Heterogeneous Tumor Evolution Initiated by Loss of pRb Function in a Preclinical Prostate Cancer Model

Reginald Hill, Yurong Song, Robert D. Cardiff, and Terry Van Dyke

Abstract

Because each change in the evolution of a cancer is predicated on the effects of previous events, a full understanding of selective changes and their effect on tumor progression can only be understood in the context of appropriate initiating events. Here, we define the effect of pRb function inactivation in prostate epithelium on both the initiation of prostate cancer and the establishment of selective pressures that lead to diminished Pten function and tumor evolution. Using genetically engineered mice, we show that inactivation of the pRb family proteins (Rb/p107/p130) induces epithelial proliferation and apoptosis and is sufficient to produce prostatic intraepithelial neoplasia (PIN) lesions. Over time, adenocarcinomas develop in all mice with no evidence of neuroendocrine tumors. Apoptosis is dependent on Pten function and not p53, unlike other epithelial cell types tested previously. Consequently, Pten hemizygosity reduces apoptosis by 50%, accelerating progression to adenocarcinomas with heterogeneous composition. Heterogeneity is associated with concurrent Pten haploinsufficiency and focal selective progression to complete Pten loss, which yields distinct tumor properties. Given that this analysis models the apparent timing of highly penetrant events in human prostate cancer, observed effects may recapitulate the natural evolution of prostate cancer development. (Cancer Res 2005; 65(22): 10243-54)

Introduction

Prostate cancer is a heterogeneous and multifocal disease that arises from multiple genetic and epigenetic aberrations (1, 2). The heterogeneity of prostatic lesions in humans confounds interpretation of genetic correlations, impedes accurate diagnosis, and hampers the development of appropriate treatment strategies. A common early aberration in human prostate cancer, allelic loss, or reduced expression of Rb occurs in 25% to 50% of prostate tumors (3–6), including in early lesions (7). Furthermore, overexpression of p16INK4a expression, an upstream regulator of pRb, has been observed in up to 80% of prostate cancers (8).

Several genetically engineered mouse (GEM) lines have been generated in an attempt to model and understand human prostate cancer. However, the effects of pRb inactivation, which often requires suppression of potentially compensatory functions of related proteins p107 and p130, have not been fully explored in prostate. The SV40 early region (SV40 large T and small t antigen) induces prostate hyperplasia, adenocarcinoma, invasive carcinoma, and metastasis in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model when expressed under control of the rat small probasin (9) or rat prostatic steroid binding protein (C3; ref. 10) promoters. In addition, probasin-driven expression of only the large T antigen causes hyperplasia, adenocarcinoma, and invasive carcinomas (11). Large T antigen inactivates all pRb family proteins (pRbα) but also inactivates p53 and p300 and may have additional tumorigenic functions (12). In addition, small t antigen interferes with the phosphatase PP2A. Thus, although such models have been useful in providing an avenue to examine prostate tumors in the mouse, the individual roles of the pRb, p53, and other pathways could not be assessed.

Prostate epithelium from Rb−/− embryonic pelvic viscera, when recombined with wild-type rat urogenital mesenchyme under the kidney capsule of recipient animals, becomes hyperplastic in 40% of grafted samples (13). In addition, deletion of a conditional Cre-dependent Rb-null allele specifically in prostate epithelium results in the development of focal hyperplasia (14). These studies establish that inactivation of Rb alone in mouse prostate can have a modest proliferative effect but does not predispose to tumorigenesis. Given the modest responses in these models, it is possible that p107 and/or p130 perform compensatory/redundant functions in the prostate epithelium, masking the effects of complete functional loss.

By dominantly expressing a domain of SV40 large T antigen (121 NH2-terminal amino acids; T121) that inactivates the pRbα, leaving p53 and other targets functional, we have directly assessed the effect of pRb inactivation in specific cells of transgenic mice. Using this approach in other epithelial cell types, we showed that pRbα inactivation induces aberrant proliferation and apoptosis and is sufficient for tumor initiation. In choroid plexus (15, 16) and mammary gland (17) epithelia, most of the apoptosis is mediated by p53 activation, and tumor progression is dependent on p53 inactivation, a process that occurs naturally during tumor evolution based on the selective pressure imposed by the p53 apoptotic function (15, 16).

Whereas pRb inactivation is believed to be an early event in prostate tumorigenesis, p53 allelic loss (18) or mutation (19) occurs in more advanced prostate cancers. Thus, p53 loss is a candidate for selective pressures imposed by pRb inactivation in the progression of prostate cancer. However, Pten alteration, also known to facilitate cell survival, is one of the most frequent events associated with human prostate cancer progression (reviewed in ref. 20). Studies in the mouse have examined the effects of conditional Pten loss (21, 22) or Pten heterozygosity alone or in combination with TRAMP (23), NKX3.1 loss (24), or p27 loss (25) with clear effects on prostate (see Discussion). However, the
natural evolution of Pten alterations in the context of pRb pathway mutations, which seem to precede Pten changes in human prostate cancer, have not been explored. In this report we use genetically engineered mice to elucidate the roles of prostate epithelium-specific pRb functional loss, its effect on subsequent tumor progression including its relation to p53 activity, and the role of Pten alteration in prostate tumor evolution. We report both mechanistic analyses as well as the establishment of a fully penetrant mouse model for the initiation and progression of prostate cancer.

Materials and Methods

Derivation of TgAPT121 transgenic mice. The 458-bp ARR2PB promoter region (26), which is regulated by androgens and activated during puberty, was isolated and cloned upstream of a 2.4-kb KpnI-SalI fragment of the d1137 plasmid (27), which encodes the truncated SV40 COOH-terminal pRb-binding domain inactivate pRb, p107, and p130 (12). Interaction with p53 and p300 binding as well as an additional transforming function map to the COOH half of T antigen and are thus not present in T121. An EcoRI fragment containing the transgene (4 ng/μL) was injected into fertilized eggs harvested from B6D2F1 mice (The Jackson Laboratory, Bar Harbor, ME) as described previously (15). Resulting and subsequent generation TgAPT121 transgenic mice were identified by PCR amplification of a 160-bp fragment using primers 5′-GAACTTTCAGCTAATGACC-3′ and 5′ GCATCCGAGCAGTCCAAAG-3′ and digit-derived genomic DNA as template. Cycling profile was 94°C for 2 minutes; 35 cycles of 94°C for 20 seconds, 62°C for 45 seconds, and 72°C for 45 seconds; and final incubation of 72°C for 2 minutes. TgAPT121 mouse lines were maintained by crossing to nontransgenic B6D2F1 mice and therefore are designated as B6D2-Tg(APT121)Tvd(APT121).

Transgenic breeding strategies. To study the effect of p53 inactivation on prostate tumorogenesis, TgAPT121 mice were mated to p53 nullizygous mice (p53tm1Tyj; The Jackson Laboratory). p53 genotypes were determined by PCR using two reactions (28): neomycin primer 5′-TCCTCGT-GCTTTACGGGTATC-3′, p53 primer 5′-TATACTCAGGCGCGCCT-3′, 525-bp product; the endogenous p53 allele; substituting 5′-ACAGCGTGGTGG-TACCTTACG-3′ for the neo primer, 475-bp product. Cycling variables were as for the T121 reaction. We produced male mice with the genotypes TgAPT121:p53+/−, TgAPT121:p53−/−, and TgAPT121:p53+/−, and nontransgenic male littermates (p53+/−, p53−/−, or p53−−−) served as controls.
To study the effect of Pten hemizygosity, TgAPT121 mice were mated to Pten heterozygous mice [C57BL/6J-Tg(Pten); The Jackson Laboratory]. Pten genotypes were determined by PCR using two reactions, one of which amplifies the neomycin insertion (as above) and the other amplifies the endogenous Pten allele (29; exon 5 PT3, 5'-TTGAGCCTCAATAC- CCACACAG-3' and PT6, 5'-CAAGCTTCATTCCCTTGCTTG-3') and neo sequences (NEO3, 5'-CGACACCACACCGAACAAC-3' and NEO4, 5'-CGATG- GATCCGAAAGC-3'). PCR conditions were 30 cycles of 30 seconds at 95°C, 30 seconds at 54°C, and 30 seconds at 72°C.

Histopathology and apoptosis assays. A portion of each prostate sample was fixed overnight in 10% phosphate-buffered formalin, transferred to 70% ethanol, and then embedded in paraffin. Samples were sectioned for 10 successive layers at 5-μm intervals and stained with H&E for histopathologic examination as described previously (15). Apoptosis levels were assessed using the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) assay as described previously (15). Differences in apoptosis levels between mice with different genotypes were evaluated by t test (P < 0.05 is considered of statistical significance).

Immunohistochemistry and immunofluorescence. Immunohistochemical analysis was done on formalin-fixed paraffin sections. Antigen retrieval for all antibodies was done by boiling the slides in citrate buffer (pH 6.0; Zymed, South San Francisco, CA) for 15 minutes. Endogenous peroxidase activity was quenched with a 10-minute incubation in 3% H2O2 in methanol. Antibodies were anti-cytokeratin-8 (1:100, sheep polyclonal, PB182; Binding Site, Birmingham, United Kingdom), smooth muscle actin (SMA; 1:1,000, mouse A2537; Sigma, St. Louis, MO), anti–phosphorylated histone H3 (1:100, rabbit 06-570; Upstate, Waltham, MA), anti-SV40 (monoclonal Ab2, 1:100; Oncogene, Cambridge, MA), anti–Pten (1:200, polyclonal; NeoMarkers, Fremont, CA), and anti–phosphorylated Akt (p-Akt; Ser473, 1:200; Cell Signaling, Beverly, MA). Detection for all antibodies was done using the Vector ABC Elite kit and a Vector V-Diaminobenzidine kit for substrate detection (Vector Laboratories, Burlingame, CA). Immunofluorescence followed the same protocol, except that signal amplification used the TSA Plus Fluorescence System (Perkin-Elmer, Wellesley, MA). Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted using Vector Hardset Mounting Media (Vector Laboratories).

Western immunoblotting analysis. Protein expression levels were assayed as described previously (15). Flash-frozen tissue samples were homogenized in lysis buffer [50 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, and 1% NP40] using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland). Total protein (100 μg) was electrophoresed through a 16% precast polyacrylamide denaturing gel (Bio-Rad, Hercules, CA) and then transferred to polyvinylidene difluoride membrane (0.1-μm pore size; Millipore, Bedford, MA; 15 V for 30 minutes) following manufacturer's instructions. The membrane was preincubated in 5% milk-TBS-Tween buffer for 1 hour at room temperature followed by incubation with primary antibody against the NH2 terminus of SV40 T antigen (PAh416 supernatant; ref. 30; at a dilution of 1:1 at 4°C overnight). The membrane was then washed following incubation with horseradish peroxidase–conjugated goat anti-mouse IgG (1:10,000 dilution; Amersham Biosciences, Little Chalfont, United Kingdom) for 1 hour at room temperature. The enhanced chemiluminescence method (Enhanced Chemiluminescence Plus kit, Amersham Biosciences) was used for autoradiography.

Histologic classification of TgAPT121 prostates. H&E-stained sections were initially reviewed by R.H., Y.S., and T.V.D. and were then classified by R.C., a veterinary pathologist with extensive experience in murine prostate pathology, using a pathologic classification scheme developed at the University of California, Davis. The criteria used to assess the prostates included nuclear size, nuclear shape, nuclear density, nucleolar size, nuclear chromatin, and nuclear shape. The prostates were classified as normal, hyperplastic, and neoplastic.

Results

Generation of mice with prostate epithelium–specific inactivation of pRb family protein function. We generated four founder mice carrying a transgene in which the prostate-specific ARR2PB promoter, a modified version of the rat probasin promoter, drives expression of T121 (Fig. 1A; see Materials and Methods). This promoter, shown to direct high-level expression to prostate secretory epithelial cells of transgenic mice, is activated on sexual maturity and due to androgen dependence (26). Transgene transmission was observed in three lines, and all three lines displayed identical phenotypes. Extensive studies focused on the representative TgAPT121-15 line (designated TgAPT121).

T121 expression in TgAPT121 prostates. Consistent with the specificity of the probasin promoter, by immunoblotting (see Materials and Methods), T121 was expressed in the prostate and not in other organs, including liver, kidney, heart, spleen, lung, and testes (Fig. 1B). Moreover, T121 expression, detectable in mice as early as 6 weeks of age, was restricted to prostate epithelium as shown by immunofluorescence (Fig. 1C). The extent of expression varied depending on the lobe, with levels highest and most extensive in the dorsal, anterior, and ventral lobes and little to no expression in the lateral lobe (Supplementary Fig. S1B). No T121 expression was detectable in the seminal vesicle and testes, although vas deferens smooth muscle cells and the ampullary gland epithelial lining were sometimes positive (Supplementary Fig. S2A and S2B). This expression may cause the sterility observed in aging male TgAPT121 mice.

pRb family protein disruption in prostate epithelium induces murine prostatic intraepithelial neoplasia associated with aberrant proliferation and apoptosis. Compared with littermate controls (Fig. 2A, a), the prostates of TgAPT121 mice showed extensive abnormal gland architecture beginning as early as 6 weeks of age when focal hyperplasias with nuclear atypia (increased nuclear-to-cytoplasmic ratio, nuclear enlargement and elongation, and hyperchromatism) were evident (Fig. 2A, b). By 12 weeks of age, all TgAPT121 mice displayed characteristic traits of murine prostatic intraepithelial neoplasia (mPIN; ref. 31; including epithelial layer stratification that manifests itself as tufting, micropapillary, and cribriform growth patterns; Fig. 2A, c and d, arrowheads, and Fig. 3). This phenotype was accompanied by hypercellularity of the fibromuscular stromal cell layer (Fig. 2A, d, star). Thus, pRb inactivation alone is sufficient for cell-autonomous induction of mPIN.

To determine the direct effects of pRb functional inactivation in prostate epithelium that produced mPIN, we assessed prostates from 8-week-old TgAPT121 mice and control littermates for levels of proliferation and apoptosis. Previously, we showed that T121 induces aberrant proliferation accompanied by p53-dependent apoptosis when targeted to mammary gland (17) and choroid plexus (15, 16) epithelia. To determine whether the same mechanism was responsible for mPIN induction, prostates were analyzed in situ for proliferation by immunohistochemistry for phosphorylated histone H3, a marker of M phase. Use of this marker ensured that cell cycle activity, and not just E2F target gene expression, was quantified. Apoptosis was measured by TUNEL assays (Fig. 4). As in the other epithelial cells, pRb inactivation in prostate epithelium induced widespread aberrant proliferation (Fig. 4A and C, compare a and b) reflected by a 6-fold increase in mitotic index compared with normal. Results were the same when assessing Ki-67 positivity, a marker of S phase (data not shown). Increased proliferation was accompanied by a 5- to 6-fold increase in apoptosis compared with normal (Fig. 4B and C, compare f and g). These responses were limited to cells expressing the transgene and were thus induced cell autonomously.

Prostate epithelial apoptosis is regulated by Pten and not by p53. In cell types where p53-dependent apoptosis accompanies...
aberrant proliferation induced by pRb inactivation, tumor progression occurs on loss of p53, a result of the strong selective pressure for the suppression of apoptosis (15–17). To determine whether apoptosis was dependent on p53 in prostate, we generated TgAPT121; p53+/−/C0 and TgAPT121; p53/C0/C0 mice and quantified the prostate apoptotic index. In an important demonstration of cell specificity, the apoptotic index in prostate was unaffected by p53 inactivation (Fig. 4B and C, i and j). A clear 50% reduction in the apoptotic index in the Pten heterozygotes indicates that Pten regulates the apoptosis in prostate and further that apoptosis levels are highly sensitive to a reduction in the amount of Pten present. Such sensitivity could account for the apparent haploinsufficiency effect of Pten in mouse prostate lesions noted previously (23).

Proliferation rates in prostate epithelium were unaffected by p53 deficiency or Pten heterozygosity (Fig. 4A and C, a-e). Thus, inactivation of pRb function in prostate epithelium induces Pten-mediated apoptosis without affecting proliferation levels. The fact that proliferation induction, often associated with Pten inactivation, is not observed is entirely consistent with reports that such effects are mediated upstream of pRb (33). In TgAPT121 prostate, pRb function has been inactivated by T121; thus, upstream effects would be inconsequential.
Tumor progression and heterogeneity in TgAPT121 mice. Although the TgAPT121 adenocarcinomas did not become grossly invasive within 22 months of observation, microinvasion was present (Fig. 2A, e, arrow). This stage was accompanied by a desmoplastic host response (Fig. 2A, e, arrowhead) with loosening and separation of a widened stromal cell layer from neighboring epithelial cells. This condition often accompanies invasive cells and has been used as a criteria for the recognition of microinvasive lesions in genetically engineered mouse models (31).

By 16 weeks, TgAPT121 mice developed focally invasive well-differentiated adenocarcinomas characterized by groups of small, closely packed acini (Fig. 2A, f, arrow) that had broken through the basement membrane into the stroma beneath a luminal cell layer (Fig. 2A, f, arrowhead, and Fig. 3). In noninvasive lesions in TgAPT121 mice, the normally continuous smooth muscle layer is detectable by immunofluorescence for SMA (Fig. 2B, b, arrow). The invasive lesions of TgAPT121 mice show a desmoplastic stromal response, and the resulting breakdown of this layer can be visualized indirectly by the loss of staining for SMA (Fig. 2B, c, arrow). The fact that these more severe microinvasive populations and adenocarcinomas coexist in the prostate with the continued presence of mPIN (Fig. 3) means that selective changes are likely required for the progression from mPIN to a more advanced lesion. The multifocal composition of the prostates of TgAPT121 mice affords the opportunity to compare the underlying mechanisms producing each lesion.

Immunohistochemistry positivity for cytokeratin-8, a low molecular weight keratin present in most epithelia, including prostate (34), confirmed that the lesions of TgAPT121 mice were of luminal epithelial cell origin (Fig. 2B, a). Other prostate cancer mouse models using the full SV40 large T and small t antigens frequently develop both neuroendocrine carcinomas and adenocarcinomas (9, 11), the former of which only rarely becomes detectable by reduction of Pten levels. (Fig. 2A, f, arrowhead, and Fig. 3). In noninvasive lesions in TgAPT121 mice, the normally continuous smooth muscle layer is detectable by immunofluorescence for SMA (Fig. 2B, b, arrow). The invasive lesions of TgAPT121 mice show a desmoplastic stromal response, and the resulting breakdown of this layer can be visualized indirectly by the loss of staining for SMA (Fig. 2B, c, arrow). The fact that these more severe microinvasive populations and adenocarcinomas coexist in the prostate with the continued presence of mPIN (Fig. 3) means that selective changes are likely required for the progression from mPIN to a more advanced lesion. The multifocal composition of the prostates of TgAPT121 mice affords the opportunity to compare the underlying mechanisms producing each lesion.

Immunohistochemistry positivity for cytokeratin-8, a low molecular weight keratin present in most epithelia, including prostate (34), confirmed that the lesions of TgAPT121 mice were of luminal epithelial cell origin (Fig. 2B, a). Other prostate cancer mouse models using the full SV40 large T and small t antigens frequently develop both neuroendocrine carcinomas and adenocarcinomas (9, 11), the former of which only rarely becomes detectable by reduction of Pten levels. (Fig. 2A, f, arrowhead, and Fig. 3). In noninvasive lesions in TgAPT121 mice, the normally continuous smooth muscle layer is detectable by immunofluorescence for SMA (Fig. 2B, b, arrow). The invasive lesions of TgAPT121 mice show a desmoplastic stromal response, and the resulting breakdown of this layer can be visualized indirectly by the loss of staining for SMA (Fig. 2B, c, arrow). The fact that these more severe microinvasive populations and adenocarcinomas coexist in the prostate with the continued presence of mPIN (Fig. 3) means that selective changes are likely required for the progression from mPIN to a more advanced lesion. The multifocal composition of the prostates of TgAPT121 mice affords the opportunity to compare the underlying mechanisms producing each lesion.

The timing of appearance and persistence of prostate lesions is graphed as a function of time based on data presented in Table 1. H&E-stained prostate sections were examined and each sample was scored for the presence of each pathologic abnormality in the dorsal, ventral, and anterior lobes of TgAPT121 mice. TgAPT121;Pten+/− and Pten−/− prostates from mice at 6 to 12 weeks of age (n = 5, 5, and 4, respectively), 13 to 35 weeks of age (n = 10, 5, and 4, respectively), and >36 weeks of age (n = 18, 10, and 6, respectively). The earliest time examined was 6 weeks. TgAPT121 prostates progress from diffuse hyperplasia with interspersed dysplasia to mPIN at 12 weeks. Older mice show development of well-differentiated adenocarcinomas at 16 weeks. Pten heterozygosity accelerates the onset of mPIN and adenocarcinoma in TgAPT121 mice. In addition, morphologically distinct areas of adenocarcinoma progression also appear by 17 weeks. Pten−/− littermate prostates appear normal until 37 weeks when they show limited focal hyperplasia without atypia.

Figure 3. Prostate tumor progression in TgAPT121 mice is accelerated and diversified by Pten heterozygosity. The timing of appearance and persistence of prostate lesions is graphed as a function of time based on data presented in Table 1. H&E-stained prostate sections were examined and each sample was scored for the presence of each pathologic abnormality in the dorsal, ventral, and anterior lobes of TgAPT121 mice. TgAPT121;Pten+/− and Pten−/− prostates from mice at 6 to 12 weeks of age (n = 5, 5, and 4, respectively), 13 to 35 weeks of age (n = 10, 5, and 4, respectively), and >36 weeks of age (n = 18, 10, and 6, respectively). The earliest time examined was 6 weeks. TgAPT121 prostates progress from diffuse hyperplasia with interspersed dysplasia to mPIN at 12 weeks. Older mice show development of well-differentiated adenocarcinomas at 16 weeks. Pten heterozygosity accelerates the onset of mPIN and adenocarcinoma in TgAPT121 mice. In addition, morphologically distinct areas of adenocarcinoma progression also appear by 17 weeks. Pten−/− littermate prostates appear normal until 37 weeks when they show limited focal hyperplasia without atypia.

The invasive lesions of TgAPT121 mice were accelerated in TgAPT121;Pten−/− mice. By 8 weeks of age, TgAPT121;Pten−/− prostates displayed pronounced cribriform and tufting of epithelial cells (Fig. 5A), characteristics that do not appear in TgAPT121;Pten+/− mice until 12 weeks of age. These mPIN lesions increased in severity with age as the entire gland became filled with cribriform structures (Fig. 5B) or back-to-back glandular growth by 14 weeks (Fig. 5C). Thus, the onset of mPIN and adenocarcinoma in TgAPT121;Pten−/− mice was accelerated compared with that in TgAPT121 mice (Fig. 3; Table 1) likely due to the direct reduction of apoptosis (Fig. 3). However, prostate abnormalities unique to the TgAPT121;Pten−/− genotype also appeared. Pale cell carcinomas, so named for their high cytoplasmic-to-nuclear ratio, began to appear in the prostates of TgAPT121;Pten−/− mice at ~17 weeks. These atypical structures filled the lumen with a characteristic back-to-back glandular pattern and could arise adjacent to more hyperchromatic atypical glands (Fig. 5D). At 17 weeks, prominent large focal adenocarcinomas with abundant cytoplasm and abnormal glandular growth patterns also appeared (Fig. 5E). These large masses appeared to be focal expansions of atypical regions but displayed a completely distinct morphology. Along with areas of local microinvasion, TgAPT121;Pten−/− tumors have also been invasive to distant sites in the urogenital system, such as the seminal vesicle (Fig. 5F).

Selective Pten inactivation and activated Akt modulation contribute to prostate cancer heterogeneity. To determine if loss of functional Pten expression was responsible for the lesion progression observed in TgAPT121;Pten−/− prostates, we examined...
expression of Pten by immunofluorescence. We used this strategy rather than assaying for genomic loss due to the high frequency of Pten epigenetic inactivation observed in human cancers (36). TgAPT121 mice expressed Pten in prostate epithelium throughout their life span (data not shown), and TgAPT121;Pten+/− mice expressed Pten in the majority of prostate epithelial cells. However, focally progressed adenocarcinomas in the prostates of TgAPT121;Pten+/− mice lacked detectable Pten expression (Fig. 6A, a and b). The distinction was evident as the transition from one cell morphology to another directly correlated to Pten expression status (Fig. 6B, a and b). Thus, Pten heterozygosity not only leads to tumor acceleration but also increases the probability of complete Pten loss leading to dramatic changes in cellular and lesion morphology. This indicates that a threshold level of Pten is necessary for tumor suppression, with a myriad of effects possible on decreased Pten activity.

To determine whether levels of Pten protein correlated with functional activity, we examined the presence and localization of p-Akt using immunofluorescence. Because Akt is a downstream target of the Pten-regulated phosphatidylinositol 3-kinase (PI3K) pathway, its phosphorylation is an indicator of Pten loss. Interestingly, although p-Akt was not detectable in nontransgenic prostates (Fig. 7A, a), cytoplasmic p-Akt was induced in Pten-positive lesions of TgAPT121;Pten+/− (Fig. 7A, b) and TgAPT121;Pten+/− (data not shown) mice, possibly indicating an intermediate level of Akt activation on pRb inactivation. However, in focally progressed lesions of TgAPT121;Pten+/− prostates that showed loss of Pten expression, p-Akt was detectable specifically associated with cell membranes (Fig. 6B, c). This staining resembles that observed in focally abnormal regions of Pten+/− prostates at ∼1 year of age (Fig. 7A, c), also coincident with loss of Pten expression (data not shown). The appearance of membrane-associated p-Akt in TgAPT121;Pten+/− prostates was associated with a dramatic further reduction in apoptosis compared with that in surrounding Pten-positive glands (Fig. 7B and C). In contrast, there was no significant change in proliferation rates based on Ki-67 staining (data not shown). This is consistent with the fact that epithelial T121 expression is sustained throughout tumor progression (data not shown). Thus, the transition from Pten hemizygosity to Pten loss occurs consistently in the natural evolution of these prostate cancers, likely due to a selective reduction in apoptosis, and correlates

![Image](cancerres.aacrjournals.org)
with membrane localization of p-Akt. It is likely that, once Pten is lost, effects in addition to apoptosis reduction contribute to tumor progression in that such loss is associated with distinct morphologic changes and increased invasiveness, contributing to the heterogeneity of tumors initiated by pRb loss.

Discussion

We directly assessed the effect of complete pRb functional inactivation in murine prostate epithelium by cell-specific expression of T121 in transgenic mice. T121, an N-terminus fragment of SV40 large T antigen, binds to and inactivates pRb.

Table 1. Phenotype summary of TgAPT121 and TgAPT121;Pten<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Age (wk)</th>
<th>n</th>
<th>Dyp (%)</th>
<th>mPIN (%)</th>
<th>WD adeno (%)</th>
<th>Phy (%)</th>
<th>Also present</th>
</tr>
</thead>
<tbody>
<tr>
<td>APT121</td>
<td>6-12</td>
<td>5</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-36</td>
<td>10</td>
<td>100</td>
<td>100</td>
<td>70</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;36</td>
<td>18</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>44</td>
<td>22% SCH*</td>
</tr>
<tr>
<td>APT121;Pten&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6-12</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>60</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-36</td>
<td>5</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>90% PA&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>&gt;36</td>
<td>8</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>33</td>
<td>100% PA&lt;sup&gt;†&lt;/sup&gt;, 5% SCH*</td>
</tr>
<tr>
<td>Pten&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>6-12</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-36</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;36</td>
<td>6</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Isolated hyperplasia&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

NOTE: Dyp, diffuse dysplasia with nuclear atypia (nuclear enlargement and condensed chromatin) with focal stromal hyperplasia; mPIN, characterized by atypical cells showing tufting, micropapillary, and cribriform growth patterns. Further atypical progression includes nuclear elongation, higher hyperchromatism, and increased mitotic and apoptotic rates. WD adeno, well-differentiated adenocarcinoma; invasive neoplastic state with increased quantity of small back-to-back glands made of atypical cells, less hyperchromatism than mPIN, and a more rounded morphology. Phenotype is accompanied by penetration of cells through fibromuscular layer into the surrounding stroma and may exhibit a desmoplastic response. Phy, phyllodes-like tumor, neoplastic mass composed of epithelia and a characteristically large stromal cell involvement.

*SCH, small cell hyperplasia.
†Pale cell carcinoma.
‡PA, adenocarcinoma progressions exhibiting loss of Pten expression.
§Focal hyperplasia without nuclear atypia.
p107, and p130, eliminating the problem of redundancy and compensation by p107 and/or p130. We show that pRb inactivation is sufficient to induce aberrant proliferation and abundant apoptosis in prostatic luminal epithelial cells, the target of the probasin promoter used to drive transgene expression. These activities both cause the development of mPIN and establish the selective pressure for tumor progression. Within their natural lives, TgAPT121 male mice display slow progression to well-differentiated prostate adenocarcinoma. However, hemizygosity for Pten both accelerates this overall phenotype by a direct reduction in apoptosis and facilitates further tumor heterogeneity via selective Pten inactivation. This work establishes several important points. First, the TgAPT121 mouse likely models the initiation of prostate cancer when pRb pathway aberrations are present; such changes are observed with high frequency in early stages of the human disease (see Introduction). Second, the specific pathway(s) affected during tumor progression is dictated by the selective pressures imposed by the cellular responses to early pRb functional loss. In the prostate epithelium, Pten-regulated apoptosis is the major tumor suppression response. However, the specific pathway managing this apoptotic response is cell specific; in other epithelial cell types analyzed previously, apoptosis is p53 dependent. Third, with Pten hemizygosity resulting in a 50% reduction in apoptosis, with further reduction on Pten loss, there is a strong selective pressure for Pten inactivation in prostate cancer evolution. Finally, significant tumor heterogeneity can be generated by alterations in Pten levels alone once neoplasia is initiated by cell cycle disruption. Given the high frequency of Pten alterations associated with advanced prostate lesions in humans, this mechanism is likely to play a role in the generation of prostate cancer heterogeneity.

Inactivation of pRb, p107, and p130 initiates prostate cancer and the predisposition to disease progression. It has been difficult to obtain direct evidence for the consequences of full pRb functional inactivation in the prostate due to functional redundancy and compensation by p107 and/or p130 and because of embryonic lethality of Rb and combined nullizygotes (reviewed in ref. 37). Indeed, all three proteins seem to be expressed in the prostate (38). Studies focusing on just pRb inactivation used embryonic prostatic rescue where tissue recombinants were created by grafting pelvic organ rudiments from Rb−/− mouse embryos to wild-type rat urogenital mesenchyme (rUGM) under

Figure 6. Tumor progression with selective loss of Pten and activation of membrane-associated Akt. A, a representative region of focal adenocarcinoma progression (a) adjacent to dysplastic cells (arrows) in TgAPT121;Pten+/− prostate. Immunofluorescence for Pten in an adjacent section (b) shows that Pten is expressed in dysplastic cells (arrows) with loss of Pten expression in adjacent adenocarcinoma. Green, Pten signal; red, DAPI; yellow, merged image. B, a representative H&E-stained section showing a transition where focal adenocarcinoma develops from neoplastic areas with back-to-back gland structure is shown in (a). Immunohistochemistry (IHC) for Pten (b; red/brown) shows loss of cytoplasmic staining in regions of focal progression. In such regions of Pten loss, cells express membrane-bound p-Akt staining (c; green).
the renal capsule of adult male nude mouse hosts (13). Forty percent of non-hormone-treated grafts developed focal hyperplasia; progression to PIN and adenocarcinoma was noted only after stimulation with the hormones testosterone and 17β-estradiol. However, similar hormone treatment alone has been shown to induce dysplasia in Noble rats (39). Recently, prostate epithelium-specific deletion of conditional Rb-null alleles (14) was shown to induce limited hyperplasia without PIN. Compared with those studies, our findings show that, in addition to pRb deficiency, inactivation of p107 and/or p130 leads to the development of a much more severe phenotype than pRb deletion alone, indicating functional redundancy or compensation in prostate epithelium.

A new model of prostate cancer initiation. Several transgenic mouse models have been generated in which SV40 large T antigen is expressed in prostate epithelium, often along with small t antigen (9, 11, 40, 41). Although these models have been successful in producing prostate lesions that initially resemble the human disease, thus providing important insight, they have not been useful for addressing roles of individual lesions in prostate tumorigenesis. This is because, in addition to inactivating the pRb, large T antigen inactivates the p53 tumor suppressor and also binds p300. Moreover, transforming functions not yet fully understood have been reported (reviewed in ref. 12). In addition, small t antigen further disrupts PP2A function.

Here, we use a small (121 amino acids) defined fragment of SV40 large T antigen to dominantly inactivate pRb, p107, and p130, which offers the unique ability to study the consequences of complete loss of pRb function without additional effects of large T antigen or small t antigen. We show that the inactivation of pRb/p107/p130 is sufficient to induce widespread prostate epithelial proliferation, accompanied by apoptosis, and the development of mPIN without the need for secondary alterations. Thus, cell-specific expression of T121 produces a strong single-allele initiation event for prostate tumorigenesis and offers the ability to study the molecular events necessary to facilitate disease progression.

Another significant finding in our studies is that the neuroendocrine phenotype prevalent in other SV40-based models (9, 11) is not observed in TgAPT121 or TgAPT121;Pten+/− mice (data not shown).
shown), indicating that functions of large and/or small antigen independent of pRb and p53 inactivation are involved in this phenotype. That the model we describe does not develop neuroendocrine tumors is significant, because the extensive formation of neuroendocrine tumors is not reflective of the human disease (35).

**Lesion heterogeneity in TgAPT121 mice.** Advanced human prostate carcinomas are generally multifocal and heterogeneous, with lesions of various stages and grades occurring in close proximity to each other. This feature represents a major impediment to accurate diagnoses and therapeutic design. Furthermore, such heterogeneity compounds the difficulty of understanding human cancer, because the progressive stages of human cancer are often not accessible and are rarely available from a single individual, and biopsy alone cannot sample the extent of genomic changes as they correlate to histologic grade or clinical outcome. Defining the precise roles that genomic changes play in prostate cancer is imperative in understanding and managing this disease but requires an appropriate animal model in which cancer initiation reflects the events observed in humans.

We show here that the TgAPT121 model offers an avenue for exploring the molecular and cellular basis for heterogeneity observed in humans. In human cancer, because pRb pathway alteration likely precedes many events explored previously in the mouse, the outcome of these events in the absence of pRb pathway effects may not reflect the true nature of their development in humans. Because these lesions develop slowly over time, the TgAPT121 model affords the opportunity to study how these alterations naturally evolve to cause tumor heterogeneity by analyzing distinct pathologic stages. For example, when TgAPT121 males are aged, in addition to prostate hyperplasias, dysplasias, mPIN, and adenocarcinomas, some prostates develop small cell hyperplasias as well as "phylloides-like" tumors, characterized by both epithelial and stromal proliferation. These lesions occur adjacent to one another, allowing the opportunity to compare the underlying mechanisms producing each phenotype. Additionally, the TgAPT121 mouse can be bred to a variety of genetic backgrounds to determine which events in combination produce tumors most like those seen in humans, in the process generating preclinical animal models that are based on early events observed in human prostate cancer. Such studies should facilitate future preclinical diagnostic and therapeutic analyses.

**Roles of Pten.** In this report, we addressed the effect of Pten alteration along with or subsequent to inactivation of pRb as it seems to occur in human prostate cancer. We initially focused on Pten for three reasons. First, we show here that Pten regulates the apoptosis accompanying the proliferative response to pRb functional loss in prostate epithelium. Thus, selective pressure for compromised Pten function is imposed by inactivation of pRb. Second, Pten mutation or reduction in expression is observed in the majority of advanced human prostate cancers but not in early lesions (see Introduction). In fact, heterogeneity in Pten expression levels within tumors is observed frequently in advanced prostate cancers, with complete loss correlating to increased tumor grade (42). Finally, because alteration of Pten is common in advanced prostate cancer, several laboratories have recently examined the effects of reduced or inactivated Pten function in mouse prostate, although not in the context of pRb function loss (21–25, 43). These studies clearly show that Pten loss, and indeed reduced Pten levels, can have a significant causal effect in prostate tumorigenesis. However, they do not address the selective pressures that lead to such changes, nor do they define the effect of Pten changes with preexisting early changes found in human lesions.

Prostate hyperplasia and carcinoma have been reported in Pten+/− mice alone (29, 43, 44). Although the frequencies may vary due to classification differences and the use of different background strains, it is clear that Pten heterozygous male mice develop prostate hyperplasia or mPIN late in life (~9–14 months). Based on pathologic guidelines proposed by the Mouse Models of Human Cancer Consortium Prostate Pathology Committee (31), the Pten+/− cohort in the current study developed focal hyperplasia at ~10 months with no occurrences of mPIN as late as 17 months. Previously, the Pten+/− phenotype was shown to be enhanced in p27-null mice, such that latency was changed from mPIN appearance at 9 months to carcinoma at 3 months (25). In TgAPT121 mice, where pRb function is inactivated in prostate epithelium, Pten heterozygosity led to a 50% reduction in apoptosis and an accelerated onset of mPIN, establishing a clear mechanism for haploinsufficiency effects. In the TRAMP model, Pten heterozygosity accelerates tumorigenesis at a significantly faster rate than TRAMP mice with two Pten alleles, with Pten loss of heterozygosity showing further acceleration (23). Because we show that apoptosis induced by pRb inactivation in prostate is unaffected by p53 inactivation (as would be elicited by T antigen), it is likely that the mechanism for Pten haploinsufficiency that we define here for TgAPT121 mice (apoptosis reduction) is the same in TRAMP. Studies with a hypomorphic Pten allele also show that levels of Pten function show distinct effects on prostate lesions with lower levels resulting in more advanced lesions (22). Instead of mPIN at 12 months as seen in Pten+/− mice, Pten+/null mice developed mPIN at 2 to 3 months. This is also when mPIN is detected in TgAPT121:Pten+/− mice.

In addition to the acceleration of prostate abnormalities by Pten hemizygosity in association with reduced apoptosis, the selective pressures likely imposed by the remaining Pten-dependent apoptosis led to a high frequency of localized Pten loss. Loss of Pten subsequent to loss of pRb function generates further tumor heterogeneity in TgAPT121:Pten+/− prostates, with an additional reduction in apoptosis along with significant morphologic changes and increased invasiveness. Loss of Pten expression resulted in membrane localization of p-Akt, a known consequence of increased phosphatidylinositol 3,4,5-triphosphate (PIP3) resulting from Pten inactivation (ref. 45; Fig. 8). In cultured cells, activation of Akt can inhibit apoptosis by several mechanisms, including phosphorylation of the proapoptotic forkhead and activation of pro-caspase-9 (46). Whether Akt activation also accounts for the morphologic and invasive changes associated with selective inactivation of Pten will require further experimentation, including the combined derivation of TgAPT121 primary prostate cultures and use of pathway inhibitors.

Recently, prostate epithelial–specific homozygous deletion of Pten alone was shown to induce hyperplasia by 4 weeks of age and mPIN by 6 weeks (21). Activation of Akt by Pten inactivation can trigger proliferation through the induction of cyclin D (47), which, in complex with cyclin-dependent kinase (CDK) 4/6, inactivates pRb (33), or inhibition of p27, which suppresses cyclin E/CDK2 activity (48). Thus, experimental inactivation of Pten inactivation in prostate epithelium may trigger a proliferative response similar to that seen in our studies with pRb functional inactivation, which is downstream of each of these targets. It is also possible that the genetic background contributed to hyperplastic induction in that
This study, pRb functional inactivation by T121 in prostate epithelium leads to increased cellular proliferation, an apoptotic response, and neoplastic growth that gives rise to mPIN. Over time, well-differentiated adenocarcinomas develop. The apoptosis induced by pRb inactivation is dependent on Pten function, and hemizygous reduction of Pten results in a 50% reduction in apoptosis and acceleration of PIN and adenocarcinoma development. In sporadic human cancers, cells with reduced Pten levels resulting from allelic mutation or silencing would thus contribute to tumor progression by accelerating lesion growth and the time to progression. A further increase in tumor heterogeneity ensues when selective pressures (i.e., the remaining Pten-dependent apoptosis) favor the expansion of cells lacking Pten. Loss of Pten further activates PI3K- and PIP3-regulated pathways as evidenced by membrane localization of p-Akt, resulting in decreased apoptosis, morphologic change, and increased invasiveness.

Figure 8. Model for prostate tumor progression when initiated by pRb inactivation. As shown in this study, pRb functional inactivation by T121 in prostate epithelium leads to increased cellular proliferation, an apoptotic response, and neoplastic growth that gives rise to mPIN. Over time, well-differentiated adenocarcinomas develop. The apoptosis induced by pRb inactivation is dependent on Pten function, and hemizygous reduction of Pten results in a 50% reduction in apoptosis and acceleration of PIN and adenocarcinoma development. In sporadic human cancers, cells with reduced Pten levels resulting from allelic mutation or silencing would thus contribute to tumor progression by accelerating lesion growth and the time to progression. A further increase in tumor heterogeneity ensues when selective pressures (i.e., the remaining Pten-dependent apoptosis) favor the expansion of cells lacking Pten. Loss of Pten further activates PI3K- and PIP3-regulated pathways as evidenced by membrane localization of p-Akt, resulting in decreased apoptosis, morphologic change, and increased invasiveness.

In the Wang et al. study, widespread prostate-specific Pten inactivation induced invasive carcinoma by 9 weeks with metastasis to lymph nodes and lung observed by 12 to 29 weeks in ~50% of mice. In the current study, by 17 weeks of age, TgAPT121;Pten+/- Pten-positive adenocarcinomas progressed to morphologically distinct and invasive Pten-deficient lesions that expressed membrane-localized p-Akt. As with experimentally induced Pten inactivation, cells within these lesions were enlarged with a large cytoplasmic content and grew in disorganized patterns. Although such lesions were clearly invasive, obvious metastases have not been observed up to 68 weeks of age. Because these lesions presumably progress from a single cell that inactivates Pten, more time may be required to develop metastasis compared with prostates in which Pten is inactivated at an early age in a large fraction of cells. Alternatively, the genetic background may influence the frequency and/or timing of metastasis.

Cell specificity in responses to pRb family protein loss dictates distinct paths for tumor progression. This report shows the critical importance of cell type specificity in cancer mechanisms. We showed previously that pRb/p107/p130 inactivation in two distinct epithelia, mammary gland (17) and brain choroid plexus (15, 16), induced aberrant proliferation and apoptosis as shown here for prostate epithelium. However, in these tissues, apoptosis is p53 dependent and tumors progress with selective inactivation of p53. The apoptosis induced in the suppression of prostate tumor growth is not p53 dependent but instead regulated by Pten. Thus, although the biological effect of pRb inactivation is similar in all three epithelial cell types, the pathways on which selective pressure is imposed is distinct in prostate. Although some studies have suggested that Pten may be a downstream transcriptional target of p53 (50), our study shows that this is not the case in prostate in vivo. If Pten were inducing apoptosis through activation of p53, a deficiency in either factor would result in reduced apoptosis. Loss of p53, however, had no effect on apoptosis levels, clearly distinguishing the effects of these two tumor suppressors. Thus, because Pten and p53 regulate distinct pathways, their loss will yield distinct characteristics for tumor progression, including both the tumor cell and the microenvironment changes that impose selective pressures for subsequent events. Inherent in this observation of cell specificity is the notion that targeted diagnostic and therapeutic development be based on the molecular evolution of a given cancer and not extrapolated from mechanisms uncovered in unrelated tissues or cell types. For prostate cancer, the effects of pRb inactivation and subsequent progression to Pten inactivation that occur in the TgAPT121;Pten+/- model should provide a useful preclinical model with which to both explore mechanisms of tumor progression and develop clinically relevant translational studies.

Acknowledgments

Received 5/10/2005; revised 6/27/2005; accepted 8/16/2005.

Grant support: National Cancer Institute grant 5-RO1CA46283 (T. Van Dyke).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 USC Section 1734 solely to indicate this fact.

We thank Huoy Lim and Shannon Meyer for expert technical assistance, the University of North Carolina at Chapel Hill Histopathology Core Facility for processing slides used in these studies, the University of North Carolina at Chapel Hill Division of Laboratory Animal Medicine for excellent animal care, and the members of the Van Dyke laboratory for many insightful discussions.
References


Heterogeneous Tumor Evolution Initiated by Loss of pRb Function in a Preclinical Prostate Cancer Model

Reginald Hill, Yurong Song, Robert D. Cardiff, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/22/10243

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2005/11/14/65.22.10243.DC1

Cited articles
This article cites 50 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/22/10243.full#ref-list-1

Citing articles
This article has been cited by 14 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/22/10243.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.