Deficiency of pRb Family Proteins and p53 in Invasive Urothelial Tumorigenesis

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Abstract

Defects in pRb tumor suppressor pathway occur in ~50% of the deadly muscle-invasive urothelial carcinomas in humans and urothelial carcinoma is the most prevalent epithelial cancer in long-term survivors of hereditary retinoblastomas caused by loss-of-function RB1 mutations. Here, we show that conditional inactivation of both RB1 alleles in mouse urothelium failed to accelerate urothelial proliferation. Instead, it profoundly activated the p53 pathway, leading to extensive apoptosis, and selectively induced pRb family member p107. Thus, pRb loss triggered multiple fail-safe mechanisms whereby urothelial cells evade tumorigenesis. Additional loss of p53 in pRb-deficient urothelial cells removed these p53-dependent tumor barriers, resulting in late-onset hyperplasia, umbrella cell nuclear atypia, and rare-occurring low-grade, superficial papillary bladder tumors, without eliciting invasive carcinomas. Importantly, mice deficient in both pRb and p53, but not those deficient in either protein alone, were highly susceptible to subthreshold carcinogen exposure and developed invasive urothelial carcinomas that strongly resembled the human counterparts. The invasive lesions had a marked reduction of p107 but not p130 of the pRb family. Our data provide compelling evidence, indicating that urothelium, one of the slowest cycling epithelia, is remarkably resistant to transformation by pRb or p53 deficiency; that concurrent loss of these two tumor suppressors is necessary but insufficient to initiate urothelial tumorigenesis along the invasive pathway; that p107 may play a critical role in suppressing invasive urothelial tumor formation; and that replacing/restoring the function of pRb, p107, or p53 could be explored as a potential therapeutic strategy to block urothelial tumor progression. [Cancer Res 2009;69(24):OF1–9]

Introduction

Urothelial carcinoma presents an interesting paradigm of tumor initiation and progression via divergent phenotypic and molecular pathways (1–6). About 70% of the carcinomas arise as low-grade, papillary tumors that are confined to the urothelial compartment. These tumors often occur at multiple loci in the bladder, and despite surgical removal and perioperative chemotherapy, they recur time and again over the lifetime of the afflicted individuals. However, the chance for these tumors to advance to the muscle-invasive stage is relatively small and the 5-year survival rate approaches 95% (7, 8). The rest (∼30%) of the urothelial carcinomas are high-grade and muscle-invasive at diagnosis. In spite of radical cystectomy in conjunction with debulking chemotherapy and/or radiotherapy, >50% of these tumors eventually spread to distant organs. The 5-year survival rate for patients with distant metastasis is only ∼6% (9). Longitudinal studies indicate that most of the muscle-invasive urothelial carcinomas have no prior history of low-grade superficial papillary tumors and they may have arisen de novo or have derived from flat, high-grade carcinoma in situ lesions (10, 11). Therefore, the two major urothelial carcinoma variants do not appear to represent a continuum of tumor progression from early to late stages but rather they seem to result from distinct mechanisms of tumor initiation (1–6).

Emerging evidence from humans and animal models suggests that two distinct sets of genetic alterations drive urothelial tumorigenesis along divergent pathways. In human low-grade, noninvasive urothelial carcinomas, gain-of-function mutations of ras pathway components, particularly ras itself or its upstream-acting fibroblast growth factor receptor 3b, are exceedingly common (4, 12). Mutations of these two genes seem always mutually exclusive (13); together, they account for up to 90% of this urothelial tumor variant (2). Consistent with this, urothelium-specific expression of a constitutively active Ha-ras oncogene in transgenic mice elicits urothelial hyperplasia, approximately half of which evolves, over a 28-month period, to low-grade, superficial papillary carcinomas that bear strong resemblance to the human counterparts (14). Doubling the transgene dosage in the same transgenic line dramatically shortens the tumor latency, provoking early-onset urothelial carcinomas without triggering tumor invasion (15). Finally, human patients with Costello syndrome, which is caused by germ-line mutations in the Ha-ras gene, are prone to developing early-onset, low-grade, noninvasive urothelial carcinomas (16). Collectively, these data indicate that overactivation of the ras signaling pathway is a principal cause of low-grade, noninvasive urothelial carcinomas.

Much less is known about what triggers the muscle-invasive urothelial carcinomas despite their high mortality rate. Among the numerous genomic, genetic, and epigenetic alterations, those affecting RB1 and p53 tumor suppressor genes are by far the most common (17). Although inactivating mutations of RB1 are rare, reduced or loss of RB1 expression accounts for 40% to 50% of the invasive carcinomas and is strongly associated with poor clinical outcome. Interestingly, long-term survivors of hereditary...
retinoblastomas that harbor RB1 mutations were highly susceptible to developing high-grade urothelial carcinomas (18). As for p53, its loss-of-function mutations occur in up to 60% of the muscle-invasive urothelial carcinomas (19) and are often associated with disease progression (20). Furthermore, pRB and p53 abnormalities coexist in 40% to 50% of the muscle-invasive urothelial carcinomas; together, they predict a more aggressive tumor behavior and poorer patient survival than carcinomas bearing abnormalities in only one gene (21–23). Therefore, defects in pRB and p53 have been synonymous with, and have been speculated to play crucial roles in, the invasive urothelial tumorigenesis.

Despite the strong clinical correlation, little experimental evidence exists to either prove or refute the presumed importance of pRB and/or p53 deficiency in invasive urothelial tumorigenesis. Mice with RB1 ablated in all tissues die embryonically (24), thus precluding studies on whether RB1 deficiency is tumorigenic for urothelium. The lethality also makes it impossible to decipher how other members of pRB (pocket) family including p107 (RB2) and p130 (RB3) respond to pRB loss in the urothelium. Mice globally deficient for p53 survive to term, but they succumb to thymic lymphomas and soft-tissue sarcomas ages 3 to 7 months when urothelial cells remain normal (25). Because of these constraints, it has been impossible to discern whether urothelial defects of pRB or p53 are tumor-initiating or are merely tumor-promoting. It is also unclear whether these two genetic defects intersect at certain point of the multistage urothelial tumorigenesis. Although urothelial expression of an SV40 large T antigen in transgenic mice elicited urothelial carcinoma in situ and invasive carcinomas (26, 27), it cannot be ruled out that this was due to the broad effects of this oncogene on inactivating not only pRB and p53 but also pRB family members p107 and p130 (28).

To address these issues, we ablated RB1 and p53 genes alone or in a combination in mouse urothelia, taking advantage of our urothelium-specific knockout system and the availability of loxP-flanked (“floxed”) RB1 and p53 transgenic mice (29, 30). We studied the urothelial responses to the loss of these tumor suppressors and the tumorigenic potential of these genetic defects under normal conditions and carcinogenic stress. Our findings shed light on the combinatorial factors necessary for driving the invasive urothelial tumorigenesis and the cell type specificity and context dependence of tumor suppressor deficiency.

Materials and Methods

Generation and characterization of conditional knockout mice. UPII-Cre transgenic mice that expressed Cre recombinase in urothelium-specific manner (31) were crossed with “floxed” RB1 mice where exon 19 was floxed with two loxP sites (29), and additional crosses produced homozygous mice for both UPII-Cre and “floxed” RB1 alleles. UPII-Cre mice were also crossed with “floxed” p53 mice, where exons 5 and 6 were floxed by two loxP sites (30). Further crosses produced homozygous mice for both UPII-Cre and “floxed” p53 alleles. The two double transgenics were intercrossed to produce homozygous mice for all three alleles (UPII-Cre, “floxed” p53, and “floxed” RB1). Genotyping of UPII-Cre transgene was done with Southern blotting and that for “floxed” RB1 and p53 alleles with PCR (29, 30). Four groups of mice were used: (a) UPII-Cre only mice, (b) UPII-Cre/“floxed” RB1 (RB1+/−), (c) UPII-Cre/“floxed” p53 (p53−/−), and (d) UPII-Cre/“floxed” p53/“floxed” RB1 (RB1+/−/p53−/−). Cre-mediated recombination of the “floxed” genes was assessed on DNA and RNA levels using PCR. All animal experiments were conducted in accordance with regulations for the Humane Use of Animals for Scientific Research and under active protocols approved by Institutional Animal Care and Use Committee.

Results

Loss of pRB function in mouse urothelium is not tumorigenic, owing to a compensatory rescue by multiple secondary tumor defenses. Despite a close association of pRB deficiency with advanced human urothelial carcinomas, it was unknown whether pRB deficiency alone can transform the urothelial cells. By expressing Cre recombinase under the control of a mouse ur- oplakin II promoter (UPII-Cre) in mice where exon 19 of the RB1 gene or cDNA corresponding to exons 5 and 6 of the p53 gene. The cDNAs were transcribed into antisense and sense cRNAs in the presence of digoxigenin-conjugated UTP. Mouse bladders were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm thick) were digested in protease K for 15 min, incubated with the probes for 16 h, reacted with anti-digoxigenin antibody conjugated with alkaline phosphatase, and developed in a nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate solution (Hoffmann-La Roche).

Quantitative real-time PCR. The expression of pRB and p53 pathway effectors was assessed by real-time PCR using a LightCycler RNA Amplification kit (Roche Diagnostics). Double-stranded urothelial cDNA was used for PCR at 95°C for 15 min for the first cycle, 95°C for 15 s, 53°C to 58°C for 20 s, and 72°C for 30 s for 50 cycles. The products were detected by direct incorporation of SYBR Green I into newly synthesized DNA. The relative abundance was expressed as a ratio to β-actin.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Deparaffinized sections of mouse urinary bladders were incubated in a DNA labeling solution (APO-BrdU TUNEL Assay Kit; Invitrogen) for 1 h at 37°C. The sections were then incubated with an anti-bromodeoxyuridine solution for 30 min.

Western blot analysis. Urothelial proteins were dissolved in a lysis buffer containing 10% SDS, 20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 5 mmol/L β-mercaptoethanol, and a cocktail of protease inhibitors. Proteins (60 μg) were resolved by SDS-PAGE, electrotherophoretically transferred onto polyvinylidene fluoride membrane, incubated with primary antibodies and then with peroxidase-coupled secondary antibodies, and developed using an enhanced chemiluminescent method (Amersham Biosciences). The primary antibodies were anti-E2F1 and anti–casepase-3 (Cell Signaling Technology); anti-MAD2 (ProteinTech Group); anti-p19, anti-p53, anti-p21 (Abcam); and anti-p107, anti-p130, anti-E2F4, anti-MDM2, anti-Bak, and anti-Bax (Santa Cruz Biotechnology). Antibody against mitogen-activated protein kinase (Cell Signaling Technology) served as a loading control.

Carcinogen treatment of knockout mice. Five groups of mice (4–5 months old) were treated with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN; TCI America): (a) UPII-Cre (n = 23), (b) RB1−/− (n = 16), (c) p53−/− (n = 20), (d) RB1−/−/p53−/− (n = 8), and (e) RB1−/−/p53−/− (n = 22). BBN was supplied ad libitum in the drinking water (final concentration = 0.01%) for 10 weeks. No difference in water consumption was observed among different groups.

Immunohistochemistry. Deparaffinized bladder sections were micro- waved in citrate buffer (pH 6.0) for 20 min to unmask the antigens and then incubated with primary and secondary antibodies conjugated with horse-radish peroxidase and developed in a solution containing hydrogen pero- xide and 3,3′-diaminobenzidine tetrahydrochloride. The primary antibodies were those for Western blotting and also included those against Ki-67 (Abcam), 34βE12 (Dako), E-cadherin (Santa Cruz Biotechnology), and matrix metalloproteinase-9 (R&D Systems).

In situ hybridization. Site-specific probes were used to determine the truncation of RB1 and/or p53 in urothelium. Briefly, reverse transcription-PCR amplified the cDNA corresponding to exon 19 of the RB1 gene or cDNA corresponding to exons 5 and 6 of the p53 gene. The cDNAs were transcribed into antisense and sense cRNAs in the presence of digoxigenin-conjugated UTP. Mouse bladders were fixed in 4% paraformaldehyde and embedded in paraffin. Sections (4 μm thick) were digested in protease K for 15 min, incubated with the probes for 16 h, reacted with anti-digoxigenin antibody conjugated with alkaline phosphatase, and developed in a nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate solution (Hoffmann-La Roche).

urothelial-specific truncation of RB1 (Fig. 1B, left). *In situ* hybridization using a deletion site–specific riboprobe (corresponding to the exon 19) detected no mRNA of the wild-type RB1 in any urothelial layer of the RB1−/− mice (Fig. 1B, bottom right) as opposed to the RB1+/+ mice where RB1 was detected in all layers (Fig. 1B, top right). Consistent with the previous finding that deletion of exon 19 is functionally equivalent to inactivation of the entire RB1 gene (29), urothelial truncation of RB1 led to a marked induction of pRb down-stream effectors E2F1 and MAD2 (32), as evidenced by real-time reverse transcription-PCR (C, left), Western blotting (C, right), and immunohistochemistry (D) of pRb effectors, showing induction of E2F1 and MAD2 in the RB1−/− mice. Mean and SD from 8 mice for each genotype (C, left). L, bladder lumen. All panels are of the same magnification; bar, 50 μm (D, top left).

Unexpectedly, urothelial pRb inactivation failed to elicit hyper-proliferation or tumorigenesis after 28 months of follow-up of a cohort of 80 RB1−/− mice. The urothelia of the null mice were not thickened (Fig. 2A, top right) compared with the wild-type control (Fig. 2A, top left) and lacked expression of proliferative marker Ki-67. Instead, the pRb-deficient urothelial cells, particularly those in the superficial layer, exhibited condensed nuclei and increased intercellular space (Fig. 2A, top right). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay and anti-caspase-3 staining revealed many apoptotic cells in pRb-deficient urothelium (Fig. 2A). These data provide compelling evidence that not only did RB1 deficiency fail to induce urothelial proliferation or tumorigenesis, it also triggered apoptosis.

To determine the molecular mechanism(s) whereby RB1 deficiency led to the above consequences, we evaluated the key components of the p53 pathway and the pRb family. We found p19, p53, and p21 to be upregulated in the RB1-deficient urothelial cells (Fig. 2B-D), with p21 protein increase being most dramatic (Fig. 2C). Proapoptotic molecules Bak and Bax and activated caspase fragments were also markedly upregulated (Fig. 2C), thus explaining the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling results (Fig. 2A). Additionally, we observed a significant induction of p107, but not p130, albeit both members of the pRb family (Fig. 2B-D). Coinciding with the p107 induction was the increased expression of E2F4, which binds p107 and acts as a transcriptional repressor/growth inhibitor (33). These findings unravel several fail-safe mechanisms with which the urothelial cells escape tumorigenesis during pRb deficiency.

**Figure 1.** Conditional inactivation of RB1 in mouse urothelium. A, targeting strategy. Top, UPII-Cre transgene in which a mouse uroplakin II promoter restricts Cre recombinase expression to urothelium; middle, RB1 allele in which its exon 19 was flanked by two loxP sites (filled triangles); bottom, recombined RB1 allele with exon 19 deleted on urothelial Cre expression. Arrows, primers for detecting RB1 truncation. B, left, PCR detection of truncated RB1 in urothelium. Note a 260-bp truncated (Δ) RB1 DNA and a 200-bp truncated RB1 mRNA in UPII-Cre/Cre/RB1lox/lox transgenic mice (or RB1−/− mice; lanes 3 and 4) and the absence of such truncated species in UPII-Cre/Cre only mice (or RB1+/+; lanes 1 and 2). B, right, *in situ* hybridization. Antisense cRNA probe corresponding to exon 19 of RB1 reacted with urothelia of a RB1+/+ mouse (top) but not that of a RB1−/− mouse. C and D, real-time reverse transcription-PCR (C, left), Western blotting (C, right), and immunohistochemistry (D) of pRb effectors, showing induction of E2F1 and MAD2 in the RB1−/− mice. Mean and SD from 8 mice for each genotype (C, left).
Additional loss of p53 in RB1-deficient urothelial cells blunts p53-dependent responses and elicits late-onset urothelial hyperproliferation. Because pRb deficiency activated the p53 pathway and urothelial apoptosis, we sought to dampen such “rescuing” effects to promote urothelial proliferation. We crossed the UPIICre/Cre/RB1flox/flox with another mouse strain where exons 5 and 6 (encoding the DNA-binding domain) of the p53 gene were flanked with two loxP sites (Fig. 3A, left; ref. 30). Additional intercrosses among the littermates yielded mice deficient for RB1 (RB1^{−/−}), p53 (p53^{−/−}), or both (RB1^{−/−}/p53^{−/−}) in the urothelia. PCR (Fig. 3A, right) and in situ hybridization (Fig. 3B) proved urothelial truncation of p53 and/or RB1 DNA and RNA. The functional effect of p53 deficiency was evident based on significant reduction of p53 downstream effectors p21 and MDM2 (Fig. 3C). Like pRb deficiency, p53 deficiency alone did not enhance urothelial proliferation despite a long-term (up to 30-month) follow-up (Fig. 3D). Approximately 10% of the mice deficient for both p53 and pRb developed urothelial hyperplasia (Fig. 3D, top right) compared with wild-type mice (Fig. 3D, top left) and p53^{−/−} only mice (Fig. 3D, top middle). Nearly 20% of the p53^{−/−}/RB1^{−/−} mice also exhibited nuclear abnormalities, particularly in the superficial urothelial layer, with large, irregularly shaped nuclei and dense chromatin (Fig. 3D, bottom left). Finally, 2% of p53^{−/−}/RB1^{−/−} mice developed low-grade, superficial papillary bladder tumors (Fig. 3D, bottom middle and right). No invasive urothelial tumor was observed throughout the 28-month observation period. These results indicate that, although deficiency of both p53 and pRb increases urothelial proliferation in aging animals, it is inadequate to trigger a high frequency of full-fledged urothelial tumors, let alone the invasive ones.

Deficiency of both pRb and p53, but not either protein alone, predisposes urothelium to subthreshold chemical
Figure 3. Spontaneous urothelial lesions in conditional p53/RB1-null mice. A, left, coinactivation of pRb and p53 in mouse urothelium. Line 1, UPII-Cre transgene; line 2, p53 allele whose exons 5 and 6 were flanked by loxP sites; line 3, recombined p53 allele on urothelial Cre expression; line 4, floxed RB1 allele; line 5, recombined RB1 allele on urothelial Cre expression. Right, PCR analyses of truncation of p53 and/or RB1 on DNA and RNA levels. Four major genotypes generated from multiple intercrosses were chosen for all studies: UPIICre/Cre (or p53+/+; RB1+/+; lanes 1 and 2), UPIICre/Cre/p53flox/flox (or p53−/−; RB1+/+; lanes 3 and 4), UPIICre/Cre/RB1flox/flox (or p53+/+; RB1−/−; lanes 5 and 6), and UPIICre/Cre/p53flox/flox/RB1flox/flox (or p53−/−; RB1−/−; lanes 7 and 8). WT, wild-type; Δ, truncated version; PD, p53 pseudogene. Note a 500-bp truncated p53 DNA and an 85-bp truncated p53 mRNA in p53−/−/RB1+/+ mice (lanes 3 and 4) and p53−/−/RB1−/− mice (lanes 7 and 8). Also note a 260-bp truncated RB1 DNA and a 200-bp truncated RB1 mRNA in p53−/−/RB1+/+ mice (lanes 5 and 6) and p53−/−/RB1−/− mice (lanes 7 and 8). B, in situ hybridization. Antisense cRNA probe corresponding to exons 5 and 6 of p53 gene (p53 Probe) hybridized to the urothelium of wild-type mice but not to those of p53-null mice or p53/RB1-null mice. Antisense cRNA probe corresponding to exon 19 of RB1 gene (RB1 Probe) hybridized to the urothelium of wild-type mice but not to those of RB1-null mice or p53/RB1-null mice. C, Western blotting of p21 and MDM2 showing that pRb deficiency greatly induced p21 (lanes 5 and 6; also see Fig. 2), but this induction was abrogated by p53 inactivation (lanes 7 and 8). D, H&E images of urinary bladders from a 15-mo-old wild-type mouse showing normal urothelial morphology (top left), an age-matched p53−/− mouse also showing normal morphology (top middle), a 12-mo-old p53−/−/RB1−/− mouse showing urothelial hyperplasia (top right), and a 28-mo-old p53−/−/RB1−/− mouse exhibiting low-grade, superficial papillary tumors. All panels are of the same magnification; bar, 50 μm (D, top left).
carcinogenesis. We next examined whether mice deficient for pRb and p53 were more susceptible to BBN, a bladder-specific carcinogen, than the wild-type counterparts or mice deficient for either protein (Supplementary Table S1). We treated groups of animals with BBN in the drinking water at a dose (0.01%) and time-frame (10 weeks) that were incapable of eliciting urothelial tumors in the wild-type mice (35). BBN is a human-relevant carcinogen and a metabolite of N-nitrosodibutylamine found in tobacco, food, and industrial products (36). Inflammation and edema were evident in all groups (Fig. 4). Urothelia of wild-type mice were non-neoplastic (Fig. 4A, top left) as were those from mice deficient for either p53 (Fig. 4A, top middle) or RB1 (Fig. 4A, top right). Heterozygous mice for both floxed p53 and floxed RB1 (p53+/−/RB1+/−) also exhibited nonneoplastic urothelia (Fig. 4A, bottom left).

Figure 4. Susceptibility of the p53/RB1-null mice to a subthreshold treatment of a bladder-specific carcinogen, BBN. A, H&E-stained cross-sections of the urinary bladders from age-matched (3-mo) wild-type (top left), p53−/− (top middle), RB1−/− (top right), and p53+/−/RB1+/− (bottom left) mice all exhibiting slight urothelial dysplasia with inflammation and edema in the lamina propria. Bottom middle and right, a p53−/−/RB1−/− double-null mouse exhibiting an invasive tumor. Bar, 200 μm (bottom middle) and 50 μm (top left representing all other panels). B, characteristics of BBN-triggered muscle-invasive urothelial carcinomas. Invasive lesions in mice null for p53 and pRb (top left) showed strong staining for basal cell keratins (top right consecutive section). Overexpression of Ki-67, decreased expression E-cadherin (E-cad), and overexpression of matrix metalloproteinase-9 (MMP9) were also observed in invasive tumor lesions (T, bottom) compared with their paired wild-type controls (WT). Bar, 100 μm (B, top left representing the top two panels) and 50 μm (B, bottom left representing all bottom panels).
striking contrast, 50% of the homozygous mice deficient for both p53 and pRb (p53−/−/RB1−/−) developed early-onset, muscle-invasive urothelial tumors (Supplementary Table S1; Fig. 4A, bottom middle and right, and B, top). The invasive tumor cells were positive for basal cell–specific keratins (Fig. 4B, top right), establishing their urothelial origin. Whereas normal urothelial cells lacked Ki-67, this proliferation marker was markedly induced in the invasive tumor cells (Fig. 4B, bottom). The invasive lesions had a profound reduction of E-cadherin compared with normal urothelium (Fig. 4B, bottom). Finally, whereas normal urothelium was negative for metalloprotease-9, invasive cells expressed large amounts of this protease (Fig. 4B, bottom). These results reveal many features of mouse invasive urothelial tumors that mirror highly aggressive muscle-invasive urothelial carcinomas in humans (2) and indicate that the combined deficiency of p53 and pRb is cooperative and necessary for promoting invasive urothelial tumorigenesis.

**Selective downregulation of p107 in BBN-induced invasive urothelial carcinomas.** Our finding that inactivating pRb in urothelium induced p107 prompted us to examine whether these two tumor suppressors collaborate to inhibit urothelial tumorigenesis and whether their coinactivation could synergize with p53 deficiency to trigger invasive carcinomas. Western blotting and immunohistochemistry showed that, unlike normal urothelium where p107 was constitutively expressed (Fig. 5A, lanes 1 and 2, and B, top left) and unlike p53−/−/RB1−/− mice where p107 was strongly induced (Fig. 5A, lanes 3 and 4), p107 was significantly reduced in the BBN-treated, pRb/p53-deficient invasive carcinoma cells (Fig. 5A, lanes 5 and 6, and B, middle left and bottom left). However, p130 remained at high levels (Fig. 5B, middle right and bottom right). These results suggest that the selective loss of p107 may play an important role in driving pRb/p53-deficient urothelial cells to form invasive carcinomas.

**Discussion**

Pocket family proteins work in concert to keep normal urothelium in a quiescent state. Urothelium is one of the slowest cycling epithelia in the body, with a turnover rate of ~200 days and a tritium-thymidine labeling index of <0.01% (37). This remarkably low self-renewal rate is physiologically important because a stable urothelium is a necessity to maintain an effective permeability barrier (38). It is unclear how the urothelial cells are kept in a quiescent state despite constant exposure to carcinogens and mitogens (39). Based on our data, we conclude that the pocket family proteins play a key role in holding urothelial growth in check. All members of this family are significantly expressed in urothelial layers (Figs. 2 and 5). Conversely, E2F1, which drives cell cycle forward by transcribing multiple growth-promoting genes, is kept at a low level (Fig. 1). Given that pRb family proteins share significant structural and functional properties (40), these proteins may be redundant to ensure that the urothelial cells remain quiescent when challenged by growth stimuli. As we observed, pRb abrogation led to a marked upregulation of p107 and its transcriptional repressor E2F4 (Figs. 2 and 6). This may represent a compensatory urothelial response to pRb loss to restore the balance of growth inhibition. Because p107 is a transcriptional target of E2F1 (41), p107 induction may be a result of E2F1 overexpression associated with pRb deficiency. Clearly, pRb family proteins work in a highly coordinated manner to restrict urothelial proliferation under normal and pRb-deficient conditions. We speculate that the loss of more than one pRb family member would be required to release the primary and the secondary blockade on urothelial growth, leading to proliferation. This notion is supported by our prior observation that all pRb family proteins are dramatically downregulated in low-grade, superficial papillary urothelial carcinomas in transgenic mice that expressed an activated Ha-ras (42). Downregulation of the entire pRb family proteins may be a prerequisite for urothelial tumorigenesis. It would be worthwhile to extend these observations through transgenic inactivation of all pRb family members in the urothelium.

**Concurrent defects of pRb and p53 are critical for promoting, but not initiating, invasive urothelial carcinomas.** We coinactivated pRb and p53 in the urothelium not only because the pRb deficiency strongly induced the p53 pathway (Fig. 2) but also because their concurrent defects are closely correlated with the invasive urothelial carcinomas in humans (43). We were surprised to find, however, that defects in both genes failed to trigger
invasive urothelial carcinomas. Our results challenge the prevailing
theory based largely on the clinical correlative data that defects of
pRb and p53 cause the invasive urothelial carcinomas. Rather, the
combined deficiency of these two tumor suppressors is necessary
to promote invasive urothelial tumors. When mice homozygously
null for pRb and p53 were fed with 0.01% BBN for 10 weeks, 50% of
the mice developed muscle-invasive urothelial carcinomas that
strongly resembled the human counterparts morphologically and
biochemically (Supplementary Table S1; Fig. 4). Few, if any, of such
carcinomas occurred in identically treated mice that were homo-
zygously null for pRb only or for p53 only or in mice heterozygously
null for both pRb and p53. These results indicate a pivotal collab-
orative role of the complete loss-of-function of both pRb and p53
in the promotion, but not in the initiation, of invasive urothelial
carcinomas (Fig. 6).

Several mechanisms may underlie the collaborative effects be-
tween pRb and p53 deficiency. First, pRb loss in urothelium pro-
vokes a robust, p53-mediated apoptotic response (Fig. 2). This
response was muted when pRb-deficient urothelial cells were also
made p53-deficient (Fig. 3). Urothelial cells defective for both p53
and pRb are therefore much less capable than those defective for
pRb only to respond to genotoxic agents such as BBN in mounting
an apoptotic response. Instead, the pRb/p53 double-deficient ur-
othelial cells carrying carcinogen-damaged DNAs exit cell cycles
 uncontrollably via defective G1-S and G2-M checkpoints transpired
by the pRb loss. Second, the collaborative effect could be due to a
strong urothelial induction of MAD2 due to pRb loss (Fig. 1).
MAD2 is a key kinetochore checkpoint protein and a downstream
target of E2F1 (32). Whereas the normal level of MAD2 prevents
premature cell cycle progression through the anaphase, excessive
amounts can lead to abnormal chromosomal segregation and an-
euploidy. Recent transgenic studies show that MAD2 overexpres-
sion leads to tumorigenesis, implicating MAD2 as an oncogene
(44). Because p53 deficiency also leads to genome instability (45),
these effects could be additive during BBN treatment.

It should be noted that, although pRb loss activates p53 pathway
(Fig. 2), this compensatory response is not reciprocal. The loss of
p53 in urothelium did not induce the expression of pRb family pro-
teins, their E2F partners or downstream effectors. Therefore, ur-
othelial cells may be more resistant to pRb deficiency but more
vulnerable to p53 deficiency. Such differential vulnerability to tu-
mor suppressor loss may be applicable to other epithelia as well.

p107 deficiency as a potential missing link in invasive ur-
othelial tumorigenesis. The fact that even combined p53 and
pRb inactivation failed to trigger muscle-invasive urothelial car-
cinomas suggests that additional genetic defects are required.
Three lines of evidence suggest p107 deficiency as a potential miss-
ning link. First, p107, but not its family member p130, is highly
upregulated in pRb/p53 double-deficient urothelial cells (Fig. 2;
data not shown), suggesting that p107 plays a critical tumor-
suppressive role during pRb/p53 deficiency (Fig. 6). Second, in
BBN-treated pRb/p53 double-knockout mice where 50% of the

8 Unpublished observation.
animals developed muscle-invasive carcinomas, p107 was significantly downregulated (Fig. 5), whereas p130 remained at high levels. This selective loss of p107 suggests that this protein is an important target for inactivation by BBN. Third, as we have shown previously, SV40T antigen, which functionally disables not only pRb and p53 but also pRb family proteins including p107 (28), was capable of inducing high-grade carcinoma in situ and invasive urothelial carcinomas (26, 46). A similar collaborative effect among pRb, p53, and p107 deficiencies was observed in the retina where deficiency of all three tumor suppressors, but not any combination of the two, induces retinal dysplasia or retinoblastoma (47). Together, the principle we showed regarding the collaborative relationships among pRb, p53, and p107 may be applicable to the tumorigenic processes in many cell types (Fig. 6). Finally, our results suggest that pRb, p107, and p53 together could be a more reliable prognostic indicator than a combination of p53 and pRb for patients with invasive urothelial carcinomas.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References