8, and synaptophysin, which are typical in the primary neoplasms (Fig. 3).

Taken together, these results indicate that p53 and Rb do cooperate in prostate carcinogenesis leading to highly aggressive, poorly differentiated, and metastatic carcinomas consisting of cells with both luminal epithelial and neuroendocrine differentiation.

Effects of androgen ablation on p53- and Rb-deficient prostate neoplasia. Because androgen ablation is the most used treatment for advanced prostate cancer, we examined the effect of castration on prostate cancer progression in p53<sup>−/+;</sup> Rb<sup>−/−</sup> mice. As prostate neoplasms were reproducibly detected at postnatal day 160, mice were castrated at that time and the immediate response of neoplastic cells to androgen withdrawal (3 days post-castration), as well as the long-term survival of mice, was assessed. Residual tumor masses were readily detected 3 days post-castration although many cells were undergoing apoptosis as per morphologic assessments and activated caspase-3 assays (Fig. 4A). At the same time, it was found that androgen ablation did not change the proliferation index of tumor cells (Fig. 4B).

Interestingly, castration did not increase the life span of the mice (Fig. 4C). Neoplasms remained positive for CK8 and androgen receptor after prolonged castration. However, in neoplastic cells of castrated mice, the androgen receptor expression level was lower than that in the noncastrated mice. Furthermore, the results of synaptophysin immunostaining indicated that neoplastic cells in castrated mice might be more prone to neuroendocrine differentiation (50 ± 20%, n = 4, versus 70 ± 20%, n = 5, P = 0.03).

To determine the onset time of androgen depletion independence, p53<sup>−/+;</sup> Rb<sup>−/−</sup> mice (n = 6) were castrated at postnatal day 60, when they have PIN, and sacrificed at postnatal day 160. By that time, neoplasms were found in all the castrated mice similar to those of noncastrated p53<sup>−/−;</sup> Rb<sup>−/−</sup> mice, indicating that either androgen ablation-resistant cell population exists even at the earliest stages of carcinogenesis or the resistance is acquired very early.

Thus, p53- and Rb-deficient prostate neoplasms, while responding to androgen ablation initially, became androgen depletion independent from the early stages of carcinogenesis due to selection of androgen ablation-resistant cells and neoplastic progression is associated with an increase in neoplastic cells with neuroendocrine differentiation.

Figure 3. Metastasis of prostate cancer in p53<sup>−/+;</sup> Rb<sup>−/−</sup> mice. A, areas of metastatic prostate neoplastic cells (arrows) in the lung (top left), liver (top right), regional lymph node (bottom left), and adrenal gland (bottom right). B, metastatic cells in the liver contain (arrows) androgen receptor and synaptophysin. A, H&E; B, ABC Elite method, hematoxylin counterstaining. Bar, 50 μm (A and B).
Transcriptome alterations in p53- and Rb-deficient mouse prostate neoplasms are similar to those of aggressive human prostate carcinomas. Expression of androgen receptor and epithelial and neuroendocrine markers, as well as acquired androgen resistance, as detected in our mouse prostate carcinoma model, is a common feature of metastatic human prostate carcinomas. To further determine the similarity between mouse and human prostate carcinomas, the patterns of gene expression in p53- and Rb-deficient mouse prostate neoplasms were compared with those in age-matched nonrecombinant mouse prostates. In agreement with our immunohistochemical results, expressions of genes indicating epithelial and neuroendocrine phenotype were detected. In sum, 475 genes were found to have expression differences between nonrecombinant prostate and prostate tumor tissue at \( t \)-test significance level of 0.00001. Among those genes, 197 genes were down-regulated and 278 genes were up-regulated in the neoplasms (Fig. 5A). Notably, of the 18 genes known to have expression alterations in aggressive human prostate cancer, 16 were similarly affected in our mouse model (Fig. 5B and C and Supplementary Table S2). Among those, Nkx3.1, myosin heavy chain 11, and \( \text{Kai1} \) were down-regulated and \( Bmi1, Ezh2 \), and \( Pim1 \) were up-regulated.

Recently, 17 signature genes (8 up-regulated and 9 down-regulated) associated with tumor metastasis were identified in various human cancers (29). Ten of these genes were present on our Affymetrix chip and all of them had similarly altered expression, with four (\( \text{Lmnb1, Pttg1, Col1a1, and Hnrpab} \)) being up-regulated and six (\( \text{Myh11, Nrfa1, Mt3, Actg2, Mylk, and Cnn1} \)) being down-regulated in p53- and Rb-deficient mouse prostate neoplasms. To date, we have not identified bone metastases but we did find an increase in expression of osteopontin, a marker associated with the presence of bone metastasis in human prostate cancer and also strongly detected in Pten mouse model (30, 31). Taken together, the changes in gene expression in p53- and Rb-deficient mouse prostate tumors seemed to be similar to the changes occurring in aggressive human prostate carcinomas, many of which have alterations in the p53 and Rb pathways.

Prostate cancer associated with p53 and Rb deficiency has consistent pattern of genomic alterations. To determine whether any specific genetic aberrations were associated with p53- and Rb-deficient prostate neoplasms, comparative genomic hybridization array analyses were done (Fig. 6A). Three chromosomal regions with consistent gene copy number alterations were identified. Significant gains were detected at locus 4qC3, which contains the \( Nfib \) gene, and at locus 4qD2, which contains the \( L-myc \) oncogene, in four of five neoplasms for each locus. A large chromosomal loss at 14qA2-qD2 was also observed in all analyzed tumor samples. This locus encompasses a region orthologous to human chromosome 8p21, which is frequently lost in human prostate cancer and contains the tumor suppressor \( Nkx3.1 \) (reviewed in ref. 15). We validated both gains by real-time quantitative PCR (Fig. 6B). Corresponding changes in the expression of \( Nfib, L-myc, \) and \( Nkx3.1 \) were confirmed using Affymetrix gene expression arrays (Fig. 6C) and immunohistochemical staining.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effects of androgen ablation on prostate carcinogenesis in \( p53^{PE}\); \( Rb^{PE}\) mice. A and B, apoptotic (A) and proliferating (B) prostatic neoplastic cells identified by detection of caspase-3 and Ki67 (arrows), respectively, in either intact 165-day-old \( p53^{ PE}\); \( Rb^{ PE}\) mice (Intact) or their littermates euthanized 3 days after castration (Castration). Quantitative analysis indicates that prostate tumor cells undergo increased apoptosis (3.03 ± 0.77% versus 1.3 ± 0.34% in mice without castration; \( n = 3, P = 0.022 \)) after castration but have no change in proliferation Ki67 (50.4 ± 2.92 versus 48.8 ± 2.68; \( n = 3, P = 0.71 \)). C, survival of intact and castrated \( p53^{ PE}\); \( Rb^{ PE}\) mice. All mice were euthanized when they became moribund. Castration was done at postnatal day 165. Median survivals are 209 and 226 days for castrated (\( n = 9 \)) and intact (\( n = 30 \)) mice, respectively. Log-rank \( P = 0.879 \), for comparison of the survival curves. A and B, ABC Elite method, hematoxylin counterstaining. Bar, 50 \( \mu \)m (A and B).
In summary, p53- and Rb-deficient prostate neoplasms consistently carry three additional genetic alterations, which are likely to represent secondary genetic alterations important for neoplastic progression.

**Discussion**

According to earlier studies with mouse models, conditional inactivation of Rb or transgenic expression of mutant p53 results in development of prostatic hyperplasia and intraepithelial neoplasia (9, 10), which is consistent with our data. However, contrary to our work, prostate epithelium-specific conditional knockout of p53 using PB-Cre4 mice did not result in any obvious phenotype by 18 months of age in one study (8). These seemingly conflicting data may be due to the differences in either genetic backgrounds or the extent of p53 deletion (exon 2-10 in our study versus exon 7 in other study). In any case, none of the data thus far showed that inactivation of either p53 or Rb alone is sufficient for development of invasive carcinoma in natural settings.

Using the Cre-loxP approach, our study shows definitively that combined deficiency for p53 and Rb acts synergistically in prostate carcinogenesis, causing invasive and highly metastatic carcinomas. This observation strengthens the earlier observations based on p53 and Rb binding to viral antigens (reviewed in refs. 14, 15) and shows that inactivation of Rb without the loss of p107 and p130 functions is sufficient for initiation of carcinogenesis.

One of the main functions of Rb is the control of cell cycle progression at G1-S, and inactivation of Rb facilitates uncontrolled cell proliferation (reviewed in ref. 12). p53 regulates a gene expression program, which leads to either cell cycle arrest or apoptosis after a number of insults, including inappropriate cell cycle progression due to Rb inactivation. These functions may explain the observed synergistic effect of combined p53 and Rb deficiency on carcinogenesis. We have directly tested the requirements for the loss of p53 and Rb during carcinogenesis by using compound mice carrying a single wild-type copy of either gene. None of the mice deficient for Rb but carrying a single wild-type copy of p53 developed carcinomas. At the same time, heterozygosity for Rb led to full malignant progression and the resulting carcinomas consistently lacked the remaining wild-type copy of Rb. These observations indicate that p53 loss of function represents an initial event permissive for uncontrolled cell proliferation due to

(Fig. 5C and data not shown). In summary, p53- and Rb-deficient prostate neoplasms consistently carry three additional genetic alterations, which are likely to represent secondary genetic alterations important for neoplastic progression.

![Figure 5](image-url)

**Figure 5.** Transcriptome alterations in prostate carcinomas of p53PE-/-; RbPE-/- mice. A, expression profile of top 475 genes differentially expressed between normal and neoplastic prostate cells at t-test significance level of 0.00001. B, changes in expression of prostate cancer “signature” genes are similar between mouse and human neoplasms in 16 of 18 genes. *, genes with dissimilar expression. C, down-regulation of Nkx3.1 and up-regulation of Ezh2 and Ect2 genes (B, arrows) in mouse prostate cancer (Cancer) as compared with the normal prostate epithelium (WT). A and B, Affymetrix GeneChip Murine Genome U74Av2 array; C, ABC Elite method, hematoxylin counterstaining. Bar, 50 µm (C).
Rb deficiency and the loss of the remaining wild-type copy of Rb is essential for progression of carcinogenesis in our model. Further studies based on temporally discreet inactivation of p53 and Rb should test these possibilities.

The majority of proliferative lesions of mouse prostate reported thus far seem to arise from transit-amplifying cells of the luminal epithelium. Among them are mouse prostate carcinomas associated with c-myc overexpression or Pten inactivation (31, 32) and some of the SV40 Tag–induced neoplasms (33). The latter have been reported to progress to carcinomas with neuroendocrine differentiation. Other neoplasms initiated by expression of SV40 Tag sometimes derive from neuroendocrine cells and express neuroendocrine markers but fail to express androgen receptor or epithelial markers. These neoplasms are mostly similar to human neuroendocrine carcinomas (34). In our model, neoplasms are phenotypically diverse and contain cells expressing luminal epithelium marker CK8, androgen receptor, and neuroendocrine markers synaptophysin and chromogranin A, but not the basal cell marker CK5. A large part of neoplastic cells coexpress synaptophysin with both CK8 and androgen receptor, indicating that these cells either originate from bipotential stem cells or acquire a significant degree of phenotypical plasticity during carcinogenesis. Interestingly, loss of Rb function has been implicated in a number of neoplasms exhibiting neuroendocrine differentiation (35, 36). At the same time, inactivation of the whole family of Rb proteins leads to well-differentiated adenocarcinomas without neuroendocrine differentiation (17). Further studies should address the cell of origin of cancers in our model, as well as evaluate the reasons for specific cellular phenotypes associated with Rb deficiency.

In humans, malignant epithelial neoplasms of the prostate include adenocarcinomas and neuroendocrine carcinomas (25). Adenocarcinomas exhibit mainly glandular differentiation and represent the majority of human cases. Neuroendocrine carcinomas, such as small-cell carcinomas, are diagnosed on the basis of their morphologic criteria, which can be confirmed by subsequent immunohistochemical detection of neuroendocrine markers, such as synaptophysin and chromogranin A. Interestingly, although distinct from a neuroendocrine morphology, human prostate adenocarcinomas exhibit focal positivity for neuroendocrine markers in the range of 30% to 100% of cases with ~10% of cases being extensively positive (37). Neuroendocrine differentiation increases in advanced tumors and androgen depletion–independent tumors (38). The mechanisms of neuroendocrine
differentiation and the development of androgen depletion-independent tumors remain poorly understood (34, 39) and our genetically defined model should be most useful to address these issues.

Few mouse models have been reported to encompass the full spectrum of neoplastic progression from PIN to invasive carcinoma and metastasis. The most fully characterized of these are based on prostate-specific expression of the SV40 Tag or its large T antigen in prostate epithelial cells. Examples of such models are transgenic adenocarcinoma of mouse prostate (TRAMP; ref. 40) and probasin-large T antigen (LPB-Tag; ref. 41). However, SV40 Tag is not naturally associated with human prostate cancer and, as previously discussed, targets many pathways, some of which may play no role in metastatic progression in humans. Furthermore, SV40 Tag-based models are commonly driven by an androgen-dependent probasin promoter and therefore are poorly suited for studying mechanisms of androgen resistance in advanced cancers. Other metastatic models of sporadic prostate cancer are the Pten conditional knockout (31) and Nkx3.1$^{+/−}$/Pten$^{+/−}$ compound mice (42). Unfortunately, less than half of neoplasms are metastatic and most metastases are only detectable microscopically.

Our model progresses to extensive metastases in the liver, lung, and regional lymph nodes, sites commonly targeted for metastasis in the human disease. Importantly, it recapitulates most aspects of human prostate cancer at the molecular level, including expression of both epithelial and neuroendocrine markers. Of the 18 genes known to have expression alterations in aggressive human prostate cancer, 16 were similarly affected in our mouse model. Furthermore, expressions of all 10 signature genes, which associated with metastasis in human cancer (29) and present in our microarray, were similarly affected in the model. Thus, our model should be particularly useful in gaining further understanding of the processes that govern metastasis in human prostate cancer.

Interestingly, prostate carcinomas associated with p53 and Rb deficiency in our model had changes in expression of two Myc signature genes, Nkx3.1 and Pim-1, which were similar to those observed in the Myc-driven mouse prostate cancer model (32). At the same time, two markers known to be up-regulated in Pten tumors, Psca and clusterin, were not similarly affected in our model, indicating that carcinogenesis associated with p53 and Rb deficiency may have distinct mechanisms. Accordingly, variations in expression of these genes have been reported in human prostate carcinomas (43, 44).

Using comparative genomic hybridization assay, we identified a limited but consistent set of genetic alterations associated with prostate carcinogenesis in our model. Importantly, amplification and overexpression of L-myc occurs in a number of human cancers, including small-cell lung cancer (45). Almost all small-cell lung cancers are p53 and Rb deficient. NFIB is a recurrent translocation partner for HMGIC in pleomorphic adenomas (46) and is located within an amplicon at 9p23-24 in squamous cell carcinoma of the esophagus (47). Mice lacking Nfib have major neuroanatomical defects, including callosal agenesis, aberrant hippocampus and pons formation, loss of specific midline glial populations, and defects in lung development (48). Our results raise an intriguing possibility that progression of neoplasm associated with inactivation of p53 and Rb may depend on activation of Nfib and L-myc across different species, at least in the context of some cell lineages. The loss of chromosomal region 8p21, where the putative tumor suppressor gene NKX3.1 is located, occurs in ~80% of human prostate cancers, and loss of NKX3.1 expression in human prostate cancers correlates with tumor progression (49). In the mouse, targeted deletion of Nkx3.1 leads to PIN (50). Nkx3.1 loss of function has also been reported to correlate with Pten inactivation (31) and is a common secondary event in c-myc-driven prostate carcinogenesis (32). Our model should be a valuable addition to available repertoire of mouse models suitable for studies addressing the mechanisms of Nkx3.1 loss of function on genetic, transcriptional, and translational levels as a function of particular genetic and cell lineage settings.

Taken together, the reported model of prostate carcinogenesis associated with p53 and Rb deficiency should be a very useful tool for understanding of prostate cancer pathogenesis, interrogation of gene-phenotype relationships, and validation of diagnostic, preventive, and therapeutic approaches in preclinical settings.

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