Pten Deficiency Activates Distinct Downstream Signaling Pathways in a Tissue-Specific Manner

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Abstract

PTEN deficiency predisposes to a subset of human cancers, but the mechanism that underlies such selectivity is unknown. We have generated a mouse line that conditionally deletes Pten in urogenital epithelium. These mice develop carcinomas at high frequency in the prostate but at relatively low frequency in the bladder, despite early and complete penetrance of hyperplasia in both organs. Cell proliferation is initially high in the bladder of newborn Pten-deficient mice but within days is inhibited by p21 induction. In contrast, proliferation remains elevated in Pten-deficient prostate, where p21 is never induced, suggesting that p21 induction is a bladder-specific compensatory mechanism to inhibit proliferation caused by Pten deletion. Furthermore, the AKT/mammalian target of rapamycin growth pathway, which is highly activated in Pten-deficient prostate, is not activated in bladder epithelium. Our results reveal alternative downstream signaling pathways activated by Pten deficiency that lead to tissue-specific susceptibilities to tumorigenesis. (Cancer Res 2006; 66(4): 1929-39)

Introduction

The PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumor suppressor gene is one of the most frequently mutated/deleted genes in human cancer (1–4). Germ line PTEN mutations have been found to occur in 80% of patients with classic Cowden syndrome, a heritable multiple hamartoma syndrome characterized by a high risk of breast, thyroid, and endometrial carcinoma and occasional incidence of other types of cancers, including bladder and renal cell carcinoma. The types of cancer arising in Cowden syndrome patients exhibit strong tissue specificity.

Homozygosity for the null mutation (Pten-/-) in mouse models results in early embryonic lethality, indicating that the function of PTEN is critical for normal development (5–7). Pten heterozygous mice (Pten+/-) develop a spectrum of neoplasias in organs, such as the breast, thyroid, endometrium, and prostate, which closely resembles the situation in humans with PTEN mutation (5, 8–10). Somatic PTEN mutations in humans occur with a wide distribution of frequencies in sporadic primary tumors, with the highest frequencies in endometrial carcinomas and glioblastoma (11). Pten mutation is among the most common genetic alterations reported in human prostate cancers, with loss of heterozygosity (LOH) of PTEN found in 30% of primary prostate cancers (12–14) and 63% of metastatic prostate cancer tissue samples (6). In contrast, the frequency of Pten mutation in bladder cancer is considerably lower than that of prostate cancer (15, 16). The mechanism of tissue specificity in tumor predisposition as a result of Pten loss is not clear.

A major substrate of PTEN is phosphatidylinositol 3,4,5-trisphosphate (PIP3), a lipid second messenger produced by phosphatidylinositol 3-kinase (PI3K; ref. 17). PTEN dephosphorylates PIP3 at the D3 position of the inositol ring to produce phosphatidylinositol 4,5-bisphosphate (PIP2). In the absence of PTEN activity, PIP3 concentrations are increased, leading to enhanced phosphorylation and activation of the survival-promoting factor AKT/PKB, whose activity is stimulated by PIP3 (18–21). Interaction of AKT with PIP3 results in an activating conformational change of AKT and its translocation from the cytoplasm to the plasma membrane, where it is further activated through phosphorylation. Activation of AKT has been shown to regulate a vast array of cellular responses, including cell proliferation, adhesion, growth, and survival (17, 22). AKT substrates include FOXO3a, TSC2 (Tuberin), and GSK-3β, among others (23). AKT has been shown to phosphorylate FOXO3a, thereby preventing the nuclear translocation of FOXO3a. The phosphorylated FOXO3a transcription factor is thus unable to up-regulate proapoptotic target genes, and thus, cell survival is promoted (24). On the other hand, the phosphorylation of TSC2 by AKT has been shown to be important for regulation of cellular metabolism and growth. Specifically, phosphorylation of TSC2 by AKT disrupts complex formation of TSC2 with TSC1 (Hamartin), preventing the complex from inhibiting the small G protein Rheb, which in turn activates the mammalian target of rapamycin (mTOR). Thus, mTOR activates the 70-kDa ribosomal S6 kinase (p70S6K) and inhibits elongation-initiation factor 4E binding protein-1 (4E-BP1), both events that result in increased translation (25). Increased cell growth and cell size are direct consequences of abnormal AKT activation due to Pten deficiency (26).

There have also been reports that AKT can affect cell proliferation and survival by regulating protein levels and subcellular localization of the cyclin-dependent kinase (cdk) inhibitor (CKI) p21. Studies have shown that AKT can increase p21 levels by enhancing protein stability, either through direct phosphorylation of p21 (27) or through inhibition of GSK-3β, which can phosphorylate p21 and increase its degradation (28). AKT has also been shown to induce cytoplasmic localization of p21 in cells overexpressing HER-2/neu, thereby blocking the cell growth inhibitory activity of nuclear p21 (29). In the nucleus, p21 binds to cyclin E/cdk2 complexes, inhibiting kinase activity, but p21 facilitates cyclin D association with cdk4/6 (30); thus, its effect on cell cycle progression may be dependent on the ratio of the cyclins, cdks, and p21 present in the cell. p21 can inhibit DNA replication...
by binding to and inhibiting the proliferating cell nuclear antigen (31). p21 also protects cells from apoptosis through multiple mechanisms, including inhibition of caspase-3 activation (7).

In this study, we address the tissue specificity of tumor formation caused by Pten deficiency in conditional Pten mutant mice induced by Fabpl-Cre–mediated deletion. Our results show that Pten deficiency in different tissues elicits drastically different downstream signaling pathways and cellular responses that may be an important basis for the differential propensities for tumor formation following Pten loss in different tissues.

Materials and Methods

Mice. Mice homozygous for floxed Pten exon 5 (Ptenloxp/loxP; ref. 32) were crossed to mice transgenic for Fabpl-Cre (33). F1 Ptenloxp/loxP; Fabpl-Cre–mice were crossed with Ptenloxp/loxP mice to obtain Ptenloxp/loxP;Fabpl-Cre–mice. P21+/− mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and crossed to the Pten conditional knockout mice. Littermate controls lacking the Fabpl-Cre transgene were used in all experiments. When bladder epithelium was needed for protein or RNA analysis, the epithelial layer was peeled away from the muscle in isolated bladders using forceps under a dissecting microscope.

PCR analysis. Mice were genotyped by PCR as described (32, 33) or with the JAX protocol. For detection of exon 5 deletion, organs were harvested and incubated overnight in digestion buffer [100 mmol/L Tris (pH 8), 1 mmol/L EDTA, 0.2% SDS, 200 mmol/L NaCl, 100 μg/mL proteinase K]. DNA was phenol/chloroform extracted and ethanol precipitated. The same primers used to genotype the loxp mice were used to detect Cre-mediated recombination at the Pten locus.

Immunohistochemistry. Tissues were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C, 16 to 24 hours. The next day, tissues were rinsed in PBS and stored in either PBS or 70% ethanol until they were paraffin embedded, sectioned, and mounted on slides. Some slides were stained with H&E. For immunohistochemistry, slides were deparaffinized, immersed in 0.5% hydrogen peroxide in PBS, boiled in 10 mmol/L citrate buffer (pH 6) in a microwave oven for 10 minutes, and blocked in 3% donkey serum/0.2% Triton X-100/PBS for 1 hour. Primary antibodies were diluted in block as follows: E-cadherin (10 μg/mL; Zymed, South San Francisco, CA), phospho-AKT Ser473 (1:50; Cell Signaling Technology, Beverly, MA), phospho-histone 3 Ser10 (1:1,000; Upstate, Beverly, MA), p21 (1:25; BD Biosciences, San Jose, CA), Ki-67 (1:50; DAKO, Carpinteria, CA), phospho-activin A receptor type IIB Ser145 (1:1,000; Upstate, Charlottesville, VA), pan-uruplakin (1:1,000; gift from Tung-Tien Sun), b-catenin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), p63 (1:100; gift from Frank McKeon). Most slides were washed and incubated with biotinylated secondary antibodies, and signals were amplified with an avidin-biotin complex kit and detected with a 3,3'-diaminobenzidine kit (Vector Labs, Burlingame, CA). Slides are counterstained with hematoxylin, dehydrated, and mounted. Slides incubated with b-catenin antibody were stained with Cy3-conjugated secondary antibodies (Jackson Immunomagnetic, West Grove, PA) and counterstained with Hoechst dye.

Immunoblots. Tissues were flash frozen in liquid nitrogen and stored at −80°C until processing. To prepare protein lysates, tissues were dounced in cold lysis buffer [20 mmol/L HEPEs (pH 7.2), 5 mmol/L EDTA, 2 mmol/L EGTA, 5 mmol/L NaF, 40 mmol/L β-glycerophosphate (pH 7.2), 1 mmol/L sodium orthovanadate, 1× protease inhibitor cocktail (Roche, Indianapolis, IN), 0.5% NP40] and spun at 13,000 rpm for 10 minutes. The supernatant was collected, and an equal volume of 2× SDS electrophoresis sample buffer was added. Samples were run on SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA), and blocked in 3% milk/TBST. Primary antibodies were diluted in either 3% milk/TBST [phospho-FKHRL1 (FOXO3a) Thr32]200, 1:1,000 (Upstate); glyceraldehyde-3-phosphate dehydrogenase, 1:1000 (US Biological, Swampscott, MA); p27, 1:1,000 (Santa Cruz Biotechnology); Akt, 1:1000] or 5% bovine serum albumin/TBST [p21, 1:500 (BD Biosciences); the rest of the antibodies were from Cell Signaling Technologies and diluted 1:1000: 4E-BP1, phospho-mTOR Ser2448, phospho-S6 Ser240/244, phospho-akt Ser472, phospho-70S6K, phospho-p70S6K Thr389] and added to the blots for 1 hour at room temperature. Secondary antibodies were horseradish peroxidase–conjugated goat antibodies (Southern Biotechnology Associates, Birmingham, AL). Signals were detected with enhanced chemiluminescence (Amersham Biosciences, Piscataway, CA) on scientific imaging film (Kodak, Rochester, NY).

Cell size measurement. Sections that were double-stained for b-catenin/Hoechst were photographed at the Nikon Imaging Facility (HMS, and pictures were taken at 20× using Metamorph Orca 100 software. Average cell size was defined by dividing bladder epithelial cell area of a given region on a section by the total number of nuclei in the area.

RNA preparation and reverse transcription-PCR. Tissues were processed as directed in the RNasea Midiprep protocol (Qiagen, Valencia, CA). RNA was quantitated on a spectrophotometer. RNA (6 μg) was put in a reverse transcriptase reaction with 0.4 mmol/L deoxynucleotide triphosphates (dNTP), Rnasin, and Moloney murine leukemia virus-RT (Promega, Madison, WI) in reaction buffer. Reactions were incubated 1 hour at 37°C, then 1 minute at 95°C to inactivate enzyme. PCR reactions with the cDNA samples were done with 0.2 mmol/L dNTPs and with Ex Taq (Takara, Shiga, Japan). Primer concentrations were 0.5 μmol/L each for the p21 primers and 1 μmol/L each for the b-actin primers. Primer sequences were p21Fwd (5'-CGGTTGAACTTTGACCTGT-3'), p21Rev (5'-TCTGCG-CTTGGAGTGATAGA-3'), b-actinFwd (5'-TCACTGAGTGTACCTGACATT-3'), and b-actinRev (5'-CCTAGAAGACATTGGGTGACAGTG-3'). Cycling conditions for the b-actin PCR were 94°C for 3 minutes, then 25 cycles of 94°C for 1 minute, 55°C for 1 minute, then 72°C for 1 minute followed by a final 10 minutes at 72°C. Cycling conditions for the p21 PCR were 94°C for 3 minutes, then 25 cycles of 94°C for 1 minute, 55°C for 1 minute, then 72°C for 1 minute followed by a final 10 minutes at 72°C.

Results

Generation of tissue-specific Pten-deficient mice by Fabpl-Cre. Mice that were homozygous for a floxed exon 5 encoding the catalytic domain of the Pten gene (32) were bred with mice carrying the Fabpl-Cre transgene (generously provided by Jeffrey Gordon). It has previously been shown that in this line of Fabpl-Cre transgenic mice, Cre is expressed in intestinal epithelium, and in urothelium of the bladder, kidney, and ureter (33). To determine the efficiency of the Fabpl-Cre–mediated Pten deletion in different tissues, we harvested various tissues from Ptenloxp/loxp, Fabpl-Cre–mice and prepared DNA to test for the presence of Cre-mediated recombination at the Pten locus by PCR (Fig. 1A). The results showed that Cre-mediated deletion of Pten exon 5 was not limited to the organs previously described (33) but in fact occurred in a wide variety of urogenital organs. Recombination could be detected in the colon, kidney, ureter, bladder, prostate, seminal vesicle, and vagina at 14 days of age (Fig. 1A) and also in the penis (data not shown) when examined at later ages. In contrast, we found that there was no detectable recombination in other organs, such as the heart, spleen, liver, brain, lung, and uterus. Pten is therefore specifically deleted in a subset of the lower abdominal reproductive and excretory organs of Ptenloxp/loxp, Fabpl-Cre–mice.

To verify that Pten protein levels were diminished in the bladder and other tissues that underwent Cre-mediated deletion of Pten, we did immunoblots against Pten using tissue protein lysates. We found a marked decrease in Pten in bladder epithelium from 8- and 28-day-old Ptenloxp/loxp, Fabpl-Cre–mice (Fig. 1B).

Fabpl-Cre–mediated Pten deletion leads to tumor development in urothelial cells. It was soon apparent that the Ptenloxp/loxp; Fabpl-Cre+ mice induced by Fabpl-Cre–mediated deletion caused accelerated death. The results showed that Cre-mediated deletion of Pten in Ptenloxp/loxp; Fabpl-Cre+–mice led to tumor development in urothelial cells. It was then apparent that those mice died at an accelerated rate compared with their Ptenloxp/loxp, Fabpl-Cre–mice and were specifically developing benign and malignant neoplasias in the same organs where Pten was deleted. We monitored the survival of the mice over a...
period of 13.5 months and found significantly increased mortality in male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice compared with that of male $\text{Pten}^{\text{loxP/loxP}}$ mice (Fig. 1C). The survival of $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice was sex influenced: 75% of female $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice survived by 13.5 months of age, but only 45% of the male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice were still alive by the same age. With respect to the male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice, one third of the deaths was attributed to inability to urinate due to urinary tract blockage, with accompanying hydronephrosis of the kidney. Such mice seemed largely asymptomatic until 1 day before the sudden onset of death due to failure to urinate. Some of these mice had urethral cancers, but excessive hyperplasia in the urinary tract or abnormal prostate growth may also have been responsible for blocking urine flow in other cases. Two thirds of the deaths of the male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice were due to other types of tumor growth, as described below. The female $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice also developed tumors in all the organs they possessed which underwent Cre-mediated $\text{Pten}$ deletion, but they never lost the ability to urinate. Because some of the male mice that died due to urine blockage may have developed tumors that would have killed them later in life, it is not feasible to compare death rates due to tumors alone in males compared with females.

A spectrum of tumors as well as hyperplasia were detected in the $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice in organs that underwent Cre-mediated deletion of $\text{Pten}$. These tumors included urothelial carcinoma of the bladder, ureter, and kidney; squamous cell carcinoma of the vagina and rectum; adenocarcinoma of the colon; and carcinomas of the prostate, seminal vesicles, and urethra (Fig. 1D and Fig. 2). Within the first year of life, we frequently found tumors of the anterior prostate, with 97% of the males developing this type of tumor (Fig. 1D and Fig. 2B). In male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice a year of age or older, the tumor had often taken over the entire prostate and seminal vesicle in a bilateral fashion. This is in contrast to $\text{Pten}^{+/+}$ mice, where only mild neoplasia in the coagulating gland was detected by 1 year of age. This illustrated a dose-dependent effect of $\text{Pten}$ deletion on tumorigenesis in the coagulating gland. Urethral tumors were also quite common, occurring in 48% of the $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice.

**Figure 1.** Tissue-specific deletion of $\text{Pten}$ and tumor development in $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}}$ mice. A, DNA was harvested from various organs from 14-day-old $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice. Samples were assayed by PCR using primers in the $\text{Pten}$ locus to detect Cre-mediated recombinated alleles (0.35-kb band) and nonrecombined alleles (1.1-kb band). B, protein lysate from bladder epithelium from 8- and 28-day-old $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ and $\text{Pten}^{\text{loxP/loxP}}$ mice was run on SDS-PAGE and blotted with an antibody for PTEN. C, survival of male and female $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ and $\text{Pten}^{\text{loxP/loxP}}$ mice was monitored over 13.5 months. A significant difference between survival curves of male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice and male $\text{Pten}^{\text{loxP/loxP}}$ mice was found ($P = 0.0002$) by log-rank test. There was also a significant difference between male $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice and female $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice ($P = 0.0018$). There was no significant difference between female $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice and $\text{Pten}^{\text{loxP/loxP}}$ mice. D, tumor types and frequencies were identified in $\text{Pten}^{\text{loxP/loxP}; \text{Fabpl-Cre}^+}$ mice over a 13.5-month study. The number of mice examined for each organ (n) is indicated. Frequency of anterior prostate tumor-bearing mice was calculated as a percentage of male mice examined, and the frequency of vaginal tumor-bearing mice was a percentage of examined female mice only.
mice by 13.5 months of age. Additionally, 41% of the Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ females developed vaginal squamous cell carcinoma (Fig. 2D). Carcinomas of the kidney, bladder, ureter, and colon (Fig. 2F) were less common but were also well represented (Fig. 1D).

We examined young Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice for abnormal cell growth and evidence of early tumor development. We found that epithelial hypertrophy and nuclear atypia were evident in bladders of Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice at birth (Fig. 3A and B), and both hypertrophy and hyperplasia were present with 100% penetrance by 6 weeks of age in bladders of Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice (Fig. 3C). We also observed characteristic vacuoles containing dead cells and enlarged and abnormally shaped nuclei in the hyperplastic bladder epithelium. Measurements of cell size on bladder sections of control Pten$^{loxp/loxp}$ and Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice stained for $\beta$-catenin expression and counterstained with Hoechst dye revealed a ~50% increase in cell size in 8-day-old Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice compared with that of their Pten$^{loxp/loxp}$ littermates (Fig. 4A).

Over time, a fraction of the hyperplasic and hypertrophic bladders in Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice progressed to high-grade urothelial carcinoma (Fig. 3D). Bladder cancer was positively identified in 22% of the Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice examined by 13.5 months of age. Although the frequency of bladder tumor formation is low in Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice, the Pten-deficient bladder carcinomas, once they are formed, progress to late-stage cancer, including invasion and metastasis. Examination of histopathology slides of bladder carcinomas revealed that the tumors in some cases invaded through the muscle wall (Fig. 3D). Furthermore, using an antibody specific for pan-uroplakin, a marker for differentiated transitional epithelium, we were able to show by immunohistochemical staining the lung metastasis of urothelial carcinomas in multiple animals, indicating the aggressiveness of the tumors that developed.

Given the full penetrance of the hyperplasia in the Pten-deficient bladder epithelium from a very early age, it is striking that the frequency of bladder cancer development is so low compared with that observed in other tissues. For example, in the anterior prostate, hyperplasia was consistently observed in the Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice at 8 days of age (Fig. 3E and F) and always progressed to carcinoma (Fig. 1D). Because the frequency of PTEN LOH in human bladder cancer is also considerably lower than some of the other tissues, such as prostate (15, 16, 34, 35), we investigated the mechanism that decreases the efficiency of tumor formation in the bladder after Pten loss. We focused on early events in the hyperplastic bladder because the urothelial carcinomas we observed were highly aggressive, suggesting that the inefficiency

Figure 2. Tumors in the Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice. A and B, H&E sections of anterior prostate from 4.5-month-old male mice. C and D, vaginal tissue from female mice. E and F, sections of H&E-stained colons from 13.5-month-old females. A, C, and E, normal tissue from Pten$^{loxp/loxp}$ mice. B, D, and F, prostatic carcinoma, vaginal squamous cell carcinoma, and colon adenocarcinoma, respectively, from Pten$^{loxp/loxp}$; Fabpl-Cre$^+$ mice. Magnification, ×200 (A-D) and ×100 (E-F).
of bladder epithelial transformation as a result of Pten loss is not due to a block in the final steps of tumor formation.

**Mechanism of hyperplasia in Ptenloxp/loxp; Fabpl-Cre+ mice.**
The hyperplasia evident in the Pten-deleted tissues prompted us to investigate whether we could detect increased cell proliferation in those sites. We stained bladder sections from 3-day-old to 16-week-old Ptenloxp/loxp; Fabpl-Cre+ and Ptenloxp/loxp mice for Ki-67, a marker of cycling cells (Fig. 4B). Although there was a large increase in frequency of Ki-67+ cells in Pten-deficient mice at 3 days of age, there was a decrease in Ki-67+ cells in bladders from the Ptenloxp/loxp; Fabpl-Cre+ mice 1 week of age and older. This was surprising, given the epithelial hypercellularity in the bladders of 4-week-old Ptenloxp/loxp; Fabpl-Cre+ mice and the previously reported increase in cell proliferation in the skin, mammary gland, and brain of Pten-deficient mice (26, 36, 37).

As a comparison, we determined the labeling ratio of Ki-67+ cells in the prostate of Ptenloxp/loxp; Fabpl-Cre+ mice, which also exhibited epithelial hyperplasia (Fig. 4C). Interestingly, we detected a clear increase in the Ki-67+ labeling ratio in the prostate of 4- to 16-week-old Ptenloxp/loxp; Fabpl-Cre+ mice when compared with that of Ptenloxp/loxp mice. This result is consistent with other Ki-67 studies in the prostates of Pten-deficient or heterozygous mice (5, 10, 38, 39). The comparison of Pten-deficient bladder and prostate suggests a possible tissue-specific divergence in the signaling pathways downstream of PTEN and could address the low tumorigenesis rates in the bladder.

We also examined the possibility that a reduction in epithelial cell apoptosis contributed to the hyperplasia we observed in the bladder. We did immunohistochemical staining for terminal deoxynucleotidyl transferase–mediated nick-end labeling–positive cells and activated caspase-3–positive cells in bladder epithelium. By both methods, the rate of apoptotic death in the urothelium was too low to be measured (data not shown). This strongly suggested that the increase in cell number in the Ptenloxp/loxp; Fabpl-Cre+ bladders was attributable to an increase in proliferation during the animals’ first week of life (Fig. 4B).

**Differential activation of PTEN downstream targets.** To determine the molecular mechanisms that lead to hyperplasia and hypertrophy in Pten-deficient bladder epithelium, we examined the phosphorylation levels of known downstream effectors of the AKT signaling pathway, such as FOXO3a, mTOR, p70S6K, and S6. We isolated bladder urothelium by cleanly dissecting away the muscularis propria and made protein lysate for immunoblotting. Surprisingly, we found minimal increase in phosphorylation levels of AKT (Fig. 5A), and no difference in the phosphorylation levels of FOXO3a, mTOR, p70S6K, or S6, the predicted downstream targets of AKT.

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**Figure 3. Urothelial hyperplasia and carcinoma in Ptenloxp/loxp; Fabpl-Cre+ mice.** A and B, bladders from Ptenloxp/loxp (A) and Ptenloxp/loxp; Fabpl-Cre+ (B) mice less than 24 hours old. C, hyperplastic bladder from 6-week-old Ptenloxp/loxp, Fabpl-Cre+ mouse. D, high-grade invasive bladder urothelial carcinoma (low magnification). E and F, developing prostate from 6-day-old Ptenloxp/loxp (E) and Ptenloxp/loxp; Fabpl-Cre+ (F) mice. Magnification: ×400 (A–C and E–F) and ×100 (D).
of AKT, in the bladder samples from Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice ranging from 5-day-old to 3-month-old mice when compared with that of age-matched Pten<sup>loxP/loxP</sup> mice (Fig. 5; data not shown). It was particularly noteworthy that there was no difference in S6 phosphorylation in bladders from 2-day-old mice (Fig. 5A), an age when there was a marked increase in proliferation and cell size in the Pten-deficient bladders. This was an unexpected result because we had established that there was a significant increase in epithelial cell size in the Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> bladders (Fig. 4A). These results suggest that the hypertrophy of bladder epithelial cell size in the Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice (Fig. 5B) was an unexpected result because we had established that there was a significant increase in epithelial cell size in the Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> bladders (Fig. 4A). These results suggest that the hypertrophy of bladder epithelial cells induced by the Pten deficiency is unlikely to be mediated through the activation of the mTOR/p70S6K pathway, a known mechanism by which the activation of AKT leads to increases in cell size (40–42).

Like the bladders in Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice, the prostates consistently became hyperplastic from a very early age. Immunoblots of prostate samples revealed that there was a clear up-regulation in the phosphorylation of AKT, FOXO3a, mTOR, p70S6K, and S6 in Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice compared with that of Pten<sup>loxP/loxP</sup> mice (Fig. 5B). Furthermore, this constitutive activation of the AKT pathway was maintained over a period of months. This is in stark contrast to the immunoblot data from the bladder samples, where no difference was detectable. The lack of Akt activation in the bladder was not due to low levels of Akt expression, as we have compared endogenous levels of total Akt in bladder and prostate and found comparable but slightly higher levels of Akt in the bladder compared with the prostate (Fig. 5C). This study suggests that the increase in cell size in the Pten-deficient bladders may not be due to a defect in differentiation.

**Activation of p21 in the bladder epithelial cells of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice.** AKT activation has been found to induce the up-regulation of p21 expression in 293 and MCF7 cells (27) and to down-regulate expression of p27 in embryonic stem cells (43). To determine if the bladder epithelium of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice has altered levels of CKIs, we compared p21 and p27 expression in Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> and Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice. We detected reduced levels of p27 (Fig. 6A) in the bladders of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice compared with that of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice. We detected reduced levels of p27 (Fig. 6A) in the bladders of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice compared with Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice. We detected reduced levels of p27 (Fig. 6A) in the bladders of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice compared with Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice. We detected reduced levels of p27 (Fig. 6A) in the bladders of Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice compared with Pten<sup>loxP/loxP</sup>; Fabpl-Cre<sup>+</sup> mice.
To examine if the levels of p21 transcripts were altered in the bladders of Ptenloxp/loxp; Fabpl-Cre+ mice, we analyzed bladder RNA samples from 3-month-old mice by reverse transcription-PCR (RT-PCR) for p21 transcript level. We detected higher levels of p21 transcripts in the RNA from the bladders of Ptenloxp/loxp; Fabpl-Cre+ mice than that of Ptenloxp/loxp mice (Supplementary Fig. S2A), which suggested that an alteration in the p21 expression as a result of Pten deficiency in bladder epithelium was at least in part due to either an increase of p21 transcription or a stabilization of p21 transcripts. AKT activation may also result in stabilization of p21 protein, as suggested by other studies (27, 28); but that cannot be the only mechanism in this case, because the RT-PCR result showed an increase in p21 transcripts.

In contrast to Ptenloxp/loxp; Fabpl-Cre+ bladders, Ptenloxp/loxp; Fabpl-Cre+ prostates showed no change in p27 or p21 levels (Supplementary Fig. S2A), while showing a clear up-regulation in phosphorylation of FOXO3a, mTOR, and S6 (Fig. 5B). These results showed a stringent tissue specificity in the activation of AKT downstream targets as a result of Pten deficiency and provides an important possible explanation for the different propensities for tumor formation in different tissues.

We did immunohistochemistry to examine the subcellular localization of p21 in bladder cells. There was a significantly increased frequency in p21-positive cells in the hyperplastic bladder epithelium of Ptenloxp/loxp; Fabpl-Cre+ mice (Fig. 6C-E). In Ptenloxp/loxp bladder epithelium, p21 expression was restricted to a fraction of the large, terminally differentiated cells localized in the superficial layers. In contrast, in Ptenloxp/loxp; Fabpl-Cre+ mice the p21-positive cell population is greatly expanded in both the superficial and intermediate layers of urothelium. Interestingly, the p21 expression in both Ptenloxp/loxp mice and Ptenloxp/loxp; Fabpl-Cre+ mice was limited to the nucleus.

Increased cell proliferation in p21+/−; Ptenloxp/loxp; Fabpl-Cre+ bladder urothelium. We were interested whether p21 induction plays a role in limiting cell proliferation in the Pten-deficient bladders at 1 week of age. To address this question, we bred p21+/− mice to the Ptenloxp/loxp; Fabpl-Cre+ mice and generated mice with four genotypes: (a) p21+/+; Ptenloxp/loxp, (b) p21−/−; Ptenloxp/loxp, (c) p21−/−; Ptenloxp/loxp; Fabpl-Cre+, (d) p21−/−; Ptenloxp/loxp; Fabpl-Cre+, Ptenloxp/loxp; Fabpl-Cre+ mice. In mice 1 and 4 weeks of age, we isolated bladders and examined the mitotic cell marker phospho-histone 3 Ser10 (Fig. 6F) and Ki-67 (Supplementary Fig. S2C) by immunohistochemistry. Intriguingly, there was a distinct increase in mitotic cell frequency in p21+/−; Ptenloxp/loxp; Fabpl-Cre+ mice compared with mice of all other genotypes in both assays. This showed that p21 induction in the Ptenloxp/loxp; Fabpl-Cre+ bladder inhibits urothelial cell proliferation in mice 1 week of age and older, but when p21 is also deleted in addition to Pten as in the p21−/−; Ptenloxp/loxp; Fabpl-Cre+ mice, the urothelial cells continue to proliferate at higher rates. The induction of p21 in response to Pten deletion may therefore be...
a type of bladder-specific tumor suppression mechanism. Experiments are in progress to determine if the bladder tumorigenesis rate in 21−/−; Ptenloxp/loxp; Fabp1-Cre cells is increased compared with that of Ptenloxp/loxp; Fabp1-Cre mice. We will note here that lysate from a bladder carcinoma arising from a 21−/−; Ptenloxp/loxp; Fabp1-Cre mouse was compared with normal and hyperplastic bladder samples for AKT and FOXO3a phosphorylation by immunoblotting (Fig. 6G). We found no increase in phosphorylation in the carcinoma. This suggests that the lack of AKT activation in the Pten-deficient bladder is maintained in tumors.

Similarly, lysates from anterior prostate carcinomas from three mice of different genotypes and age were tested for phospho-AKT and FOXO3a (Fig. 6H). We found that the increased levels of phosphorylation were maintained in the tumors.

Finally, to determine if p21 induction plays a role in suppressing activation of the AKT pathway, we made bladder epithelial lysates from 21+/−; Ptenloxp/loxp and 21−/−; Ptenloxp/loxp; Fabp1-Cre mice and immunoblotted for phospho-AKT, mTOR, and S6 (Supplementary Fig. S2D). As with the p21−/− mice, we found no difference in activation of the AKT/mTOR pathway, showing that p21 induction in Pten-deleted urothelium does not suppress activation of the AKT pathway.

Discussion

Loss of Pten expression has been strongly linked to the development of multiple types of human cancer, such as glioblastoma, breast cancer, and prostate cancer, but its importance in suppressing the development of other types of malignancies, such as bladder cancer, has been controversial (15, 16, 47, 48). An analysis of tumor predisposition in Cowden syndrome patients (PTEN+/−) also revealed biases toward the development of tumors in certain organs, such as breast and thyroid (11), although genitourinary cancers are also common. The molecular basis for such differential tumor predispositions is not understood. To address this issue, we generated mice that were conditionally deficient for Pten in urothelium of the bladder, ureter, and kidney and in epithelium of the urethra, vagina, prostate, colon, and seminal vesicle. These mice developed a spectrum of epithelial
tumors in the same organs that underwent Cre-mediated deletion at the \textit{Pten} locus. \textit{Pten}\textsuperscript{lox/lox}\textnbsp;\textit{Fabpl-Cre}\textsuperscript{+} mice died at an accelerated rate compared with \textit{Pten}\textsuperscript{lox/lox} mice or wild-type mice, due to tumors and obstructed urine flow. The tumors were characteristically aggressive and in a few cases metastasized to the lung. The aggressiveness of the tumors is consistent with the known effects of PTEN deficiency on cell motility and angiogenesis, in addition to proliferation and survival (49, 50). In future studies, the \textit{Pten}\textsuperscript{lox/lox}\textnbsp;\textit{Fabpl-Cre}\textsuperscript{+} mouse line may serve as a unique, valuable model for high-grade urothelial carcinoma, prostate adenocarcinoma, and vaginal squamous cell carcinoma.

Hyperplasia was apparent in the bladders of \textit{Pten}\textsuperscript{lox/lox}\textnbsp;\textit{Fabpl-Cre}\textsuperscript{+} mice before 6 weeks of age and increased epithelial cell size was evident even at birth, suggesting that loss of \textit{Pten} exerts an early effect on the epithelial cells. Surprisingly, however, despite the increased number of epithelial cells in the \textit{Pten}-deficient bladders, cell proliferation rates were elevated only during the first week of life, after which they dropped to normal. We also examined apoptosis rates and found very low levels of apoptosis in the bladders. This led us to conclude that \textit{Pten} deletion in the bladder was causing increased proliferation during a 1-week time span in newborn mice, leading to a hyperplasia that was maintained during the life of the animal.

A general survey of protein phosphorylation in the bladder was done with immunoblots to determine which signaling pathways had been activated by \textit{Pten} deficiency. In the \textit{Pten}-deficient bladder, we found that very few downstream targets of PI3K were phosphorylated or changed as described in other tissues or cell types. We did find that the protein levels of p27 were decreased from 2 days of age through at least 3 months of age. Previous types. We did find that the protein levels of p27 were decreased in the bladder, we found that very few downstream targets of PI3K were activated by 

The induction of p21 may be a key bladder-specific tumor suppression mechanism to limit abnormal growth in the presence of constitutive cell proliferation signals, and drugs designed to induce p21 in p21-negative tumors may be an effective strategy for chemotherapy.

It is worth noting that whereas Wu et al. (50) found increased levels of activated AKT in 55% primary bladder tumors, we did not observe any increase in activated AKT in tissue from hyperplastic \textit{Pten}-deleted bladders. We have verified that there are similar levels of AKT in the bladder compared with the prostate so the failure to detect an increase in AKT activation is not due to an absence of AKT in the bladder. It is likely that in the bladder, there is either a negative feedback loop that mitigates the effect of \textit{Pten} deletion on AKT activation, or there is a partial redundancy in PTEN function. Regardless, our results suggest that AKT/mTOR pathway inhibitors, such as rapamycin, may not be useful for the treatment of \textit{Pten}-negative bladder tumors. This is especially critical given the fact that rapamycin analogues are being tested as chemotherapeutic agents, and whereas they may be effective against certain tumor types, our study shows the necessity of recognizing tissue-specific differences when selecting drug therapy.

The striking tissue specificity exhibited by \textit{Pten}-deficient bladder and prostate epithelial cells in activating p21 or p70S6K/FOXO3a suggests that this represents two distinct choices of cell fate. What are the critical determinants for differential regulation of p21 or p70S6K? One possible determinant is the levels of AKT activation.
The lack of AKT activation in the bladders of the Pten\textsuperscript{loxP/loxP}\textsubscript{Fabpl-Cre\_mice} may explain why the p70S6K pathway was not activated. Trotman et al. (39) have shown that fine-tuning levels of activated AKT in the prostate through the use of a series of PTEN hypomorphic mice results in dose-responsive tumor progression, illustrating the exquisite sensitivity of cancer development to levels of AKT activation. A certain level of AKT activity may be required to up-regulate the p70S6K signaling pathway. Alternatively, the endogenous levels of AKT signaling pathway targets may play an important role in determining cell fate. For instance, we found that Pten deficiency in the prostate resulted in increased absolute levels of p70S6K in addition to increased activation of p70S6K, whereas in the bladder, there was no effect of Pten deletion on either (data not shown). This suggests that there is some prostate-specific mechanism that results in increased p70S6K protein levels when PTEN is absent, which may be responsible for the differential activation of this pathway. Another interesting possibility is that exogenous factors secreted by stromal cells may enhance AKT signaling in the prostate compared with the bladder. For example, You et al. (63) have shown that conditioned medium from prostate stromal cells induces the PI3K pathway. As for an increase in the PI3K pathway, we recently showed that conditioned medium from prostate stromal cells induces the PI3K pathway in primary prostate epithelial cells. As for an increase in p70S6K, there was no effect of Pten deletion on either (data not shown). This suggests that there is some prostate-specific mechanism that results in increased p70S6K in addition to increased activation of p70S6K, whereas in the bladder, there was no effect of Pten deletion on either (data not shown).

In conclusion, our study shows an interesting contrast between Pten deficiency in a tissue preferentially predisposed to cancer formation leads to the activation of FOXO3a and p70S6K pathways, whereas in the bladder which is less sensitive to tumor formation, Pten deficiency leads to decreased levels of p27, increased levels of p21, and a decrease in proliferation rates. We believe the key to the relatively low rate of tumorigenesis in the bladder lies in the fact that the cell population, which increases in response to Pten loss, is largely nonproliferative, p21 positive, and differentiated. We are currently in the process of determining if the p21\textsuperscript{−/−}/Pten\textsuperscript{loxP/loxP}\textsubscript{Fabpl-Cre\_mice} have increased rates of bladder cancer development compared with the Pten\textsuperscript{loxP/loxP}\textsubscript{Fabpl-Cre\_mice} clearly. It will be important to study the expression and mutation of both the p21 and PTEN genes in human bladder cancer samples. Understanding the specificities of downstream signaling pathways will be key to the development of effective, tailored, and tissue-specific anticancer therapies.

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