Deciphering Cancer Complexities in Genetically Engineered Mice

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Because the pRb pathway is disrupted in most solid human cancers, we have generated genetically engineered mouse cancer models by inactivating pRb function in several cell types, including astrocytes and mammary, prostate, ovarian, and brain choroid plexus epithelia. In every case, proliferation and apoptosis are acutely induced, predisposing to malignancy. Cell type dictates the pathways involved in tumor progression. In the astrocytoma model, we developed strategies to induce events in the adult brain, either throughout the tissue or focally. Both K-Ras activation and Pten inactivation play significant roles in progression. In the prostate model, adenocarcinoma progression depends on Pten inactivation. However, nonautonomous induction of p53 in the mesenchyme leads to evolution of both compartments, with p53 loss occurring in the mesenchyme. Thus, studies in these models continue to identify key tumorigenesis mechanisms. Furthermore, we are hopeful that the models will provide useful preclinical systems for diagnostic and therapeutic development.

In an effort to model and understand human cancers, we have generated several genetically engineered mouse (GEM) cancer models based on highly penetrant tumor suppressor gene aberrations observed in human tumors. Because the pRb pathway is disrupted in most solid human cancers, we have inactivated pRb function cell-specifically in the mouse by transgenic expression of T₁₂₁, a truncated SV40 T antigen that binds and inactivates pRb and related proteins p107 and p130. In numerous cell types, including mammary (Simin et al. 2004), prostate (Hill et al. 2005a,b), ovarian (C. Yin and T. Van Dyke, unpubl.), and brain choroid plexus (Symonds et al. 1994), epithelia and astrocytes (Xiao et al. 2002), inactivation of pRb, p107, and p130 leads to similar induction of aberrant proliferation and concomitant apoptosis (Fig. 1). In addition, these cellular responses have predisposed to malignancy in each case, producing fully penetrant preclinical models for cancer initiation and progression.

In exploring the genetic basis for tumor progression, we have discovered that the specific pathways involved depend on cell type. Whereas mammary and brain epithelia are similar in that p53 mediates most of the apoptotic response and its loss is the predominant mechanism for tumor progression (Symonds et al. 1994; Lu et al. 2001; Simin et al. 2004), astrocytic and prostate epithelial apoptosis depend on Pten (Xiao et al. 2002; Hill et al. 2005a). Brain and mammary epithelia are also divergent in their requirement for E2F1 for proliferation. In choroid plexus epithelium, proliferation is mediated by E2F1 activity (Pan et al. 1998), in contrast to mammary epithelium, where E2F1 activity is dispensable (M. Sardo and T. Van Dyke, unpubl.). These differences have significant impli-

	pRb _f → (T ₁₂₁)	aberrant proliferation +	apoptosis →	additional events; progression
brain (CPE)	✓	E2F1 E2F2,3	✓ p53 E2F1 → p53 → Bax	✓ p53
mammary	✓	not E2F1	✓ p53 not E2F1	✓ p53
prostate	✓		✓ Pten	✓ Pten ^l Pten
ovarian	✓		✓	✓
astrocytes	✓		✓ Pten	✓ Pten

Figure 1. Summary of cancer progression mechanisms. A similar course of events occurs in each system analyzed: T₁₂₁ blocks pRb activity and causes aberrant proliferation and concomitant increased apoptosis; a selective pressure exists for reduced apoptosis among these neoplastic cells, leading to a subsequent loss of apoptosis and acquisition of other tumor-promoting alterations. However, despite the similarity of events, cells differ in the mechanisms they employ for these functions; brain epithelial cells (CPE) and breast cells rely on p53-mediated apoptosis, whereas astrocytes and prostate epithelial cells depend on Pten-mediated apoptosis. Likewise, whereas E2F1 is required for apoptosis and optimal cellular proliferation in CPE, E2F1 is dispensable in mammary epithelial cells. These differences affect the evolution and biology of each tumor type and may ultimately influence the way we diagnose and treat these cancers.

cations for the mechanisms of tumor progression and the biological behaviors of respective tumor types.

In recent studies, we have examined the complex mechanisms of tumor progression in astrocytic and prostate cancers, including the contribution of multiple Pten-regulated pathways to multiple aspects of tumor progression, such as cell survival, invasion, and angiogenesis (Hill et al. 2005a; Xiao et al. 2005), and of stro-

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mal p53 loss to the progression of prostate tumor (Hill et al. 2005b). Thus, the complex dynamics of tumor evolution are being deciphered for these significant human cancers. Our current efforts to understand these mechanisms are discussed.

MATERIALS AND METHODS

Mice. The generation and the characterization of *TgGZT₁₂₁*, *TgG(ΔZ)T₁₂₁* (Xiao et al. 2002), and *TgAPT₁₂₁* (Hill et al. 2005a) mice have been described previously. We generated *TgG(ΔZ)T₁₂₁* by mating *TgGZT₁₂₁* mice to *Tgβ-actinCre* mice (Lewandoski and Martin 1997) to generate bi-transgenic mice, selecting for pure *TgG(ΔZ)T₁₂₁* mice in subsequent generations. Screening of the *Pten* nullizygous allele has been described previously (Di Cristofano et al. 1998). Characterization and screening of the *p53* null allele (Jacks et al. 1994), the conditional *Pten* allele (Trotman et al. 2003; Xiao et al. 2005), and the *LSL-K-ras^{G12D}* allele (Jackson et al. 2001) have been described. *TgGFAP-CreER* mice were constructed and characterized in the McCarthy lab (K. McCarthy et al., unpubl.) and are genotyped using the following parameters: primers are 5'-TGATGAGGTTTCGCAAGAACC-3' and 5'-CCATGAGTGAACGAACCTGG-3'. PCR conditions: 94°C 1.5 min, 35 cycles of 94°C 30 sec, 59°C 1 min, and 72°C 5 min, and finally 72°C 5 min.

Tamoxifen-induced Cre expression. Cre recombinase was activated in *TgGFAP-CreER* mice by daily i.p. injections of 1 mg of 4-hydroxytamoxifen (4-OHT) over 5 days. Tamoxifen was dissolved in a 1:9 mixture of ethanol and sunflower oil (100 μl), facilitated by 10-minute sonication at mid power. Vehicle-only injections served as a control.

Brain histology and immunohistochemistry. Brain tissue was fixed in formalin (10%) for 20–24 hours and then stored in ethanol (70%). Paraffin-embedded sections (5 μm thick) were processed using standard immunohistochemical methods. The antibodies and their dilution were: anti-p-Erk 42/44 (Cell Signaling, 1:50) and anti-SV40 Tag (1:200, mouse monoclonal, Oncogene). Briefly, samples were deparaffinized with Histo-Clear (National Diagnostics), then hydrated through a graded ethanol series. Boiling-mediated antigen retrieval was performed using citrate buffer (pH 6.0) and microwave heating for 2 minutes at high power followed by 7 minutes at low power. Endogenous peroxidases were quenched with H₂O₂ (3%). Immunohistochemistry was performed using the Vectastain ABC kit with the NovaRED (Vector Laboratories, Burlingame, California) peroxidase substrate as chromogen. Samples were counterstained with Harris hematoxylin (Sigma, St. Louis, Missouri). A subset of samples were stained with ABC-AP substrate kit (Vector Laboratories) and counterstained with Nuclear Fast Red.

Prostate histopathology and in situ assays. Prostate samples were prepared and analyzed as reported previously (Hill et al. 2005a,b).

Laser capture microdissection and LOH analysis.

Laser capture microdissection (LCM) of H&E-stained sections was performed using a Leica AS LMD (Leica Microsystems Inc., Bannockburn, Illinois) as described previously (Hill et al. 2005b). Briefly, formalin-fixed paraffin-embedded tissue sections were mounted onto Glass Foiled PEN slides (Vashaw Scientific, Atlanta, Georgia). Cells were collected in 50 μl of lysis buffer (10 mM Tris-HCl, pH 8.0, 1% Tween-20) with addition of 5 μl of proteinase K (100 mg/ml), and incubated at 55°C overnight. Proteinase K was inactivated at 99°C for 10 minutes, and 5–10-μl aliquots were used for PCR analysis. The primers for semi-quantitative PCR and quantitative real-time PCR analysis were as described previously (Hill et al. 2005b). The data were analyzed using SDS 2.1 software (Applied Biosystems, Foster City, California) and standard protocols (<http://www.appliedbiosystems.com>) as previously described (Hill et al. 2005b).

Statistical analysis. Binomial exact test was performed using SAS 9.1 (Cary, North Carolina) to determine whether loss of the wild-type *p53* allele was statistically significant in tumor stroma of *TgAPT₁₂₁;p53^{+/-}* and *TgAPT₁₂₁;p53^{+/+}* mice. The probability of random wild-type *p53* allele loss in the tumor stroma was arbitrarily set at 1%. However, results remain significant ($p < 0.0001$) even if the probability of random loss is as high as 10%.

RESULTS

An Astrocytoma Model and New Systems for Inducing T₁₂₁

Astrocytoma is the most common primary brain tumor in humans and is rarely amenable to standard therapies. We have engineered mouse models to gain insight to the mechanisms of this poorly understood and often lethal disease, because to fully understand the complexities of such pathologies requires investigation in vivo. The vast majority (70–80%) of high-grade anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV, GBM) harbor mutations in the pRb pathway (Henson et al. 1994; Ueki et al. 1996) in addition to other genetic alterations such as p53 (~30%) and PTEN loss-of-function (~50%) and EGFR gain-of-function (50%) mutations (Louis and Cavenee 1997; Maher et al. 2001; Reilly and Jacks 2001). Such high frequencies indicate that these defects are often concurrent with pRb pathway loss. Therefore, we designed experiments to investigate the combined effects of these perturbations in the defined experimental setting of GEM.

We used a promoter from the human gene encoding *glial fibrillary acidic protein (GFAP)* to direct T₁₂₁ expression within the astrocyte lineage of the central nervous system (CNS) (Fig. 2A) (Xiao et al. 2002). However, expression of T₁₂₁ is dependent on Cre recombinase activity, since a *lacZ* reporter gene flanked by lox P sites lies between the promoter and the T₁₂₁ gene (*TgGZT₁₂₁* mice). In *TgG(ΔZ)T₁₂₁* (generated after crossing to *Tgβ-actinCre* mice; see Materials and Methods) (Lewandoski and Martin 1997), T₁₂₁ is expressed from embryonic day

Figure 2. Conditional inactivation of pRb, p107, and p130 combined with Ras activation in adult astrocytes causes progression to high-grade astrocytoma. Two *Cre*-conditional alleles, encoding T₁₂₁ (*TgGZT₁₂₁*) and an activating K-ras point mutant (*LSL-K-ras^{G12D}*), were used to test the consequences of pRb function inactivation and Ras activation, respectively, singly and combined (A). Both genes were activated in adult mice at 2–3 months of age using a tamoxifen-inducible Cre recombinase encoded by the *GFAP-CreER* transgene to avoid confounding developmental effects. Two months post-4-OHT, mice with a combination of T₁₂₁ and activated K-ras (E–G) show a dramatic expansion of astrocytes that express T₁₂₁ (E) as compared to mice expressing T₁₂₁ alone (B–D). Ras-dependent activation of the MAPK pathway is indicated in cortical neoplastic cells by phospho-Erk staining (G) but not in those of the thalamus (F). At 4 months post-4-OHT, both angiogenesis (H) and necrosis are apparent (I) in mice with both T₁₂₁ and activated K-ras. Samples B, C, E, F, H, and I are thalamus; D and G are cortex.

11.5 onward and ultimately results in high-grade astrocytoma in adult mice with 100% penetrance (Xiao et al. 2002). Although we observed diffuse grade III astrocytomas, none had progressed to malignant grade IV astrocytoma (also called glioblastoma multiforme, or GBM). A secondary phenotype we observed in brains of these mice was an expansion of cells in the subventricular zone (SVZ), a cell population of neuronal lineage and not a glial lineage, which we hypothesize was due to the early expression of GFAP promoter in neuronal progenitor

Figure 3. Focal inactivation of pRb function in adult astrocytes by lentiviral *Cre* delivery. As an alternative to germ-line transgenesis using tissue-specific promoter elements, we introduced lentivirus harboring a self-excising-*Cre* construct (unpublished, Tal Kafri, UNC) into the frontal cortex of *TgGZT₁₂₁* transgenic mice via stereotaxic injection to initiate localized tumorigenesis. Nineteen days postinjection, T₁₂₁ (dark brown stain) can be detected along the needle track (dashed lines) in astrocytic cells (A). Six months postinjection, clusters of astrocytic cells (arrows) can be seen along the injection site (C) and as far as 1500 μ m away. Magnifications: A, C, 100 $\times$; B, D, 400 $\times$. B and D are higher magnifications of the regions boxed in A and C, respectively.

cells. To circumvent the possible effects of T₁₂₁ activity during CNS development and to better model the human disease, we crossed *TgGZT₁₂₁* mice with *TgGFAP-CreER* mice (K. McCarthy, UNC), such that Cre activity can be controlled by treatment with 4-OHT (Fig. 3A). Indeed, T₁₂₁ was inducible in astrocytes of adult mice (2–3 months of age) and yielded diffuse astrocytoma over time as observed in *TgG(Δ)T₁₂₁* mice, establishing a valuable inducible model of astrocytoma initiation.

We investigated the possibility that cooperating mutations would synergize with pRb pathway loss to facilitate tumor progression to more severe grades of disease. Although not frequently mutated, Ras is frequently activated in human high-grade astrocytomas (Bos 1989), likely through amplification or mutation of the epidermal growth factor receptor (EGFR; ~40–60% of high-grade tumors) or platelet-derived growth factor (PDGF). Thus, Ras activation generally coexists with aberrations of the pRb pathway. Previous studies showed that overexpression of mutant H-Ras in transgenic astrocytes (Ding et al. 2001), or of activated K-ras in combination with activated Akt (Holland et al. 2000), predisposed GBM-like lesions. We examined the consequences of astrocyte-specific endogenous K-ras activation alone and together with astrocytic T₁₂₁ expression. Through matings, we generated *TgGZT₁₂₁;TgGFAP-CreER, LSL-K-ras^{G12D/+};TgGFAP-CreER*, and *TgGZT₁₂₁; LSL-K-ras^{G12D/+};TgGFAP-CreER* mice (Fig 2A). At 2–3 months of age, Cre activity was induced by treating the mice with 4-OHT (see Materials and Methods) and brains were analyzed at 2 and 4 months postinduction. Activation of Ras alone in astrocytes had no apparent effects up to 4 months post 4-OHT injection (not shown), whereas activation of T₁₂₁ in astrocytes induced an astrocytic expansion within 2

months as described above (Fig. 2B). However, a more dramatic phenotype was observed when both T_{121} and $K-ras$ were activated simultaneously; by 2 months following 4-OHT injections, brains showed features characteristic of human astrocytoma, including peri-neuron and perivascular satellitosis (Fig. 2E). Neoplastic astrocytes were positive for T_{121} , and evidence for Ras-specific activation of the MAPK pathway was demonstrated by phospho-Erk IHC. Importantly, phospho-Erk was detectable in the cortex, but not in the thalamus (Fig. 2E, F), suggesting region-specific effects.

Significantly, by 4 months post 4-OHT, angiogenesis (Fig. 2H) and necrosis (Fig. 2I), both properties of GBM, were apparent when both Ras and T_{121} were activated, but not when either was singly activated (data not shown). We conclude from these results that pRb pathway loss is sufficient to predispose astrocytes to tumorigenesis, and that additional events, such as Ras activation, are required for astrocytoma tumor progression to GBM.

Somatic Induction of T_{121} Using Cre-expressing Lentivirus Vectors

The recombinase-mediated experiments outlined above provide temporal, but not spatial, control of tumor induction. Since human cancers likely evolve from a single cell, initiating tumorigenesis focally rather than in a

large cell population may provide a more authentic model of sporadic cancer, mimicking the scenario in which a transformed cell originates within a microenvironment of wild-type cells. As an alternative to germ-line transgenesis using tissue-specific promoter elements, we are developing retroviral approaches to somatically deliver Cre recombinase in adult cells in more limited regions. In one model, lentivirus harboring a self-excising-Cre gene (a kind gift of Tal Kafri, UNC) was introduced into brains of $TgGZT_{121}$ transgenic mice via stereotaxic injection to initiate local tumorigenesis by expressing T_{121} in a limited number of astrocytes. One microliter of lentivirus (10^9 IU/ml) was injected into the cortex of adult $TgGZT_{121}$ mice ($n = 4$). Nineteen days post injection, T_{121} was detected by IHC along the needle track in astrocytic cells ($n = 2$, Fig. 3A,B). To determine whether local T_{121} expression was sufficient to generate astrocytoma, the remaining mice ($n = 2$) were aged for 6 months. Despite T_{121} expression in clusters of astrocytic cells along the injection site (Fig. 3C,D), and as far as 1500 μm away, these conditions were insufficient for astrocytoma development. Although we did not observe tumors in this small cohort, the results of this pilot study show promise for this technique; therefore, we continue to explore the consequences of limited regional pRb pathway inactivation together with additional events such as $K-ras$ activation and $Pten$ inactivation, and evaluate its effectiveness as a new paradigm for modeling sporadic cancer.

Figure 4. Regional somatic $Pten$ inactivation in pRb-inactivated astrocytes. Somatic delivery of Cre (A) induces accumulation of neoplastic cells. PCNA immunohistochemical staining of brains 5 weeks after retroviral delivery. Viral-Cre delivery to $TgG(\Delta Z)T_{121};Pten^{lox/lox}$ brains induced dense neoplastic cell foci at the injection sites (D) and in the regions as far as 150 μm away (E). The highly dense neoplastic cell foci were not present at the comparable region of the uninjected contralateral hemisphere (B). Focal tumor development was not due to retrovirus infection ($TgG(\Delta Z)T_{121};Pten^{lox/lox}$ + GFP) or Cre protein ($TgG(\Delta Z)T_{121};Pten^{+/+}$ + Cre; C) alone, because no such nodules were found in these controls. The only morphologic difference observed between these brains and those not injected (data not shown) is the needle track (C, arrows). In $TgG(\Delta Z)T_{121};Pten^{lox/lox}$ brains, the apoptotic level was decreased in tumor cell clusters near injection sites ($0.69 \pm 0.04\%$), compared with contralateral regions ($3.31 \pm 1.24\%$) and distant adjacent regions (300 μm from the injection site) where injection tracks were not present ($3.51 \pm 0.20\%$). Similar levels of apoptosis were also found at the injected sites of controls ($2.50 \pm 0.53\%$ and $2.86 \pm 0.30\%$). The $TgG(\Delta Z)T_{121};Pten^{lox/lox}$ + Cre group is compared with each of the other groups; $p < 0.05$ is considered statistically significant. (Portions of this figure adapted from Xiao et al. 2005.)

In a similar experimental approach, we used somatic viral delivery to measure direct and long-term effects of depleting *Pten* locally in initiated cells already deficient in pRb pathway function, reminiscent of the clonal changes acquired by human cancers during tumor progression (Xiao et al. 2005). *Pten* mutations are associated with a significant fraction of human high-grade astrocytomas (30–50%). We employed an *MSCV-Cre* viral vector (a kind gift from L. Su, UNC) to assay the effects of *Pten* loss within initiated tumor cells (Fig. 4A). Because normal astrocytes are non-dividing when terminally differentiated, we reasoned that cells expressing T_{121} would be more susceptible to retroviral gene delivery because they are highly proliferative at all ages. Indeed, tumor cells along the injection tracks of $TgG(\Delta Z)T_{121}; Pten^{loxp/loxp}$ mice had significantly reduced apoptosis ($p < 0.05$), which is evoked by pRb pathway inactivation, as compared with a distant contralateral region (Fig. 4F). Furthermore, tumor-cell density foci were detected at some distance from the injection tracks in $TgG(\Delta Z)T_{121}; Pten^{loxp/loxp}$ brains (Fig. 4E), but not $TgG(\Delta Z)T_{121}; Pten^{+/+}$ brains (Fig. 4C), indicating *Pten* loss may also facilitate tumor cell invasion.

Using primary astrocytes from $TgG(\Delta Z)T_{121}; Pten^{loxp/loxp}$ newborn mice, we showed that *Pten* inactivation by adenovirus-*MSCV-Cre* induced invasion through Matrigel. This primary cell model facilitates analysis of pathways involved in tumorigenic properties and should provide a valuable resource for therapeutic target discovery and validation. In preliminary tests using inhibitors, we showed that invasion induced by *Pten* inactivation was inhibited by both a PI3 kinase inhibitor and a pan protein kinase C (PKC) inhibitor. Furthermore, treatment of cells

with a peptide inhibitor of the atypical PKC- ζ also significantly suppressed Matrigel invasion (Xiao et al. 2005).

These studies indicate that *Pten* loss can potentiate multiple tumorigenic properties in neoplastic astrocytes initiated by inactivation of pRb function and provide both primary cell and animal models for further assessment of the pathways and mechanisms involved. Current studies are exploring the relative contributions of both *K-ras* activation and *Pten* inactivation to astrocytoma progression using each of the above-described strategies.

Complexity of Tumor Progression in a Model of Prostate Cancer

A number of useful transgenic models of prostate cancer have provided the means to investigate one of the most common epithelial cancers afflicting men (Greenberg et al. 1994; Yoshidome et al. 1998). However, because these previous strategies employed wild-type SV40 Large T antigen or the full SV40 early region, these models do not permit assessment of the individual contributions of pRb family loss versus the multiple other concomitant perturbations, some of which have demonstrated roles in tumorigenesis, such as p53 or p300 deficiency, or blocked phosphatase PP2A function. We have shown that inactivation of pRb function mediated by modified rat *probasin* promoter-driven expression of T_{121} (*TgAPT₁₂₁*) is sufficient for tumor initiation, produces widespread intraepithelial neoplasia (PIN) consisting of high rates of epithelial cell proliferation and apoptosis (Fig. 5B), and ultimately progresses to locally invasive adenocarcinoma

Figure 5. *TgAPT₁₂₁* mice develop prostate adenocarcinoma. Inactivation of pRb function leads to aberrant proliferation, and apoptosis is mediated by *Pten*, not p53. Additional events such as loss of *Pten* and/or p53 lead to heterogeneous tumor progression. Prostate sections (5 μ m) shown are from an 8-week-old nontransgenic littermate (A) and *TgAPT₁₂₁* mice of different ages (B,C). Normal prostate displays a single layer of luminal cells and a thin fibromuscular stromal cell layer (A). By 12 weeks (B), mouse PIN (mPIN) appears broadly where glands are filled with atypical cells in a cribriform pattern. More advanced lesions are present in prostates of 30-week-old *TgAPT₁₂₁* mice, including microinvasive glands with a desmoplastic response and well-differentiated adenocarcinoma (C). *Pten* heterozygosity accelerates the onset of mPIN and adenocarcinoma in *TgAPT₁₂₁* mice (D). In addition, morphologically distinct areas of adenocarcinoma progression also appear (D, 30-week-old, *star*). p53 heterozygosity does not accelerate the onset of mPIN; however, stromal tumors develop as young as 22 weeks of age (E) and consist of an abundance of stromal cells (*star*). All sections were stained with hematoxylin and eosin (H&E). (A–D, Adapted from Hill et al. 2005a; E, adapted from Hill et al. 2005b.)

with no evidence of neuroendocrine tumors (Fig. 5C) (Hill et al. 2005a). Therefore, the *TgAPT₁₂₁* mouse line provides a fully penetrant mouse model of prostate cancer initiation with which to dissect the mechanisms of tumor progression.

Roles of *Pten* in Prostate Cancer Suppression

Because apoptosis induced by pRb functional loss is mediated through different mechanisms in different cell types, we examined the pathway(s) used by *TgAPT₁₂₁* prostates with a genetic approach. Using the TUNEL assay, we quantified prostate apoptosis levels in 8-week-old mice of the following genotypes: wild type, *TgAPT₁₂₁*, *TgAPT₁₂₁;Pten^{+/-}*, *TgAPT₁₂₁;p53^{+/-}*, and *TgAPT₁₂₁;p53^{-/-}*. In *TgAPT₁₂₁* prostates, hemizyosity or nullizyosity of *p53* caused no reduction in the apoptotic index, whereas hemizyosity at *Pten* caused a 50% reduction in the apoptotic index accelerating progression to adenocarcinomas with heterogeneous composition (Fig. 6A). Het-

erogeneity is associated with concurrent *Pten* haploinsufficiency and selective focal tumor progression identifiable by concomitant *Pten* loss (Fig. 6C), the appearance of membrane-bound phospho-Akt (p-Akt)(Fig. 6D), a further reduction in apoptosis levels (Fig. 7E), and distinct morphology (Fig. 6B).

Unexpected Roles for p53 in Epithelial/Mesenchymal Interactions

Although p53 is not involved in epithelial apoptosis in *TgAPT₁₂₁* mice, we found that it plays a critical role in tumor evolution. *T₁₂₁* expression in prostate epithelium resulted in p53 induction in both epithelial and mesenchymal compartments (Hill et al. 2005b). p53 appears to

Figure 6. *Pten* mediates apoptosis in *TgAPT₁₂₁* prostate, and its loss contributes to focal tumor progression. (A) Prostates from 8-week-old non-transgenic, *TgAPT₁₂₁*, *TgAPT₁₂₁;p53^{+/-}*, *TgAPT₁₂₁;p53^{-/-}*, and *TgAPT₁₂₁;Pten^{+/-}* mice were analyzed for apoptosis levels quantified by calculating the number of TUNEL-positive cells (brown) as a percentage of the total (methyl green). *n* = 4 for each genotype. (B) A representative H&E-stained section shows a transition where focal adenocarcinoma develops from neoplastic areas with back-to-back gland structure. (C) Immunohistochemistry for *Pten* (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 (D; green). (E) Apoptosis assay was performed via TUNEL in those p-Akt expressed and nonexpressed regions (white boxes). p-Akt expressed cells, indicating loss of *Pten*, have further reduction of apoptosis. (Adapted from Hill et al. 2005a.)

Figure 7. Stromal p53 loss in *TgAPT₁₂₁;p53^{+/-}* and *TgAPT₁₂₁* tumors. Serial sections of an emerging stromal tumor in *TgAPT₁₂₁;p53^{+/-}* mice with expanding mesenchyme (star) and adjacent mPIN (arrow) are shown (A; H&E) and (B; p53 IF). p53 expression is lost in the tumor mesenchyme (B, star) and retained in the epithelium in addition to the mPIN-associated stroma (arrow). A total of 18 *APT₁₂₁;p53^{+/-}* mice with stromal tumors were examined, and all were found to have lost p53 expression in the stroma. Epithelial p53 loss subsequent to stromal p53 loss compounds heterogeneous tumor progression. In H&E-stained *TgAPT₁₂₁;p53^{+/-}* tumor sections (C), regions of dense epithelial cell growth morphologically distinct from surrounding epithelium (arrows) grow in small back-to-back circular glands (star). IF for p53 (yellow) shows that such regions no longer express p53. Representative tumors from 7-month-old *TgAPT₁₂₁;p53^{+/-}* mice are shown (D). PCR analysis on stromal DNA extracted from laser-captured microdissected (LCM) samples in *TgAPT₁₂₁;p53^{+/-}* mice showed that stromal loss of wild-type *p53* allele occurred in four of six tumors (F). Similar stromal loss of p53 expression is observed in stromal tumors arising in *TgAPT₁₂₁;p53^{+/-}* mice (*n* = 8). Real-time quantitative PCR was performed on stromal DNA extracted from LCM samples to determine the status of wild-type *p53* alleles in stromal tumors or tissues of *TgAPT₁₂₁* mice. LCM muscle samples were used as controls. Among 11 stromal tumor samples from 11 distinct animals, 7 showed loss of 1 wild-type allele of *p53*, 2 showed loss of both alleles of *p53*, and 2 retained both wild-type alleles. (Portions of this figure adapted from Hill et al. 2005b.)

suppress fibroblast proliferation in the stroma, since these cells proliferated in a *TgAPT₁₂₁ p53*-deficient, but not wild-type or heterozygous, background (Hill et al. 2005b). Indeed, all *TgAPT₁₂₁;p53^{-/-}* and *TgAPT₁₂₁;p53^{+/-}* (Fig. 5E) male mice developed large tumors composed of extensive stroma, a phenotype that develops most rapidly in the *p53* null background. Tumor fibroblasts of the *p53* heterozygous background no longer expressed *p53* (Fig. 7B), and laser capture microdissection-PCR analysis showed that the wild-type *p53* allele was selectively lost in this compartment in most tumors (Fig. 7B,F). Similar stromal loss of *p53* expression is observed in stromal tumors arising in *TgAPT₁₂₁;p53^{+/-}* mice ($n = 8$). Real-time quantitative PCR on stromal DNA extracted from LCM samples showed that among 11 stromal tumor samples from 11 distinct animals, 7 lost 1 wild-type allele of *p53*, 2 lost both alleles of *p53*, and 2 retained both wild-type alleles (Fig. 7E) (Hill et al. 2005b). Although the epithelium within these tumors initially retained *p53* expression (Fig. 7B), focal regions subsequently lost *p53* expression as well (Fig. 7D), suggesting an as yet unknown function of *p53* as tumor suppressor in this compartment. These studies demonstrate that non-cell-autonomous responses to epithelial tumorigenic lesions can create a microenvironment in which selective genetic changes occur in non-tumor cells, causing coevolution of multiple cell types within the tumor. Current studies are focused on identifying the *p53*-inductive signal(s) from initiated epithelium to the mesenchyme and on the mechanisms of *p53* tumor suppression in multiple prostate compartments.

CONCLUSIONS

By exploiting *T₁₂₁* to inactivate pRb and its related family members, we have explored the requirement of pRb tumor suppression in glial cells and multiple epithelial cell types, including brain (CPE), prostate, and breast. This has been a powerful approach to generate highly penetrant mouse models of cancer that are reflective of human tumors in which the pRb pathway is perturbed and that evolve through the accumulation of additional genetic or epigenetic aberrations. We have dissected some of the genetic pathways contributing to tumor progression both by combining the initiated transgenic models with other engineered germ-line mutations and by analyzing the natural evolution of progressing tumors. Through these experiments, we have identified differences in tumor suppressor mechanisms invoked by varying cell types. More specifically, in each of the systems studied, there is a similar course of events: *T₁₂₁* expression causes aberrant proliferation and concomitant increased levels of apoptosis, resulting in a strong selective pressure for reduced apoptosis. Tumors progress when a major tumor suppressor is inactivated, resulting in reduced apoptosis, but also in additional tumor-promoting functions. In response to pRb function loss, CPE and breast cells rely on *p53*-mediated apoptosis, whereas astrocytes and prostate epithelium depend on *Pten* activity. These differences significantly affect the dynamics of

cancer evolution, and therefore may have wide-ranging implications in the interpretation of diagnostic tumor mutation analysis and in the development of appropriately targeted therapies.

Because *T₁₂₁* so effectively evokes illicit cellular proliferation and apoptosis, we have until recently relied on gene regulatory sequences to direct *T₁₂₁* expression only in adult cells to avoid secondary developmental defects. We have further refined our strategy by engineering conditional alleles using *Cre-LoxP* technology to more finely restrict the timing and location of *T₁₂₁* expression. Building on this conditional approach, we now also employ retroviral vectors to manipulate more limited cell populations to investigate the consequences of focal events, both in tumor initiation and in events that may contribute to tumor progression. This paradigm more closely mimics natural spontaneous tumor evolution. Combined, these strategies facilitate the analysis of acute consequences to specific genetic lesions, both intrinsic and extrinsic, and the dissection of natural events in the evolution of specific tumors. We are hopeful that the GEM models produced in these studies will also provide useful preclinical systems for diagnostic and therapeutic development.

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