Differential Expression of Cell Cycle Regulators in Phenotypic Variants of Transgenically Induced Bladder Tumors: Implications for Tumor Behavior

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Abstract

Proteins controlling cell growth, differentiation, apoptosis, and oncogenic stress are often deregulated in tumor cells. However, whether such deregulations affect tumor behavior remains poorly understood in many tumor types. We recently showed that the urothelium-specific expression of activated H-*ras* and SV40 T antigen in transgenic mice produced two distinctive types of tumors strongly resembling the human superficial papillary tumors and carcinoma *in situ* of the bladder, respectively. Here we assessed the expression of a key set of cell cycle regulators in these mouse tumors and in a new transgenic line expressing a cyclin D1 oncogene in the urothelium. We found that urothelia of the wild-type and cyclin D1 transgenic mice exhibited a profile of cell cycle regulators found in quiescent (G_0) cells, indicating that urothelium overexpressing the cyclin D1 (an 8-fold increase) is reminiscent of normal urothelium and remains slow-cycling. Low-grade superficial papillary tumors induced by activated H-*ras* had no detectable Rb family proteins (Rb, p107, and p130) and late cell cycle cyclins and kinases (cyclin A, E, and CDK1), but had increased level of p16, p53, and MDM2. These data suggest that the inactivation of the Rb pathway plays an important role in H-*ras*-induced superficial papillary tumors and that oncogenic H-*ras* can induce a compensatory activation of alternative tumor suppressor pathways. In contrast, carcinoma *in situ* of the bladder induced by SV40 T antigen had increased expression of cell cycle regulators mainly active in post-G_1 phases. The fact that phenotypically different bladder tumors exhibit different patterns of cell cycle regulators may explain why these tumors have different propensity to progress to invasive tumors. Our results indicate that the transgenic mouse models can be used not only for studying tumorigenesis but also for evaluating therapeutic strategies that target specific cell cycle regulators. (Cancer Res 2005; 65(4): 1150-7)

Introduction

Bladder cancer is a very common malignancy, afflicting more than 2 million people on a global scale. In the United States, it ranks as the fifth most frequent neoplasm with about 54,000 new cases diagnosed annually (1). In the developed countries where schistosomiasis is not a problem, bladder neoplasias are predominately of the transitional cell carcinoma type, which is heterogeneous, exhibiting various histologic morphologies and biological behaviors (2–4). Seventy percent to 80% of transitional cell carcinomas present as low-grade, superficial papillary tumors that frequently recur, but rarely progress to the muscle-invasive stage. By contrast, the remaining 20% to 30% had muscle invasion at diagnosis, and they carry a high risk of spreading locally as well as advancing to incurable metastatic stage. It has been suggested that different genetic alterations underlie these different biological behaviors, although direct experimental evidence is lacking. Therefore, an improved understanding of the molecular basis underlying bladder tumor pathways is of clear importance in helping to better predict bladder tumor progression and devise optimal management strategies for different transitional cell carcinomas.

The identification of the urothelium-specific uroplakin II promoter (5) has made it possible to express specific oncogenes in the urothelium of transgenic mice. Mice expressing a constitutively activated H-*ras* oncogene developed urothelial hyperplasia and low-grade superficial papillary tumors (6). In contrast, mice expressing an SV40 T antigen, which functionally inactivates the p53 and pRb tumor suppressors, developed carcinoma *in situ* (CIS) and invasive bladder tumors (7), a finding also supported by the transgenic expression of SV40 T antigen in the urothelium driven by the cytokeratin 19 gene regulatory elements (8). These results provided direct experimental evidence supporting the notion that phenotypically different bladder tumors are caused by different genetic defects.

To further characterize the molecular events underlying urothelial tumorigenesis, we have generated transgenic mouse lines expressing cyclin D1, an oncogene that is amplified and/or overexpressed in about 20% of human bladder tumors (3). We have analyzed the molecular alterations elicited by the urothelial expression of cyclin D1, and compared those with mice expressing an activated H-*ras* or an SV40 T antigen. We found that the normal mouse urothelium exhibits the molecular characteristics of a quiescent tissue consistent with the known slow turnover of the epithelium (9, 10). Overexpression of a wild-type cyclin D1 correlates with increased levels of the cell cycle inhibitors p21 and p27 with no noticeable morphologic abnormality in the urothelium. In urothelial hyperplasia and low-grade superficial papillary tumors induced by the activated H-*ras* oncogene, there is a dramatic reduction of Rb family proteins. This is accompanied by an induction of the cell cycle inhibitor p16 and of p53 and MDM2 only in low-grade superficial papillary tumors but not in hyperplasia. In contrast, in CIS lesions induced by the SV40 T antigen, there is

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a specific increase of cell cycle regulators commonly affecting the post-G_1 phases of the cell cycle. These changes in cell cycle regulators strongly resemble those occurring in human bladder cancers and may provide a molecular explanation as to why the two distinctive variants of bladder tumors have different propensity to progress (11).

Materials and Methods

Generation of *UPII*-cyclin D1 Transgenic Mice. The mouse uroplakin II promoter was used to drive the urothelium-specific overexpression of the wild-type cyclin D1. Briefly, a 1.9-kb full-length human cyclin D1 cDNA coding region plus SV40 small tumor antigen intron and poly A signal was released from pXcycD1 (12) and inserted downstream of the 3.6-kb mouse *UPII* promoter that was previously cloned into pBluescript (5). The orientation of the chimeric gene was confirmed by restriction digestions and DNA sequencing. The *UPII-CD1* chimeric gene was excised from pBluescript by digestion with *Kpn*I-*Bam*HI, purified by agarose gel electrophoresis, dialysis, phenol/chloroform extraction and filtration through 0.22-μm filters before being injected into fertilized eggs of FVB/N inbred mice for transgenic mice production.

Southern Blot Analysis. Transgenic mice were identified by Southern blot analysis of the genomic DNA isolated from the mouse tail biopsies. DNA was digested with *Nco*I, resolved by gel electrophoresis, and hybridized with a 550-bp *Bam*HI-*Stu*I fragment located at the 3′ end of the mouse *UPII* promoter, which allowed the detection of both the endogenous *UPII* gene and *UPII-CD1* transgene.

Northern Blot Analysis. The urothelia from six bladders of each different cyclin D1 transgenic line were processed for total RNA extraction with Trizol. The RNA was resolved by agarose-formaldehyde gel (15-20 μg/lane), transferred onto nylon membrane, and probed for cyclin D1 expression with an SV40 3′ untranslated region cDNA probe.

Reverse Transcription–PCR. Trizol extracts of urothelial RNA were treated with DNAase, reverse transcribed, and subjected to PCR using primers designed to flank the small SV40 intron (66 bp) in the 3′ untranslated region of the *UPII*-cyclin D1 construct. The sense and antisense primers were GAGATGTCTCTAAATTTCGAGA and CTACCG-TAAAGAAGACTCGT. These primers yielded two PCR products that differ between spliced *UPII*-cyclin D1 cDNA (115 bp) and contaminating genomic (181 bp) cyclin D1 transgene. The PCR conditions were 30 cycles of 93°C for 30 seconds, 72°C for 45 seconds, and 58°C for 45 seconds.

Histopathology. Mouse bladders were fixed in 10% buffered formalin and embedded in paraffin. Sections (3-5 μm) were stained with H&E and examined microscopically for pathologic changes.

Preparation of Urothelial Proteins and Western Blotting. Urothelial cells were scraped from the normal mouse bladder mucosa and the

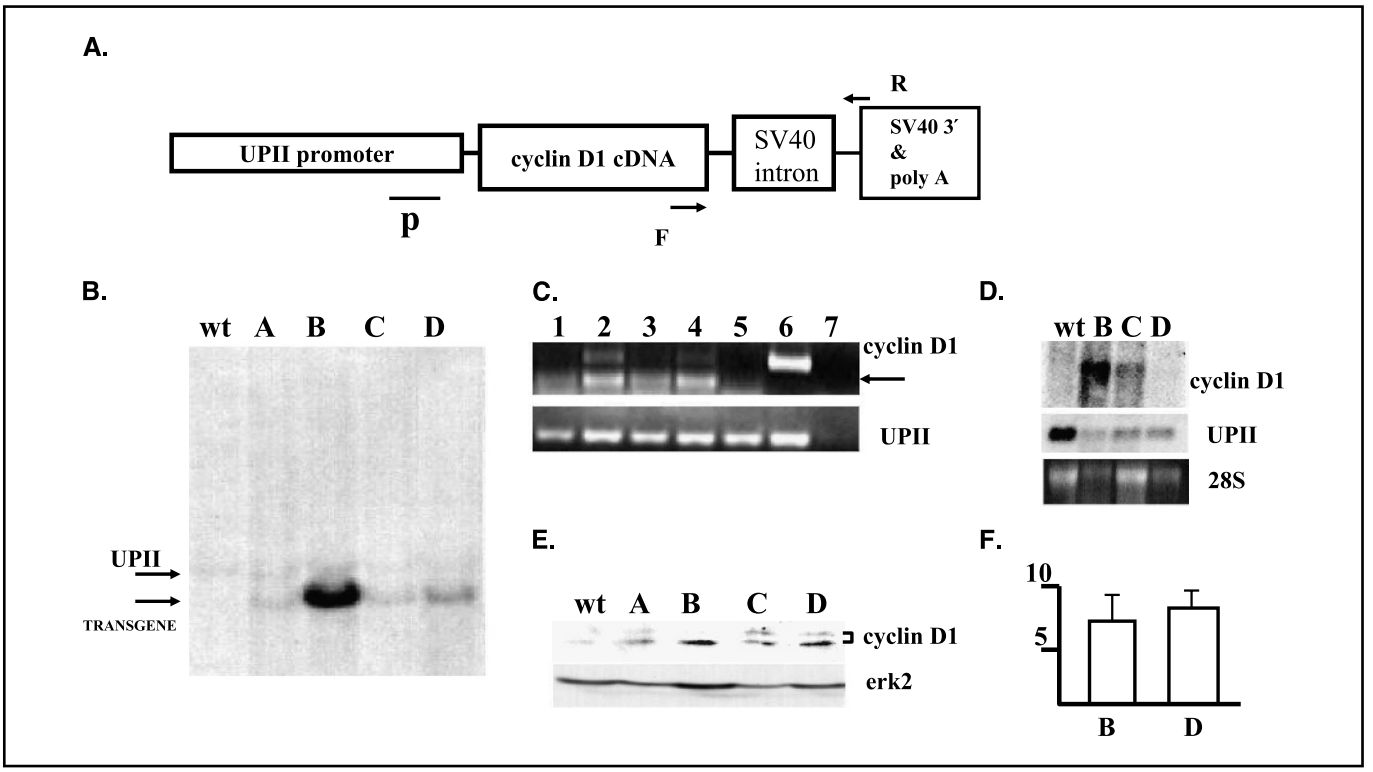

Figure 1. Generation and characterization of *UPII*/cyclin D1 transgenic mice. *A,* a chimeric gene consisting of the 3.6-kb *UPII* promoter, the human cyclin D1 cDNA, and the SV40 large T antigen small intron and polyadenylation signal. *P,* probe for Southern blotting. *R* and *F,* PCR primers *B,* Southern blot analysis. Genomic DNA from the tail of F1 mice was hybridized with a probe located at the 3′ end of the *UPII* promoter (*P* in *A*). The probe detected the endogenous *UPII* gene fragment (1.4 kb) and a predicted transgenic fragment of 1.1 kb. *wt,* wild-type. *C,* RT-PCR. *Gel lines 1 to 4* (*A, B, C, D,* transgenic urotheliums), *gel line 5* (nontransgenic urothelium), *gel line 6* (positive controls; *UPII*-cyclinD1 construct plasmid in *top* and *UPII* cDNA plasmid in *bottom*), and *gel line 7* (no RNA). *Arrow,* PCR product that corresponds to the spliced *UPII*-cyclin D1 transgenic construct mRNA. *Top bands* are generated by the control plasmid (*line 6*) and unspliced and/or contaminating genomic transgenic *UPII*-cyclin D1 DNA (*lines 1 to 4*). *D,* Northern blot analysis of total RNA from isolated mouse urothelia. *Top*, transgenic cyclin D1 mRNA expression. *Middle,* for control of RNA quality the same blot was hybridized with a uroplakin II cDNA probe. *Bottom,* ethidum bromide staining of 28S rRNA. *E,* Western blot analysis of the expression of cyclin D1 in the urothelium of different transgenic lines. Increased cyclin D1 protein expression could be observed in the transgenic urothelium. Erk2 protein expression was used as a loading control. *A, B, C,* and *D,* different transgenic lines. *F,* cyclin D1 protein overexpression in transgenic lines *B* and *D*. *Columns,* mean fold increase in cyclin D1 expression in transgenic urothelium relative to cyclin D1 protein expression in normal urothelium (7.5 ± 1.8- and 8.1 ± 1.5-fold increase in transgenic lines *B* and *D*, respectively; $n = 4$); *bars,* SE.

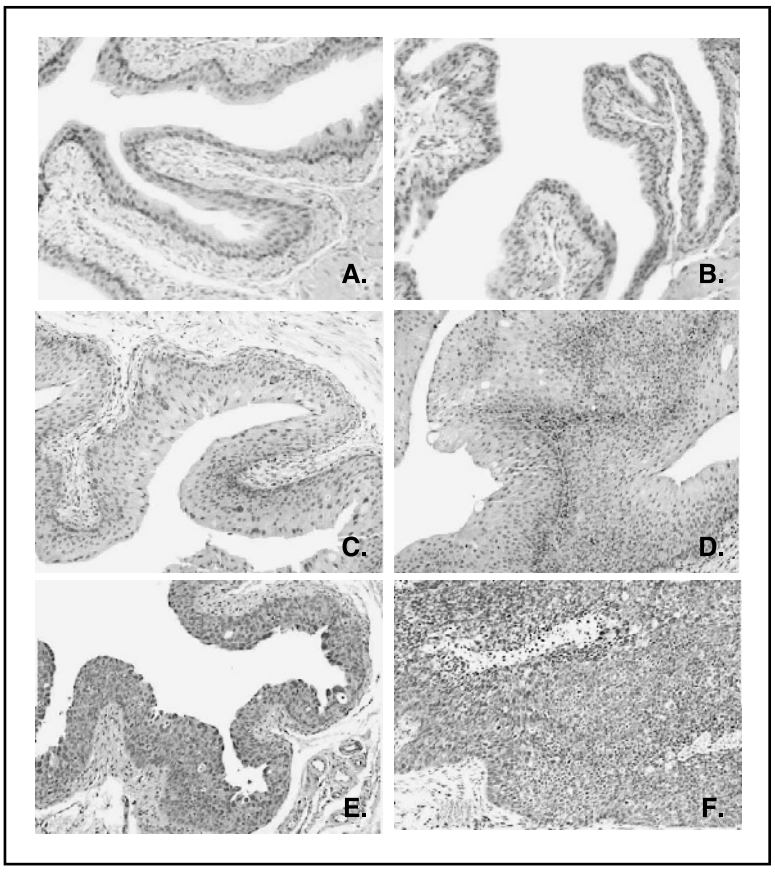

Figure 2. Phenotypic alterations of transgenic mice expressing various transgenes. *A,* cross section of the bladder of a 24-month-old nontransgenic mouse showing normal histology. *B,* urothelium of a 24-month-old cyclin D1 transgenic mouse (*line B*) also showing normal histology. *C,* urothelial hyperplasia in a 6-month-old H-*ras* transgenic mouse. *D,* low-grade superficial papillary bladder tumor in a 16-month-old H-*ras* mouse. *E,* CIS in a 5-month-old SV40 T antigen mouse. *F,* high-grade bladder carcinoma in a 15-month-old SV40 T antigen transgenic mouse. Magnifications ×100 (*A* and *B*); ×200 (*C, D, E,* and *F*).

various tumor lesions of the transgenic mice with a clinical spatula as described (13). The cells were collected by centrifugation and lysed in a buffer containing 25 mmol/L Tris-HCl, 2 mmol/L EDTA, and 2% SDS. Lysates were quickly frozen and thawed thrice, sonicated, and centrifuged at 10,000 × *g* for 10 minutes. Soluble proteins were quantitated by the bichinconic acid method (Pierce, Rockford, IL). Lysates were brought to 3 μg/μL with concentrated sample loading buffer, stored in aliquots, and kept at −80°C.

The lysates (30-45 μg of the total protein) were resolved by 7.5%, 10%, or 15% SDS-PAGE and transferred onto nitrocellulose membranes. After the membranes were reversibly stained with 0.1% Ponceau Red solution, they were blocked with 3% nonfat dry milk dissolved in TBST solution (10 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 0.1% Tween 20) and incubated for 1 hour at room temperature with the primary antibodies in TBST with 1% nonfat dry milk [the antibodies were diluted 1:100, except for p53, p53 Ser^{15}-P, MDM2 and CDK4 (1:200); cyclin D1, CDK1, cyclin A, and cyclin E (1:500); and Erk1,2 (1:5000)]. After washing in TBST solution, the secondary antibodies, diluted in 1% nonfat dry milk in TBST, were added for 1 hour at room temperature. The membranes were rinsed four times with water and twice with TBST before being developed by an enhanced chemiluminescence detection system (ECL, Pierce) according to the manufacturer's instructions. West Pico reagent was used for highly expressed proteins, whereas the West Femto reagent was used to detect low-level proteins. The antibodies were optimized with cell line extracts and/or recombinant proteins. The antibodies were obtained from the following vendors: proliferating cell nuclear antigen (PCNA; Signal Transduction, Toms River, NJ), Cdk1 (ABCAM, Cambridge, United Kingdom), Erk1,2 (Promega, Madison, WI), p16 (Upstate Biotechnology, Lake Placid, NY), p53 and P53 Ser^{15}-P (Oncogene Research Products, Boston, MA), and Rb, p21, and p27 (BD PharMingen, San Diego, CA). All other antibodies were obtained from Santa Cruz Biotechnology (Berkeley, CA). Cyclin D1 protein expression was quantified by densitometry using NIH image 1.63 software (Bethesda, MD).

Results

Urothelial Overexpression of Cyclin D1 Induced p21, p27, and PCNA. Although amplification and overexpression of the cyclin D1 gene was found in about 20% of human bladder tumors, little is known about the potential of cyclin D1 in transforming urothelial cells *in vivo*. (3). To address this issue, we generated transgenic mouse lines in which the cyclin D1 expression was driven by the well-characterized urothelium-specific promoter of the *UPII* gene (Fig. 1*A*; refs. 5–7). Four independent transgenic mouse lines were obtained (Fig. 1*B*) expressing the cyclin D1 transgene in urothelia as evidenced by reverse transcription–PCR (RT-PCR; Fig. 1*C*), Northern blotting (Fig. 1*D*), and Western blotting (Fig. 1*E*). The level of cyclin D1 protein in transgenic lines B and D was 7.5 ± 1.8- and 8.1 ± 1.5-fold higher, respectively, than that in nontransgenic urothelium (Fig. 1*F*).

Although cyclin D1 was suspected to be a growth-promoting oncogene in urothelial tumorigenesis, continued expression (up to 48 months) of this gene alone in transgenic mice did not produce any morphologic abnormalities in the urothelium (Fig. 2). At the molecular level, however, urothelia of the transgenic mice, but not nontransgenic controls, overexpressed the PCNA, a hyperproliferative marker (14). This suggests that whereas the urothelial cells had acquired the proliferative potential as a result of cyclin D1

overexpression, such an effect was only marginal and was insufficient to promote urothelial hyperplasia. Alternatively, compensatory mechanism(s) may be present in curtailing the growth-promoting effect of cyclin D1. Indeed, p21 and p27, two important cell cycle inhibitors preventing the G_1-S transition (15), were overexpressed in transgenic mice (Fig. 3*A*).

Absence of Rb Family Proteins in H-*ras* Induced Urothelial Hyperplasia and Superficial Papillary Tumors. The urothelium is among the slowest cycling epithelia, with a labeling index of 0.02% to 0.05% and a turnover rate of about 6 months (9, 10, 16). Consistent with this notion, Rb family proteins, including Rb, p107, and p130, were all in a hypophosphorylated state, thus preventing the cell from progressing through the cell cycle. Of these, p107 remained at a low level (Fig. 3*C*). In addition, cyclin-dependent kinases of early G_1 phase, such as CDK4 and CDK2, were readily detectable, whereas cell cycle proteins of later G_1 and S phase, such as CDK1, cyclin E, and cyclin A, were absent (Fig. 3*A* and *B*). These results indicate that normal urothelial cells are nonproliferating.

In contrast to the normal urothelium in which Rb family proteins were readily detectable, these proteins were largely undetectable in the urothelia of H-*ras* transgenic mice (Fig. 4*C*). Only a residual amount of hypophosphorylated p107 could be detected in two urothelial lesions (Fig. 4*C*; Table 1). The lack of protein detection was not due to inefficient nuclear protein extraction because histone deacetylase 3, another nuclear protein, was readily detected (Fig. 4*C*). Other significant alterations in cell cycle regulators included the overexpression of p16 and p53, two important tumor suppressor proteins, and overexpression of MDM2, CDK4, and CDK2 (Fig. 4*A* and *B*). In general, although there was an increased expression of growth-promoting cell cycle regulators, this increase was accompanied by an overexpression of cell cycle inhibitors, indicative of a low proliferative status of the urothelia in H-*ras* transgenic mice.

Marked Increase of Cyclins and CDKs in SV40 T–Induced Bladder Carcinoma *In situ*. Carcinoma *in situ* of the bladder induced by SV40 large T antigen had a dramatic increase in post-G_1 cell cycle regulators including cyclin A and E and CDKs 1 and 2, whereas the G_1 phase protein cyclin D1 was down-regulated (Fig. 4*A*; Table 1). Cell cycle inhibitors p21, p27, and p16 were up-regulated (Fig. 3*A* and *B*; Table 1). SV40 large T antigen binds and inactivates p53 and the Rb family of proteins (17, 18). Accumulation of p53 without induction of MDM2 (Fig. 4*B*) is consistent with p53 being inactivated by the SV40 T antigen. In addition, p53 was phosphorylated at Ser^{15} (Fig. 4*B*), indicative of DNA damage (19, 20), and perhaps reflecting the fact that SV40 T–induced tumors are genomically unstable (21). The expression level and phosphorylation status of the Rb family proteins in SV40 T–induced tumors were consistent with what occurs in a highly proliferating tissue. Moreover, these molecular changes were reminiscent of the changes that the SV40 oncogene produces in

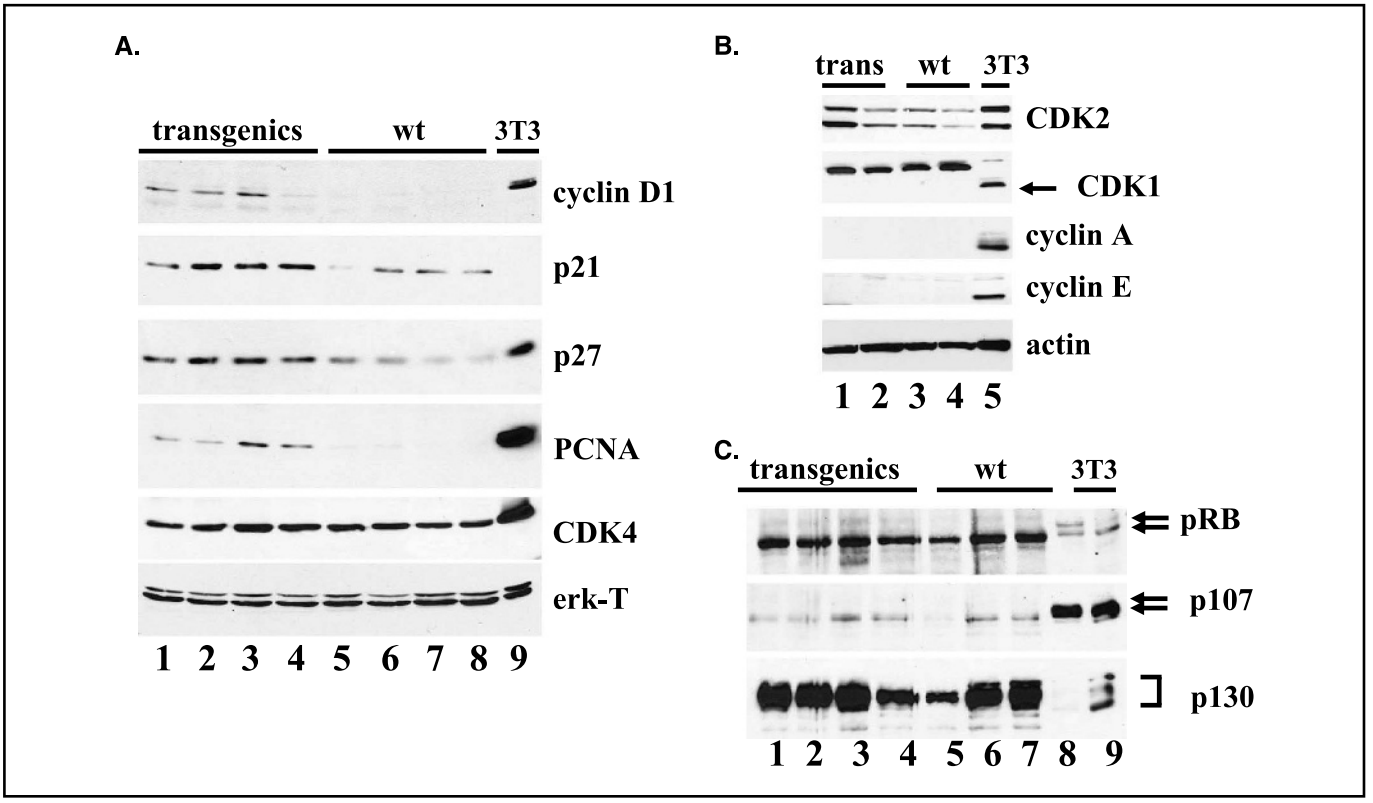

Figure 3. Western blot analysis of total urothelial proteins from wild-type mice (*wt*) and cyclin D1 transgenic lines. *A,* protein levels of early G_1 cell cycle proteins: cyclin D1, p21, p27, PCNA, and CDK4 from urothelia of *transgenic lines B and D* (*blot lines 1 and 2 and 3 and 4*, respectively; 45 μg of protein). *Blot lines 1 and 3,* protein from 15-month-old animals; *lines 2 and 4,* protein from 9-month-old animals; wild-type mice (*blot lines 5 to 8*; protein in *lines 5 to 8* was from mice at 9, 6, 15, and 2 months old, respectively). *Line 9* was loaded with 20 μg of total lysate from the cell line NIH-3T3 as a control for expression levels. *B,* levels of late G_1 and S phase cell cycle proteins CDK2, CDK1, cyclin A, and cyclin E. *Line 5,* 20 μg of NIH-3T3 lysate. *C,* protein levels and phosphorylation status of the Rb protein family, Rb, p107, and p130 (45 μg). *Lines 8 and 9,* 20-μg lysates of NIH-3T3 cells untransfected or transfected (*line 8*) with the H-*ras* oncogene as control for the phosphorylation of Rb proteins.

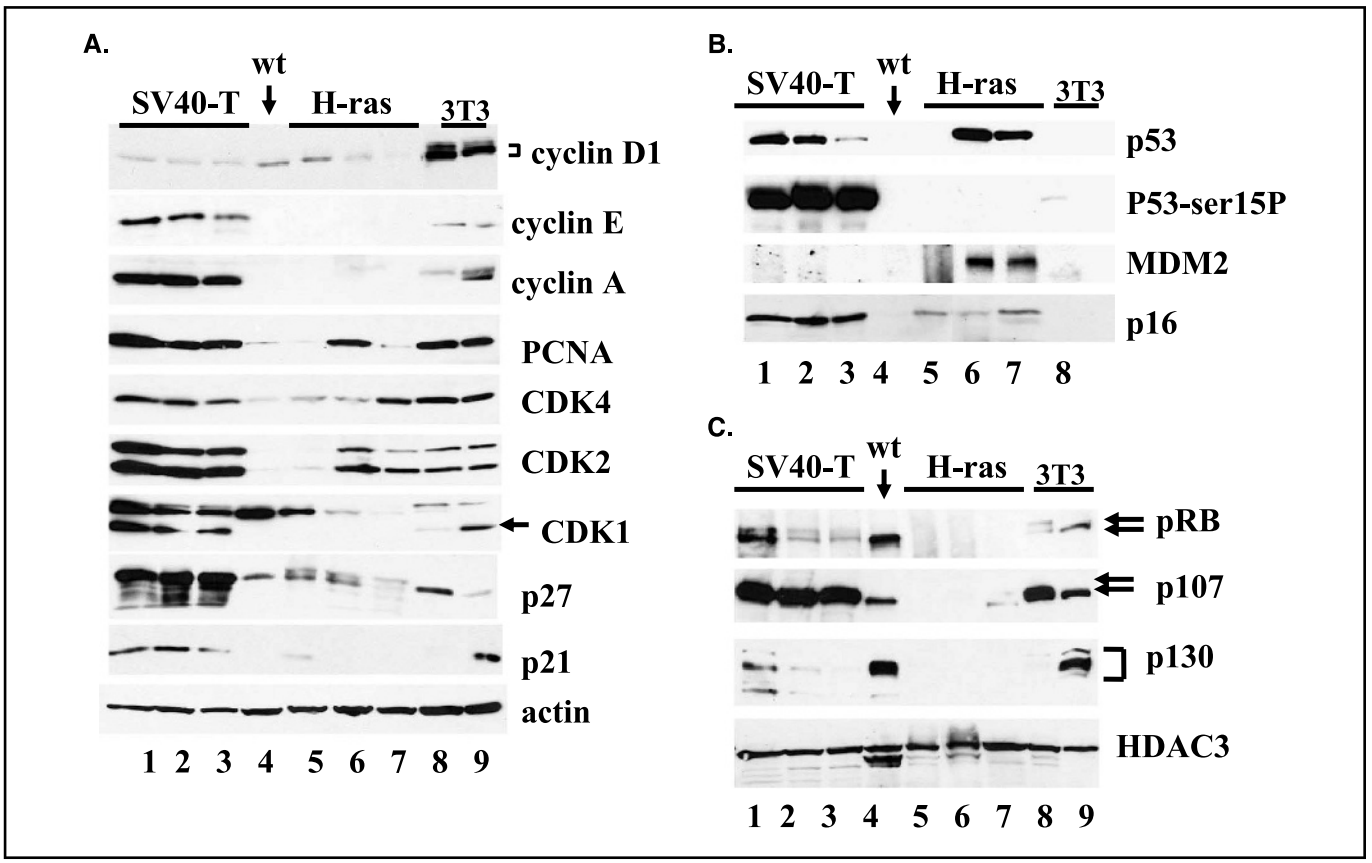

Figure 4. Western blot analysis from SV40 T antigen and H-*ras* transgenic mice with urothelial hyperplasia and tumors. *A,* levels of early and late cell cycle proteins cyclin D1, cyclin E, cyclin A, PCNA, CDK 4, CDK 2, CDK 1, p27, and p21. *Lanes 1 and 2,* SV40 T antigen high-grade superficial tumors from 13-month-old SV40 T antigen mice (30 μg). *Lane 3,* SV40 T antigen–induced CIS from an SV40 T mouse of 11.5 months (30 μg). *Lane 4,* wild-type mice (30 μg). *Lane 5,* H-*ras* induced hyperplastic urothelia; *lanes 6 and 7,* H-*ras* induced papillary tumors (30 μg). *Lanes 8 and 9,* 15 μg of NIH-3T3 and NIH-3T3 H-*ras* lysate. *B,* tumor suppressor proteins p53 and p16 (30 μg). *Lanes 1 to 7* as in *A*. *Lane 8,* NIH-3T3 lysate (15 μg). *C,* protein levels and phosphorylation status of the Rb protein family, Rb, p107, and p130. *Lanes 1 to 7* as in *A* (30 μg). *Lanes 8 and 9,* lysates of NIH-3T3 cells untransfected or transfected (*line 8*) with the H-*ras* oncogene as control for phosphorylation (15 μg).

other tissues (22). The only exception was the significant increase of hyperphosphorylated p107 in SV40 T–induced bladder tumors (Fig. 4*C*).

Discussion

Cyclin D1 Overexpression in Urothelium Up-regulates CIP/KIP Proteins p21 and p27. As a central target of mitogenic signals, cyclin D1 is frequently overexpressed in a variety of human cancers, particularly during the early stages of tumorigenesis (23). Bladder cancer is no exception as up to 20% of the cases have increased expression of cyclin D1 (3, 24). Overexpressing cyclin D1 in various tissues of transgenic models, however, have yielded a mixed outcome. For instance, overexpression of cyclin D1 in mammary epithelium resulted in mammary adenocarcinoma (25). The overexpression of cyclin D1 in both hepatocytes and enterocytes, under the control of the rat liver fatty acid–binding protein promoter, produced hepatocellular carcinomas but did not produce any detectable abnormality in the intestinal epithelium (26). Driven by the keratin 5 promoter, cyclin D1 induced epidermal hyperproliferation and thymic hyperplasia (27, 28). When expressed under the control of the EBV promoter, cyclin D1 caused dysplasia in stratified squamous epithelia of the tongue, esophagus, and forestomach (29, 30). When targeted to B lymphocytes, despite abundant transgene expression, lymphocytes were normal in cell cycle activity, size, and mitogenic responsiveness (31, 32). On the other hand, mice lacking the cyclin D1 gene have specific alterations in mammary epithelial proliferation (33, 34), most likely because cyclin D1 functions as an important mitogen and as a coactivator for the estrogen receptor (35). It is also noteworthy that in hepatocytes, cyclin D1 expression is sufficient to promote hepatocellular cell cycle progression in the absence of mitogens (36, 37). With the exception of mammary gland and hepatocytes, overexpression of cyclin D1 in other tissues, including the urothelium (Fig. 3), failed to produce frank epithelial tumors. These results indicate that the hyperproliferative responses to cyclin D1 overexpression are cell type specific.

The lack of urothelial tumor formation in the face of cyclin D1 overexpression could be due to a compensatory induction of the cell cycle inhibitors p21 and p27. It seems that the overexpression of PCNA alone, an auxiliary factor for DNA polymerases δ and ε during DNA replication and repair (38–40), is inadequate to drive urothelial cells into the S phase. Our data indicate that there was a lack of hyperphosphorylated Rb and cyclin E expression suggesting that the CDK4/cyclin D1 complex is inactive. This situation is analogous to that of hepatocytes in which transient expression of cyclin D1 promoted hepatocyte proliferation only during the first few days and the proliferative effect disappeared afterward. The

Table 1. Summary of Western blot analysis

	SV40 T1	SV40 T2	SV40 H1	wt	ras H1	ras H2	ras T2	ras T3	ras T1
cyclinD1	+/-	+/-	+/-	+	+			ND	+
cyclin A	++	++	++	-	ND	-	-	-	+/-
cyclin E	+	+	+	-	ND	-	-	-	-
CDK4	+	+	+	+/-	ND	+/-	+/-	+	ND
CDK2	+++	+++	+++	+/-	+/-	+/-	+	+	+
CDC2	++	++	++	-	-	-	-	-	-
PCNA	++	++	++	+/-	+/-	-		+/-	
p21	+	+	+/-	-	-	-	-	-	-
p27	++	++	++	+	ND	+	+	+/-	+/-
p53	+	+	+/-	-	-	-	+/-	ND	+
p53-P	++	++	++	-	ND	-	-	ND	-
p16	++	++	++	-	+	+	+	ND	+
MDM2	-	-	-	-	-	-	+	ND	+
RB-hyper	+	+	+	-	-	-	-	-	-
RB-hypo	+	+	+	++	-	-	-	-	-
p107-hyper	++	++	++	-	-	-	-	-	
p107-hypo	++	++	++	+	-	+/-	-	+/-	-
p130	+	+/-	+/-	++	-	-	-	-	-

Abbreviation: ND, not determined. −, no detectable signal. +/−, +, ++, and +++, arbitrary increasing band signal within rows. Light gray, no change; strong gray, increased, and dotted cells, decreased band signal in comparison with wild-type. White, no detectable signal. SV40 T1, SV40 T2, and SV40 H1 urothelial tumors (T1 and T2) and hyperplastic tissue (H1) from *UPII*/SV40 transgenic mice. Ras H1, H2, hyperplastic urothelium; Ras T1, T2, and T3, papillary tumors from *UPII*/ H-*ras* transgenic mice.

concomitant induction of p21 and p27 proteins suggests that endogenous antiproliferative mechanisms might have come into play, which effectively curtailed cells from undergoing uncontrolled proliferation (37).

Cell Cycle Status of Normal Urothelial Cells. Commensurate with its physiologic requirement as a permeability barrier, normal urothelium has an exceedingly low renewal rate, with a tritium-thymidine labeling index of only 0.02% to 0.05% and a turnover rate of about 200 days (16). This means that under normal conditions, most urothelial cells are not cycling or are quiescent. It is well known that the phosphorylation status of the pRb family proteins is key to regulating cell cycle progression in normal cells as well as in tumors. In the G_0 phase of normal resting cells, pRb and p107, when detectable, are found in the hypophosphorylated state, whereas p130 exists in two differently phosphorylated forms (i.e., p130 forms 1 and 2; refs. 41, 42). Our results on both the level of expression and phosphorylation status of the Rb family proteins (Fig. 3*C*) establish in molecular terms that normal urothelium is in a G_0 resting state. The expression of early G_1 phase cyclin-dependent kinases CDK4 and CDK2 and the lack of expression of late G_1 and S phase regulators such as CDK1, cyclin E, and cyclin A (Fig. 3*A* and *B*) confirm the quiescent nature of the urothelium and also explain at the molecular level the readiness of the urothelium to undergo renewal when provided with sufficient growth signals as would occur during wound healing (43).

Differential Expression of Cell Cycle Regulators in Tumors Induced by H-*ras* and SV40 T Antigen. The recently generated transgenic mouse models showed that expression of an activated H-*ras* gene in the mouse urothelium caused hyperplasia that evolves, in some cases, to low-grade superficial papillary urothelial tumors (6). In contrast, all mouse lines with low-level expression of SV40 T antigen developed bladder CIS at early ages (1-5 months), most of which evolved into high-grade superficial papillary bladder tumors before a small fraction advanced to invasion/metastasis (21). When analyzed at the molecular level, the main characteristic of the SV40 T–induced tumors is a dramatic increase of most cell cycle–related proteins. With exception of cyclin D1, all CDKs and cyclins are strongly expressed. The strong induction of late G_1 phase CDKs and cyclins suggest that the progression through the restriction point of the cell cycle is mainly driven by CDK2 and CDK1 kinases. The lack of MDM2 expression indicates that p53 is not functional in its transcription activating function, mostly likely because SV40 T antigen binds and inactivates p53 (17). The phosphorylation of p53 at serine15 is indicative of genome instability (19, 20), consistent with the frequent chromosomal aberrations in these tumors (21).

The changes we observed in the Rb family proteins are similar to what has been described in other cell types expressing the SV40 T (17, 18, 44). The only exception is the hyperphosphorylation of p107, which may reflect a tissue-specific event or may be due to the low expression level of SV40 T, which may not completely inhibit the p107 function.

This enhanced expression of proliferation-promoting proteins in SV40 T–induced tumors in the transgenic mice is consistent with what has been found in a subset of human bladder tumors. A cluster of 21 genes, including cyclin A, CDK1, and PCNA, were found to be up-regulated in high-grade human bladder tumors

(grade 3 of Ta tumors) with high recurrence rate, CIS and T2+ tumors. This gene cluster has been shown to play key roles in cell-cycle transition and mitosis, and may therefore be responsible for tumor progression (11).

By contrast, in the H-*ras*-induced hyperplasia and superficial papillary tumors, late G_1 phase proteins were absent, suggesting that proliferation is low and is confined to a small number of cells, similar to how it occurs in the normal urothelium. The tumor suppressor p16 was induced as early as in urothelial hyperplasia (Fig. 4*B*; Table 1). Also evident in tumors is the increased expression of p53 and its downstream target MDM2. The loss of expression of all three Rb family members is particularly intriguing. The loss of Rb family proteins has been reported in bladder cancer cell lines in conjunction with an increase in p16 (3, 45, 46, 47). It has been postulated that the induction of p16 down-regulates the Rb gene expression (48, 49). Other authors have observed Rb loss at the protein level (with Rb mRNA present) in cell lines derived from invasive bladder cancers in which p53 is mutated (50, 51). In these studies, RT4, the only cell line derived from a papillary tumor, showed loss of Rb expression in the absence of p53 mutation, a situation similar to what we have found in our H-*ras*-induced superficial papillary tumors (Fig. 4*C*). The difference is that the p16 gene is deleted in the RT4 cell line. The elevated expression of p16 protein in our mouse papillary tumors probably reflects the fact that our analysis is done on *in vivo* tumors, whereas the RT4 cell line had to overcome the senescence block to proliferate (3). In fact, only invasive, nonpapillary, human tumors can be cultured *in vitro* without having to go through a senescence crisis (3). Our observation of the loss of p107 and p130 expression in superficial papillary tumors in mice that do not progress to invasive bladder cancer suggests a senescence-like state in these tumors similar to that observed in triple knockout fibroblasts for Rb, p107, and p130 genes. In those cells, expression of an activated *ras* oncogene induces senescence (52). These data, together with our early results, suggest that our mouse papillary tumors are similar to human superficial papillary transitional cell carcinomas, which have a low propensity for progression due to the lack of genomic instability and the inability to bypass cellular senescence.

Unlocking the mechanisms responsible for urothelial growth and differentiation is critical for understanding both urothelial physiology and diseases. The strong similarities between mouse and human tumors, not only in phenotypes but also in cell cycle regulators, indicate the potential for these mouse models to be used in studying bladder cancer pathogenesis and in evaluating emerging therapies.

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