Conditional Loss of Uterine *Pten* Unfailingly and Rapidly Induces Endometrial Cancer in Mice

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Abstract

Etiology of endometrial cancer (EMC) is not fully understood. Animal models with rapidly and spontaneously developing EMC will help explore mechanisms of cancer initiation and progression. *Pten*$^{+/-}$ mice are currently being used as a model to study EMC. These females develop atypical endometrial hyperplasia of which ~20% progresses to EMC. In addition, tumors develop in other organs, complicating the use of this model to specifically study EMC. Here, we show that conditional deletion of endometrial *Pten* results in EMC in all female mice as early as age 1 month with myometrial invasion occurring by 3 months. In contrast, conditional deletion of endometrial *p53* had no phenotype within this time frame. Whereas mice with endometrial *Pten* deletion had a life span of ~5 months, mice with combined deletion of endometrial *Pten* and *p53* had a shorter life span with an exacerbated disease state. Such rapid development of EMC from homozygous loss of endometrial *Pten* suggests that this organ is very sensitive to this tumor suppressor gene for tumor development. All lesions at early stages exhibited elevated Cox-2 and phospho-Akt levels, hallmarks of solid tumors. More interestingly, levels of two microRNAs *miR-199a and *miR-101a* that posttranscriptionally inhibit Cox-2 expression were down-regulated in tumors in parallel with Cox-2 up-regulation. This mouse model in which the *loxP-Cre* system has been used to delete endometrial *Pten* and/or *p53* allows us to study in detail the initiation and progression of EMC. These mouse models have the added advantage because they mimic several features of human EMC.** [Cancer Res 2008;68(14):5619–27]

Introduction

Endometrial cancer (EMC) is a common gynecologic malignancy, affecting ~40,000 women and leading to ~7,000 deaths each year in the United States alone (1). The etiology of EMC is not yet fully understood, although there is evidence that endocrine and genetic factors contribute to its initiation and progression (1). EMC is categorized into two major types, type I and II, with ~85% of EMCs classified as type I. Type I EMC is divided into well-, moderately, and poorly differentiated grades, depending on t he degree of solid tumor growth. Type II EMC, while uncommon, is more aggressive (1, 2).

In human type I EMCs, the most common genetic mutations are detected in the *phosphatase and tensin homologue* (*Pten*)gene (1, 3). *Pten* mutations are observed in 30% to 80% of type I EMCs and in ~20% of complex atypical hyperplasia (CAH), a precursor to type I EMC. Mutations of *p53* are also found in type I EMC, but this alteration occurs in ~50% of poorly differentiated carcinomas and some moderately differentiated type I EMCs, suggesting that *p53* mutations are later events that contribute to progression of the disease (1). On the other hand, the majority of type II EMCs, which are more aggressive and less common, mainly contain *p53*, but not *Pten*, mutations (1, 2).

The most widely used animal model for studying EMC is *Pten* heterozygous mice (3). Although *Pten* homozygous null mice are unavailable due to their embryonic lethality, all *Pten* heterozygous females develop atypical endometrial hyperplasia with 20% progressing to well-differentiated EMC by age 10 months. The timing and incidence of hyperplasia and carcinoma vary from mouse to mouse in this model (3, 4). Furthermore, *Pten* heterozygous mice also develop other types of cancer, creating limitations to exclusively study EMC in this model. With respect to p53, both *p53* heterozygous and homozygous mice develop many types of cancers, with most homozygous mice dying by age 6 months due to development of widespread lymphoma (5–7). However, EMCs are rarely observed in *p53* null mice (5–7). Therefore, endometrial-specific *Pten* and/or *p53*-deleted mice would be more preferred models to study endometrial-specific cancer.

Pten is a dual-specificity phosphatase with phosphatidylinositol-3,4,5-phosphate (PIP_3) a major substrate. PIP_3 is dephosphorylated to phosphatidylinositol-4,5-phosphate by Pten, an event that opposes phosphatidylinositol-3-kinase (PI3K) signaling (8). Loss of Pten function, resulting in stimulation of PI3K signaling, is widely found in many types of cancers. PI3K activates Akt, a serine-threonine kinase, and phosphorylated/activated Akt regulates a variety of target molecules that control cell survival and growth. It was recently shown that introducing *Akt* deficiency in *Pten* heterozygous mice impedes tumor development including that of EMC (9), suggesting pAkt to be an immediate downstream molecule of Pten. Cox-2 is a major target of Akt signaling in cancer, overexpressed in tumors and carcinomas of the colon, breast, and lung (10). We and others have shown that human EMCs and endometria with hyperplasia express elevated Cox-2 (11–13). Moreover, Cox-2 expression is also elevated in human EMC cell lines harboring *Pten* mutations and activated Akt (14, 15). These studies collectively indicate that elevated pAkt and Cox-2 levels resulting from *Pten* mutations probably contribute to EMC development.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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In this study, endometrial-specific *Pten* and/or *p53* deletion were generated by crossing floxed *Pten* ($Pten^{loxP/loxP}$) and/or floxed *p53* ($p53^{loxP/loxP}$) mice with mice expressing Cre under the regulation of the progesterone receptor promoter ($PR^{cre/+}$; ref. 16). We found that 100% of $Pten^{loxP/loxP}/PR^{cre/+}$ ($Pten^{pr-/-}$) and $Pten^{loxP/loxP}/p53^{loxP/loxP}/PR^{cre/+}$ ($Pten^{pr-/-}/p53^{pr-/-}$) mice develop *in situ* carcinoma as early as ages 3 weeks to 1 month. Although the development of hyperplasia was similar between $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ mice, the loss of both *Pten* and *p53* exacerbated the disease state and was associated with a shorter life span. In contrast, $p53^{loxP/loxP}/PR^{cre/+}$ ($p53^{pr-/-}$) mice had apparently normal endometrial histology even through age 5 months. We found that Cox-2 and phospho-Akt (pAkt) were up-regulated in regions with hyperplasia and carcinoma in both $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ uteri. Additionally, microRNAs (miRNA) *miR-199a** and *miR-101a*, which are known to posttranscriptionally impede Cox-2 expression in the mouse uterus and human cancer cell lines (17), were down-regulated in $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ uteri. These mouse models have provided valuable information on genetic, temporal, and dynamic aspects of EMC initiation and progression. Our findings present an opportunity for further study, especially with regards to drug development focused at EMC treatment at early stages.

Materials and Methods

Mice. All mice were housed in the Institutional Animal Care Facility according to NIH and institutional guidelines for laboratory animals. $Pten^{loxP/loxP}$ mice were obtained from Jackson Laboratory, and $p53^{loxP/loxP}$ mice were obtained from the Mouse Models of Human Cancers Consortium (National Cancer Institute). *PR-Cre* ($PR^{cre/+}$; C57BL6/129SV) mice were obtained from John B. Lydon (Baylor College of Medicine). $PR^{cre/+}$ mice have Cre recombinase introduced into exon 1 of the *PR* gene (16). Therefore, Cre expression is regulated by the endogenous progesterone receptor (PR) promoter. *PR-Cre* heterozygous ($PR^{cre/+}$) mice are phenotypically indistinguishable from wild-type mice (16). $Pten^{loxP/loxP}$, $p53^{loxP/loxP}$, and $PR^{cre/+}$ mice were generated as described previously (16, 18, 19). PCR analysis of tail genomic DNA determined the genotypes of mutant mice. Standard breeding strategies were used to generate conditional deletion of *Pten* and/or *p53* using transgenic mice (20). Bilateral ovariectomy was performed under appropriate anesthesia. Because the currently used $Pten^{pr-/-}$, $p53^{pr-/-}$, and $Pten^{pr-/-}/p53^{pr-/-}$ mouse models are on mixed backgrounds, we consistently used littermate floxed (wild-type) mice in all of our experiments. This ensures that results obtained within each experimental set are meaningful.

Western blot analysis. Tissue samples were prepared as previously described (21). After measuring protein concentrations, supernatants mixed with SDS sample buffer were boiled for 5 min. The samples were run on 10% SDS-PAGE gels under reducing conditions and transferred onto nitrocellulose membranes. Membranes were blocked with 10% milk in TBST and probed with antibodies to Pten (Cell Signaling), pAkt (Cell Signaling), p53 (Cell Signaling), Cox-2 (Cayman), or actin (SantaCruz) overnight at 4°C. After washing, blots were incubated in peroxidase-conjugated donkey-anti-goat IgG, donkey-anti-rabbit IgG, or donkey-anti-mouse IgG (Jackson Immuno Research Laboratories, Inc.). For secondary antibody detection of p53, mouse-IgG Trueblot (eBioscience) was used to remove IgG signals. All signals were detected using chemiluminescent reagents (GE Healthcare). Actin served as a loading control.

Immunohistochemistry. Immunohistochemistry was performed as previously described by us (21). In brief, formalin-fixed paraffin-embedded sections (6 μm) were subjected to immunostaining using antibodies to cytokeratin 8 (CK8; Developmental Studies Hybridoma Bank), Pten, pAkt, Cox-2, Ki-67 (Lab Vision Corporation), or α-smooth muscle actin (α-SMA; Abcam). After deparaffinization and hydration, sections were subjected to antigen retrieval by autoclaving in 10 mmol/L sodium citrate solution (pH = 6) for 10 min. A Histostain-Plus kit (Invitrogen) was used to visualize antigens.

***In situ* hybridization.** cDNA clones for *Cox-2* have previously been described (22, 23). cDNA clones for *p53* were generated by reverse transcription-PCR (RT-PCR). Sense and antisense ^{35}S-labeled cRNA probes were generated using *Sp6* or *T7* RNA polymerases. Frozen uterine sections were used for *in situ* hybridization. Uteri from 10-d-old and 3-wk-old mice were placed inside a small groove made on a piece of kidney as a holding cassette because they are extremely tiny, and then snap frozen for cryosectioning. *In situ* hybridization was performed as previously described (21). Sections hybridized with sense probes did not exhibit any positive signals and served as negative controls.

RT-PCR. RT-PCR was performed as previously described (21). Primers to detect *p53* are 5′ ACAGGACCCTGTCACCGAGACC 3′ and 5′ GACCTCCGTCATGTGCTGTGAC 3′.

Northern hybridization. Northern blotting of miRNA was performed as previously described (17). Total RNA (20 μg) was resolved through a 12.5% urea-polyacrylamide gel, transferred onto GeneScreen Plus membranes (PerkinElmer), and UV crosslinked. Antisense oligonucleotide (IDT) was labeled with ^{32}P with a StarFire labeling kit (IDT). Prehybridization, hybridization, and washing were performed at 42°C.

Results

***PR-Cre* efficiently deletes endometrial *Pten* and *p53*.** Our objective was to study endometrial-specific cancer by conditional deletion of *Pten, p53*, or both in the mouse endometrium. We therefore crossed *Pten* ($Pten^{loxP/loxP}$), *p53* ($p53^{loxP/loxP}$), or *Pten/p53* ($Pten^{loxP/loxP}/p53^{loxP/loxP}$) floxed mice with $PR^{cre/+}$ mice. We have previously used $PR^{cre/+}$ mice to delete endometrial genes to examine the roles of *Hbegf* and *Bmp2* in pregnancy (20, 24). As shown in Supplementary Fig. S1, endometrial *Pten* and *p53* are deleted by PR-driven Cre activity as confirmed by conventional genotyping.

We next confirmed loss of Pten protein in $Pten^{pr-/-}$ uterine lysates by Western blotting. As shown in Fig. 1A (*top*), Pten levels were drastically reduced in $Pten^{pr-/-}$ uteri from age 3 wk with concomitant increases in Akt phosphorylation (pAkt). This observation of Akt activation with the loss of Pten is consistent with previous findings in other systems (25). We also used immunohistochemistry to monitor cell-specific down-regulation of Pten and up-regulation of Akt activation (Fig. 1*A, bottom*). Pten levels were efficiently down-regulated in uteri of $Pten^{pr-/-}$ mice from as early as age 10 days substantiating the Western blot results. Although levels of pAkt were low to undetectable in wild-type uteri with normal levels of Pten, pAkt levels were remarkably up-regulated in $Pten^{pr-/-}$ uteri in both 10-day-old and 3-week-old mice (Fig. 1*A, bottom*). Similar analyses were carried out using $Pten^{pr-/-}/p53^{pr-/-}$ uteri. Western blotting and *in situ* hybridization showed decreased p53 expression in $Pten^{pr-/-}/p53^{pr-/-}$ uteri as expected (Fig. 1*B*). Loss of both Pten and p53 in these mice was also accompanied by heightened levels of pAkt (Fig. 1*B, left*). Loss of *p53* in $p53^{pr-/-}$ uteri was confirmed by RT-PCR (Fig. 1*C*). Collectively, PR-Cre efficiently deletes endometrial Pten and p53.

Endometrial deletion of *Pten* induces EMC. Our next objective was to examine whether endometrial deletion of *Pten* in mice results in EMC. To address this question, we examined histology of $Pten^{pr-/-}$ and/or $p53^{pr-/-}$ uteri. $Pten^{pr-/-}$ uteri showed endometrial epithelial hyperplasia as early as age 10 days (Fig. 2*A*). Staining with CK8, an epithelial cell-specific marker, showed luminal and glandular epithelial hyperplasia in *Pten*-deleted uteri from mice as

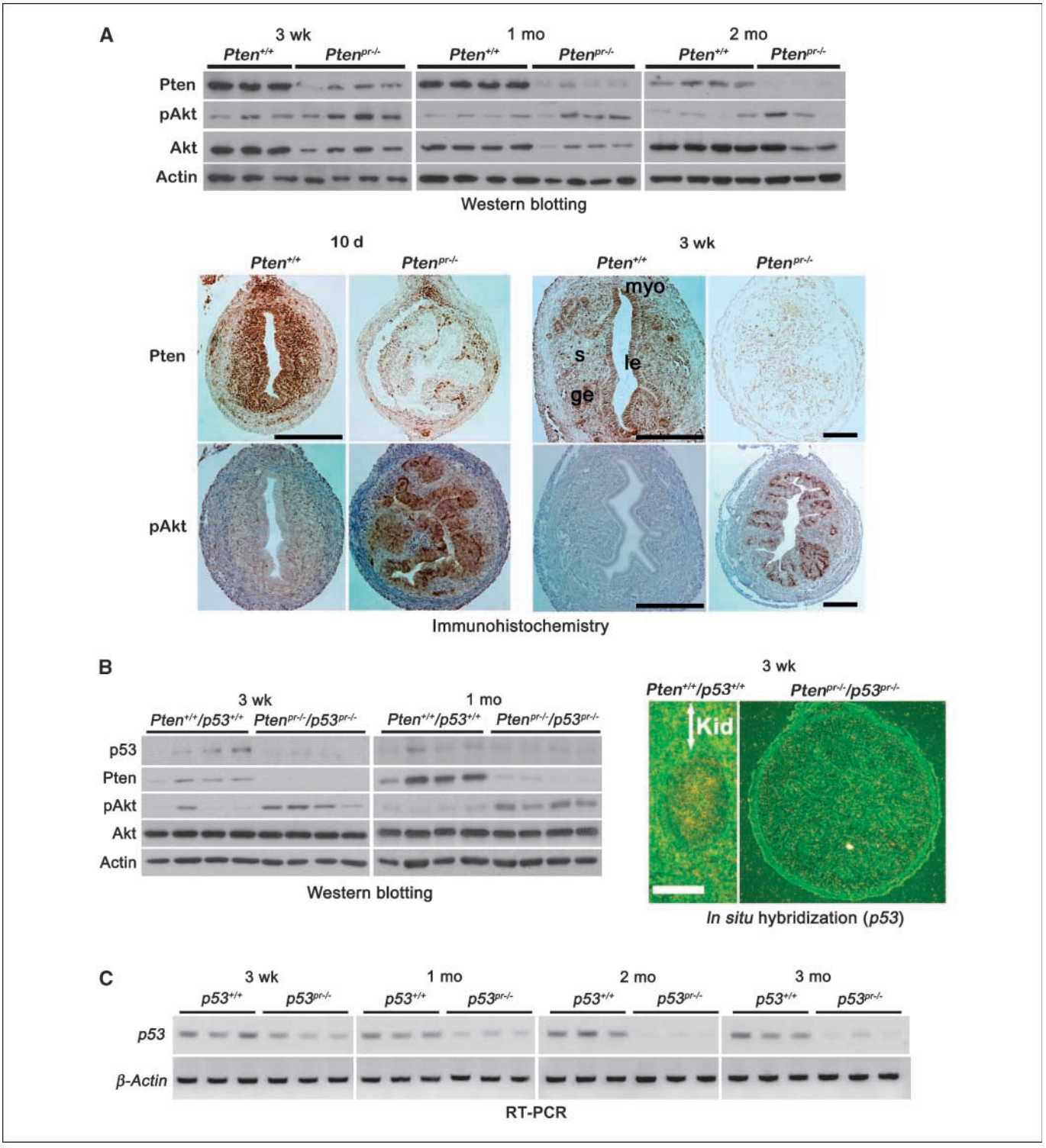

Figure 1. *PR-Cre* efficiently deletes endometrial *Pten* and *p53. A,* Western blot analysis of Pten, pAkt, and total Akt in wild-type and $Pten^{pr-/-}$ uteri (*top*). Immunohistochemistry of Pten and pAkt in 10-d-old (*10 d*) and 3-wk-old (*3 wk*) $Pten^{pr-/-}$ and wild-type uteri (*bottom*). *Bar,* 200 μm. *le,* luminal epithelium; *ge,* glandular epithelium; *s,* stroma; *myo,* myometrium. *B,* Western blot analysis of p53, Pten, pAkt, and total Akt in wild-type and $Pten^{pr-/-}/p53^{pr-/-}$ uteri (*left*). Actin serves as a loading control. *In situ* hybridization of *p53* in 3-wk-old wild-type and $Pten^{pr-/-}/p53^{pr-/-}$ uteri (*right*). Because of extremely tiny sizes of uteri, all wild-type and $Pten^{pr-/-}$ uteri from 10-d-old and 3-wk-old mice were placed into small grooves made in kidney slices to serve as cassettes for making frozen sections. *Bar,* 400 μm. *Kid,* kidney. *C,* RT-PCR of *p53* in wild-type and $p53^{pr-/-}$ uteri. *β-Actin* is a housekeeping gene.

young as 10 days. Hyperplasia progressed to carcinoma by age 1 month with invasion into the myometrium occurring by 3 months (Fig. 2*A*). Myometrial invasion was confirmed by α-SMA immunostaining (Supplementary Fig. S2). Detailed characterization of tumor types and grades are shown (Table 1). These results show that conditional deletion of endometrial *Pten* specifically results in EMC rapidly with 100% penetrance, a much more drastic phenotype than observed in mice heterozygous for *Pten* deletion.

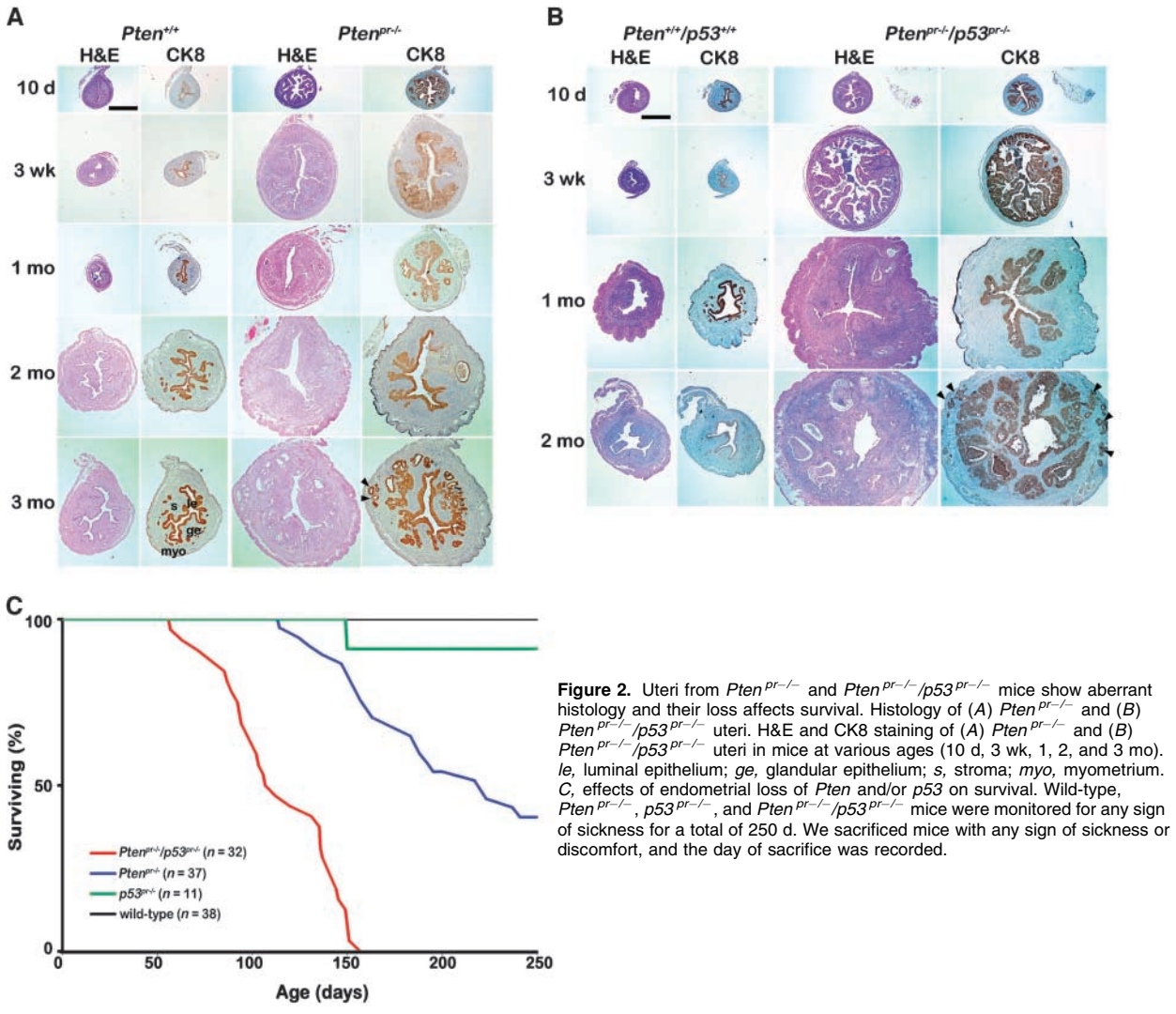

Figure 2. Uteri from *Pten*$^{pr-/-}$ and *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ mice show aberrant histology and their loss affects survival. Histology of (*A*) *Pten*$^{pr-/-}$ and (*B*) *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ uteri. H&E and CK8 staining of (*A*) *Pten*$^{pr-/-}$ and (*B*) *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ uteri in mice at various ages (10 d, 3 wk, 1, 2, and 3 mo). *le,* luminal epithelium; *ge,* glandular epithelium; *s,* stroma; *myo,* myometrium. *C,* effects of endometrial loss of *Pten* and/or *p53* on survival. Wild-type, *Pten*$^{pr-/-}$, *p53*$^{pr-/-}$, and *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ mice were monitored for any sign of sickness for a total of 250 d. We sacrificed mice with any sign of sickness or discomfort, and the day of sacrifice was recorded.

We next examined the effects of combined deletion of *p53* and *Pten*. Histologic analyses of *Pten*$^{loxP/loxP}$/*p53*$^{loxP/loxP}$/*PR*$^{cre/+}$ (*Pten*$^{pr-/-}$/*p53*$^{pr-/-}$) uteri after staining with H&E showed that *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ uteri were enlarged compared with those of *Pten*$^{pr-/-}$, with luminal and glandular hyperplasia (Fig. 2*B*). Lesions were observed as early as age 10 days similar to the time frame noted in *Pten*$^{pr-/-}$ uteri (Fig. 2*B*). Furthermore, these lesions progressed to *in situ* carcinoma by age 3 weeks (Table 1). Uteri from wild-type mice did not exhibit any pathology. We next examined *p53*$^{pr-/-}$ uteri. Although loss of endometrial *p53* expression was seen in mice as young as age 3 weeks (Fig. 1*C*), endometrial morphology and histology seemed normal through age 5 months (Supplementary Fig. S3; Supplementary Table S1).

Endometrial deletion of both *Pten* and *p53* advances mortality. In human, loss of *Pten* and *p53* increases severity of EMC development when compared with those with only *Pten* mutation (1, 2). However, we observed similar histology between *Pten*$^{pr-/-}$ and *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ uteri during EMC initiation. A close monitoring of conditionally deleted mice for sickness during the tenure of these experiments revealed that loss of both *Pten* and *p53* affects their survival as early as age 2 months, whereas loss of *Pten* alone does not affect longevity until around 5 months, with deletion of *p53* alone affecting viability even later (Fig. 2*C*). H&E staining of uterine sections from *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ uteri indicate that the cause of early death in these mice is due to excess uterine bleeding due to invasion of uterine blood vessels by tumor cells (Supplementary Fig. S4). At sacrifice, extensive blood clots on the surface of the entire uterus were visible in these mice.

Endometrial deletion of *Pten* or of both *Pten* and *p53* induces epithelial Cox-2 expression and proliferation. Cox-2 is a downstream target of pAkt signaling and associated with development of many types of cancers (14, 26). Thus, we examined Cox-2 expression in *Pten*$^{pr-/-}$ uteri. As shown in Fig. 3*A*, levels of Cox-2 protein increased in *Pten*$^{pr-/-}$ uteri compared with wild-type uteri from age 3 weeks. Interestingly, uterine levels of Cox-1 were very low to undetectable at these time points irrespective of genotype (Fig. 3*A*). Levels of *Cox-2* transcripts as determined by RT-PCR correlated well with Cox-2 protein levels (Fig. 3*B*). We also used *in situ* hybridization (Fig. 3*C*) and immunohistochemistry

(Fig. 3*D*) to determine the spatiotemporal expression of Cox-2. We observed increased *Cox-2* mRNA and Cox-2 protein levels primarily in endometrial epithelia of $Pten^{pr-/-}$ mice. As expected, Cox-2 expression was low to undetectable in wild-type uteri. This observation is similar to higher Cox-2 expression that is observed in human type I EMC (12, 13).

Heightened levels of Cox-2 protein and mRNA were also observed in $Pten^{pr-/-}/p53^{pr-/-}$ uteri (Fig. 4*A–C* and *D, top*). In contrast, levels of *Cox-2* transcripts remained very low and unaltered in endometria deleted with *p53* alone (Supplementary Fig. S5). Because Cox-2 is known to stimulate cell proliferation in other cancers, we performed Ki67 immunostaining in uterine sections of wild-type, $Pten^{pr-/-}$ or $Pten^{pr-/-}/p53^{pr-/-}$ mice at age 3 weeks. We observed increased Ki67-positive cells primarily in the epithelia of both $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ uteri compared with wild-type mice (Fig. 4*D, bottom*). Because levels of *Cox*-2 expression were lower in $Pten^{pr-/-}/p53^{pr-/-}$ uteri of 2-month-old mice, we also performed Ki67 immunostaining to determine the proliferation status (Fig. 4*D, bottom*). We found a correlation between *Cox-2* expression and proliferation index. Uteri with low *Cox-2* expression at 2 months also had reduced Ki-67 staining. This is in contrast with the observed higher *Cox-2* expression and Ki-67 staining in 3-week-old tumors (Fig. 4*D, bottom*). It is important to note, however, that immunostaining of Ki67 was noted at the leading edge of tumors in uteri of older mice. This agrees with previous studies showing that the middle of tumors is hypoxic and necrotic (27). These findings in our $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ mice are exciting and identify these two mouse models as suitable systems for studying human type I EMC.

Down-regulation of *miR-199a and *miR-101a* correlates with heightened Cox-2 expression in endometrial carcinogenesis.** miRNAs are noncoding small RNAs (19–22 nucleotides) transcribed from genomic DNAs. These small RNAs influence posttranscriptional gene expression and effect various biological events (28, 29). Recent studies have shown differential expression of various miRNAs in certain cancers compared with normal tissues. This differential expression is thought to affect the tumor suppressor genes such as p27 and cyclin G_1 (30, 31). We recently found that *miR-199a** and *miR-101a* posttranscriptionally suppress Cox-2 expression in a human cancer cell line (17). We also observed that these miRNAs colocalize with Cox-2 in the mouse uterus during implantation and regulate its protein levels at the implantation site (17). Thus, we examined whether expression levels of these two miRNAs are affected by endometrial carcinogenesis in $Pten^{pr-/-}$ or $Pten^{pr-/-}/p53^{pr-/-}$ uteri. We observed that Cox-2 overexpression in $Pten^{pr-/-}$ uteri (Fig. 3) is indeed correlated with decreased levels of *miR-199a** and

Table 1. EMCs in mice with uterine loss of *Pten* and/or *p53*

Age (d)	Genotype	No of mice examined	Uterine histology	No of mice with indicated histology (%)
10	$Pten^{+/+}$	6	Normal epithelium (no lesion)	6 (100%)
	$Pten^{pr-/-}$	6	Hyperplasia with cytologic atypia	6 (100%)
	$Pten^{+/+}/p53^{+/+}$	6	Normal epithelium (no lesion)	6 (100%)
	$Pten^{pr-/-}p53^{pr-/-}$	6	Hyperplasia with cytologic atypia	6 (100%)
21	$Pten^{+/+}$	8	Normal epithelium (no lesion)	8 (100%)
	$Pten^{pr-/-}$	8	CAH with acute inflammation	8 (100%)
	$Pten^{+/+}/p53^{+/+}$	8	Normal epithelium (no lesion)	8 (100%)
	$Pten^{pr-/-}p53^{pr-/-}$	8	CAH with *in situ* carcinoma, Fallopian tube hyperplasia	8 (100%)
30	$Pten^{+/+}$	8	Normal epithelium (no lesion)	8 (100%)
	$Pten^{pr-/-}$	8	CAH with *in situ* carcinoma	8 (100%)
	$Pten^{+/+}/p53^{+/+}$	8	Normal epithelium (no lesion)	8 (100%)
	$Pten^{pr-/-}p53^{pr-/-}$	8	Grade II carcinoma, disease more severe than $Pten^{pr-/-}$ uteri	7 (87.5%)
			Cribriform appearance of glands, invasion into myometrium	1 (12.5%)
60	$Pten^{+/+}$	8	Normal epithelium (no lesion)	8 (100%)
	$Pten^{pr-/-}$	8	Carcinoma	4 (50%)*
			CAH with focal carcinoma	1 (12.5%)
			CAH with squamous metaplasia	3 (37.5%)†
	$Pten^{+/+}/p53^{+/+}$	7	Normal epithelium (no lesion)	7 (100%)
	$Pten^{pr-/-}p53^{pr-/-}$	6	*In situ* carcinoma with cribriform pattern without invasion	3 (50%)
			Invasion into the myometrium with well to moderate differentiation	3 (50%)
90	$Pten^{+/+}$	9	Normal epithelium (no lesion)	8 (88.9%)
			Hyperplasia	1 (11.1%)
	$Pten^{pr-/-}$	9	Carcinoma	8 (88.9%)‡
			CAH with squamous metaplasia	1 (11.1%)

Abbreviation: CAH, complex atypical hyperplasia.
*One mouse showed invasion.
† Two mice showed invasion.
‡ One mouse showed extensive carcinoma; one showed well differentiated adenocarcinoma with invasion into the serosa and two showed carcinoma with squamous metaplasia.

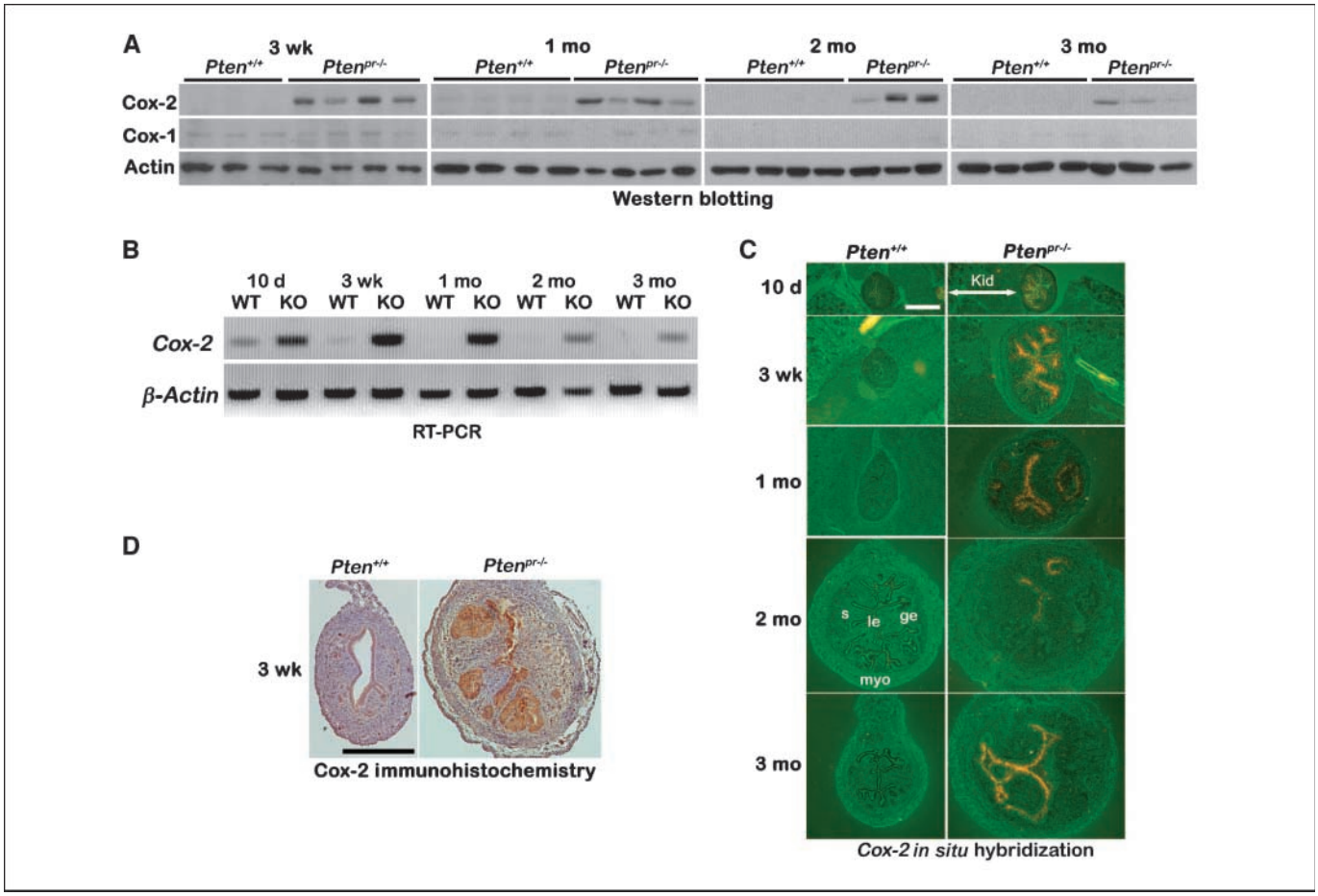

Figure 3. Heightened Cox-2 expression in *Pten*$^{pr-/-}$ uteri with EMC. *A,* Western blot analysis of Cox-2 and Cox-1 in *Pten*$^{pr-/-}$ uteri. Actin serves as control. *B,* RT-PCR of *Cox-2* in *Pten*$^{pr-/-}$ uteri. *β-Actin* is a housekeeping gene. *WT*, wild-type; KO, *Pten*$^{pr-/-}$. *C, in situ* hybridization of *Cox-2* in *Pten*$^{pr-/-}$ and wild-type (*Pten*$^{+/+}$) uteri. *Bar,* 400 μm. *le,* luminal epithelium; *ge,* glandular epithelium; *s,* stroma; *myo,* myometrium; *Kid,* kidney. *D,* immunohistochemistry of Cox-2 in uteri of 3-wk-old *Pten*$^{pr-/-}$ and wild-type (*Pten*$^{+/+}$) mice. *Bar,* 400 μm.

miR-101a expression (Fig. 5*A*). The expression of these miRNAs is also reduced in *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ uteri with elevated Cox-2 expression (Fig. 5*B*). These data suggest that *miR-199a** and *miR-101a* play roles in regulating Cox-2 expression in EMC.

Discussion

Mutations of tumor suppressor genes are linked to carcinogenesis in various organ systems. Unfortunately, systemic deletion of these genes in mice often results in embryonic lethality. The *loxP-Cre* system allows for tissue-specific and temporal deletion of tumor suppressor genes (32, 33). The goal of this study was to take advantage of the *loxP-Cre* system to generate conditional deletion of *Pten* and/or *p53* in the uterus to study the initiation and progression of endometrial carcinoma. Because there is no endometrial-specific promoter identified as of yet, *PR-Cre* mice are being widely used to delete floxed genes in the uterus (16, 20, 24). It is to be noted, however, that PR is also expressed in tissues such as oviduct, ovary, mammary gland, and pituitary. Our current study shows that EMC develops in *Pten*$^{pr-/-}$ or *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ mice within age 3 weeks to 1 month with no evidence of cancers in other tissues, including those that express PR. One reason for development of endometrial-specific cancer could perhaps be attributed to increased sensitivity of the uterus to *Pten* loss. Nonetheless, this endometrial-specific phenotype demonstrates the importance of *Pten* loss in the initiation of EMC. Studies in humans have shown that type I EMC originates mainly from the epithelial components and not the stroma; however PR is expressed in both epithelia and stroma (1). Therefore, whether EMC in *Pten*$^{pr-/-}$ or *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ mice originates from epithelial or stromal components remains to be determined. Because Pten is expressed primarily in endometrial epithelia during early neonatal development, it is speculated that cancer is initiated in this compartment due to the loss of *Pten*. Definitive answers to the specific roles of epithelial versus stromal Pten in EMC will require epithelial-specific *loxP-Cre* and stromal-specific *loxP-Cre* systems for deletion of *Pten*.

We observed that the combined loss of *Pten* and *p53* resulted in a shorter life span compared with mice with single deletion of either *Pten* or *p53*. Previous reports suggested a role for *p53* in advanced type I EMC in women (1). Therefore, we speculated that superimposing *p53* mutation on *Pten* deletion would aggravate the disease condition. Our findings of shorter life span of *Pten*$^{pr-/-}$/*p53*$^{pr-/-}$ mice supports this hypothesis, with the cause of early death attributed to excessive uterine bleeding due to invasion of uterine blood vessels by invasive tumor cells. As a note, type II EMC, which comprises only 10% to 15% of all EMCs, shows a high frequency of *p53* mutations. This is contrary to our observation

that uterine deletion of p53 does not cause cancer in mice (1). Therefore, understanding the role of *p53* mutations in EMC requires further studies.

Our findings of deletion of both *p53* and *Pten* aggravating the mortality rate are consistent with a similar study that used conditional deletion of *Pten* to induce prostate cancer (32). In this study, the investigators used $Pten^{loxp/loxp}$/*probasin-Cre* mice and showed that *Pten* deletion alone did not increase the incidence of mortality, but the combined deletion of both *Pten* and *p53* did. Their explanation for this observation was that deletion of *Pten* alone resulted in *p53* up-regulation, which protected cells from senescence (32). We also observed *p53* up-regulation primarily in endometrial epithelia of $Pten^{pr-/-}$ mice (data not shown), consistent with their explanation. Another possibility is that *PR-Cre* driven deletion of *Pten* and *p53* results in deleterious effects on immune cells. Studies have shown that T-cell or B-cell–specific deletion of *Pten* results in T-cell lymphomas or defects in class switch recombination (reviewed in ref. 34). It seems unlikely that local immune complications contribute to the progression of EMC in $Pten^{pr-/-}/p53^{pr-/-}$ mice because neither estrogen receptor nor

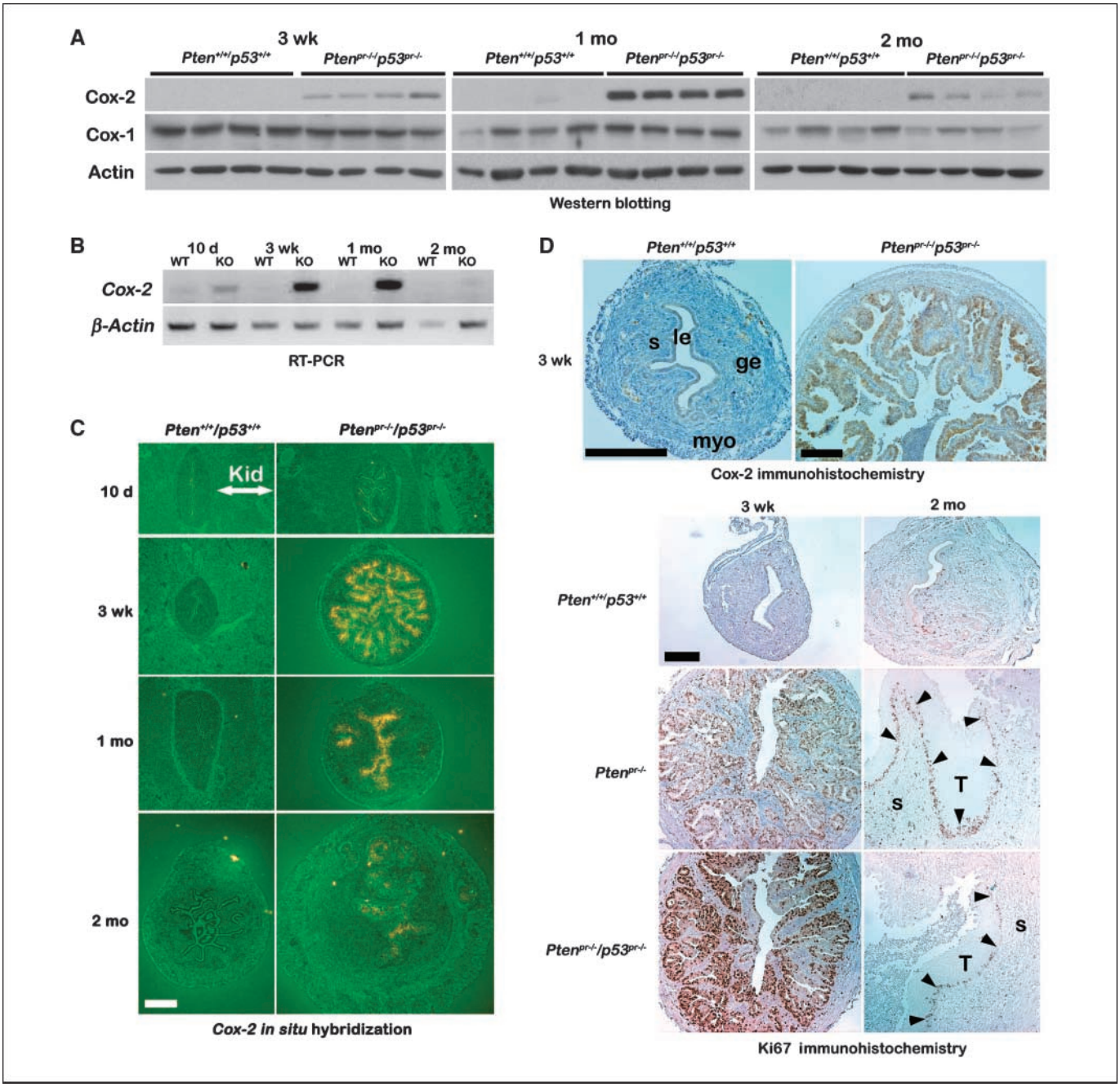

Figure 4. Cox-2 expression and cell proliferation in $Pten^{pr-/-}/p53^{pr-/-}$ uteri. *A,* Western blot analysis of Cox-2 and Cox-1 in $Pten^{pr-/-}/p53^{pr-/-}$ uteri. Actin serves as control. *B,* RT-PCR of *Cox-2* in $Pten^{pr-/-}/p53^{pr-/-}$ uteri. *β-Actin* is a housekeeping gene. *C, in situ* hybridization of *Cox-2* in $Pten^{pr-/-}/p53^{pr-/-}$ and wild-type uteri. *Bar,* 400 μm. *D,* immunohistochemistry of Cox-2 in 3-wk-old $Pten^{pr-/-}/p53^{pr-/-}$ and wild-type mice (*top*). Immunohistochemistry of Ki67 in 3-wk-old and 2-mo-old $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ and wild-type uteri (*bottom*). *Arrowheads,* leading edge. *Bar,* 200 μm. *le,* luminal epithelium; *ge,* glandular epithelium; *s*, stroma; *myo,* myometrium; *Kid,* kidney; *T,* tumor.

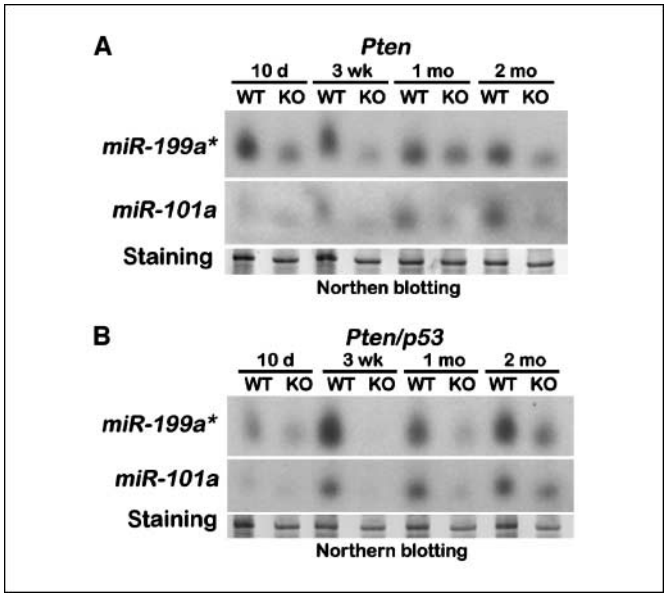

Figure 5. Levels of *miR-199a** and *miR-101a* expression are reduced in uteri with EMC. Northern blot analysis of (*A*) $Pten^{+/+}$ (*WT*) versus $Pten^{pr-/-}$ (*KO*) uteri and (*B*) $Pten^{+/+}/p53^{+/+}$ (*WT*) versus $Pten^{pr-/-}/p53^{pr-/-}$ (*KO*) uteri. Stained gels served as loading controls.

PR is expressed in uterine lymphocytes, macrophages, or natural killer cells (35, 36).

Because EMC is also known to be influenced by hormonal components (1), we ovariectomized $Pten^{pr-/-}/p53^{pr-/-}$ mice age 3 weeks to evaluate the contribution of ovarian steroid hormones. We found that ovariectomizing these mice did not minimize tumor development when examined at 2 months (Supplementary Fig. S6), suggesting limited contribution of ovarian steroid hormones to EMC progression induced by *Pten* and *p53* mutations.

Cox-2 is thought to play an important role in carcinogenesis and is overexpressed in a number of solid tumors, including colorectal, breast and lung cancers (10). Treatments with nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit Cox activity have been shown to be effective in the chemoprevention of many types of solid tumors. In fact, even their daily consumption reduces the risk of certain cancers (37–40). Our study showing elevated Cox-2 expression at the early stages of hyperplasia and carcinoma in both $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ mice suggests that NSAIDs could be considered potential treatment options for type I EMC patients at early stages. Unfortunately, recent clinical studies show that prolonged use of highly selective Cox-2 inhibitors, such as Vioxx, leads to increased myocardial infarctions and stroke. However, NSAIDs such as aspirin or naproxen show lesser side effects and are still being widely used to combat inflammatory diseases and to reduce the risk of developing cancers.

Although Cox-2 expression was elevated in the early stages of hyperplasia and carcinoma, we were initially surprised to observe its gradual disappearance with cancer progression. However, it has been shown that Cox-2 is often associated with early stages of cancer in both liver and uterine cancers, and then is down-regulated with tumor advancement (41, 42). In uterine cancers, levels of Cox-2 correlate with vascular endothelial growth factor expression, implicating their roles in angiogenesis at early stages (42). These studies suggest the potential for NSAIDs treatment to prevent recurrence of EMCs.

Our observations of increased levels of pAkt and Cox-2 at early stages of EMC suggest that the Pten-Akt-Cox-2 signaling axis is important for the initiation of tumor growth. The accelerated growth perhaps occurs due to increased cell proliferation that is evident from Ki67 staining even at age three weeks. Our results showing decreased expression of *miR-199a** and *miR-101a* with enhanced Cox-2 levels in $Pten^{pr-/-}$ and $Pten^{pr-/-}/p53^{pr-/-}$ uteri also suggests their close relationship with Cox-2 status in the uterus as we have previously shown (17). However, it is not known whether Pten directly regulates the expression of these miRNAs or their down-regulation is a consequence of the development of EMC with enhanced Cox-2 expression. Nonetheless, these miRNAs are potential targets for anticancer therapy because of their role in down-regulating Cox-2 levels.

In conclusion, the present study presents mouse models that rapidly and unfailingly produce EMC, many characteristics of which mimic human EMC. These models will help delineate other downstream signaling pathways critical to the initiation and progression of human EMC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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