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Cell-specific conditional deletion of *Pten* in the uterus results in differential phenotypes

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Abstract

Objective—Endometrial cancer (EMC) is the most common gynecological malignancy. The etiology and the cell types that are conducive to EMC are not completely understood, provoking further studies. Our objective was to determine whether deletion of *Pten* specifically in the uterine stroma and myometrium induces cancer or manifests different phenotypes.

Methods—*Pten*^{Amhr2(d/d)} mice with conditional deletion of *Pten* in the mouse uterine stroma and myometrium, but not in the epithelium, were generated by mating floxed *Pten* mice and *anti-Mullerian hormone type 2 receptor (Amhr2)-Cre* mice. The phenotypes were compared between *Pten*^{ff/ff} and *Pten*^{Amhr2(d/d)} uteri.

Results—We show that conditional deletion of *Pten* in the mouse uterine stroma and myometrium, but not in the epithelium, fails to generate EMC even at the age of 5 months. Surprisingly *Pten* deletion by *Amhr2-Cre* transformed a large number of myometrial cells into adipocytes with lipid accumulation, possibly a result of increased levels of SREBP1 and PPAR γ which regulate adipose differentiation.

Conclusions—These results provide evidence that deletion of *Pten* specifically in the stroma and myometrium does not result in EMC in female mice examined up to 5 months of age but alters the myocytes to adipocytes and mimics histologic similarities with lipoleiomyomas in humans, raising the possibility of using this mouse model to further explore the cause of the disease.

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Introduction

Endometrial cancer (EMC) is the most common gynecological malignancy. In fact, 6% of U.S. female cancer patients exhibit EMC (1). According to the American Cancer Society, nearly 42,000 new cases of EMC were diagnosed and 7,800 patients died from the disease in 2009 (1). There are several treatment options effective for the early stages of EMC, such as hysterectomy and hormonal therapy; however, only limited options remain if the cancer metastasizes (2). The etiology and the cell types that give rise to EMC are not completely understood and further studies are warranted to develop treatment options.

Animal models that spontaneously develop cancer are powerful tools for studying the mechanism of cancer initiation and progression and for developing treatment strategies. We previously reported a mouse model of endometrial carcinoma in which the floxed *phosphatase and tensin homologue (Pten)* gene was deleted by Cre expression under the control of a *progesterone receptor (PR)* promoter (3). PTEN is a tumor suppressor with the highest frequency of mutation in human EMCs (2). In our mouse model, uterine deletion of *Pten* resulted in severe complex hyperplasia of the uterine epithelium by three weeks of age with carcinoma occurring by one month. More importantly, our mouse model of EMC resembles many aspects of type I human EMCs (3).

Conditional deletion of *Pten* in the mouse uterus using *PR-Cre* results in the development of cancer in the epithelium. Given that PR is expressed in the epithelium, stroma, and myometrium, the question remains whether *Pten* deletion in the uterine stroma and/or myometrium also contributes to EMC (4). To address this question, we used *anti-Mullerian hormone type 2 receptor-Cre (Amhr2-Cre)* mice to specifically delete *Pten* in the uterine stroma and myometrium, since *Amhr2* is expressed in these tissue types, but not in the epithelium (5,6). We found that *Pten^{loxP/loxP}/Amhr2^{cre/+}* (*Pten^{Amhr2(d/d)}*) mice did not develop cancer in the uterus even at 150 days of age. Remarkably, many myometrial cells in these mice accumulated lipids and these uteri expressed markers of white adipose tissue, possibly due to the observed increases in levels of SREBP1 (sterol regulatory element binding protein 1) and PPAR γ (peroxisome proliferator-activated receptor γ) that are known to regulate adipose differentiation (7–10). Further investigation as to how myometrial cells transform into adipocytes in *Pten^{Amhr2(d/d)}* mice may provide valuable information relevant to lipoleiomyomas in humans.

Materials and Methods

Mice

All mice were housed in the Cincinnati Children's Hospital Medical Center Animal Care Facility according to NIH and institutional guidelines for laboratory animals. While *Pten^{loxP/loxP}* mice (stock number 004597, 129S4/SvJae/BALB/cAnNTac) were obtained from the Jackson Laboratory, *Amhr2-Cre* mice were obtained from Richard Behringer (MD Anderson Medical Center) and *PR-Cre* mice were obtained from Francesco DeMayo and John Lydon (Baylor College of Medicine). PCR analysis of tail genomic DNA determined the genotypes of mutant mice.

In situ hybridization

Paraformaldehyde-fixed frozen sections were hybridized with ³⁵S-labeled cRNA probes as described (3).

LacZ staining

LacZ staining was performed as previously described (11). In brief, tissues were embedded in OCT after fixation in 0.2% paraformaldehyde and infused in 30% sucrose at 4°C. Frozen sections were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside overnight at 37°C. Sections were counter-stained with eosin.

Immunohistochemistry

Immunohistochemistry was performed as previously described (12). In brief, tissues were fixed in PROTOCOL Safefix II (Thermo Fisher) and embedded in paraffin. Uterine sections (6 μ m) were subjected to immunostaining using antibodies to cytokeratin 8 (CK8; Developmental Studies Hybridoma Bank), α -smooth muscle actin (α SMA; Abcam), or PPAR γ (Santa Cruz). After deparaffinization and hydration, sections were subjected to antigen retrieval by autoclaving in 10 mM sodium citrate solution (pH = 6) for 10 min. A diaminobenzidine (DAB) kit (Invitrogen) was used to visualize antigens. Sections were counter-stained with hematoxylin.

Sudan black B staining

Frozen sections (12 μ m) were fixed in 10% formalin, treated with propylene glycol, and stained with Sudan black B solution (0.7% in propylene glycol). After staining, sections were rinsed with 85% propylene glycol followed by washing in distilled water (13,14).

Oil O Red staining

Frozen sections (12 μ m) were fixed in 10% formalin solution and stained with Oil O Red solution (0.5% in isopropanol). After staining, sections were rinsed with distilled water and mounted.

RNA extraction and RT-PCR

RNA samples were prepared and analyzed as previously described (15). In brief, total RNA was extracted with RNAeasy Protect Mini kit (Qiagen) according to the manufacturer's protocol. Samples were obtained from three *Pten*^{f/f} or *Pten*^{Amhr2(d/d)} uteri. Quantitative RT-PCRs using Taqman probes (Applied BioSystems) were performed with primer sets specific for *Adfp* (adipose differentiation-related protein), *Lpl* (lipoprotein lipase), and *Fabp4* (fatty acid binding protein 4). A probe set for 18S rRNA was used as the normalization standard. The PCR and relative quantifications were performed using 25 ng of cDNA per reaction in a real-time PCR system. Relative quantification data from quantitative PCR analysis were statistically analyzed using Student's *t*-tests.

Western blot analysis

Tissue samples were prepared as previously described (12). After measuring protein concentrations, supernatants were mixed with SDS-PAGE sample buffer and boiled for 5 min. Samples were run on 10% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked with 10% milk or 5% BSA in TBST and probed with antibodies to pAKT (Cell Signaling), AKT (Cell Signaling), SREBP1 (Santa Cruz), or ACTIN (Santa Cruz) overnight at 4°C. After washing, blots were incubated in peroxidase-conjugated donkey anti-goat or donkey anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc.). All signals were detected using chemiluminescent reagents (GE Healthcare). ACTIN and AKT served as loading controls.

Results

Conditional deletion of *Pten* in the uterine stroma and myometrium fails to induce EMC but transforms numerous myometrial cells to adipocytes

Our first objective was to study whether deletion of *Pten* specifically in the uterine stroma and myometrium induces cancer, since *Pten* is also expressed in the stroma and myometrium (Fig. 1A). For this study, we used *Amhr2-Cre* transgenic mice to delete *Pten* in the stroma and myometrium (5,6). Using *Rosa26R* mice, we confirmed that Cre induced by the *Amhr2* promoter successfully recombined *loxP* sites only in the stroma and myometrium. Recombination did not occur in the epithelium, even at 60 days of age, while *PR-Cre* very rapidly recombines *loxP* sites in the epithelium, stroma, and myometrium (Fig. 1B). Of note, the recombination was also observed in the ovary and oviduct. We examined uterine histology at 150 days of age in *Pten^{loxP/loxP}/Amhr2^{cre/+}* (*Pten^{Amhr2(d/d)}*) mice (n=9). As shown in Figure 2A, *Pten^{Amhr2(d/d)}* uteri had only uncommon foci of complex atypical hyperplasia (CAH), but not EMC, while *Pten^{Pr(d/d)}* females developed EMC (Fig. 2D). Ovarian histology in these mice appeared normal (n=7).

Remarkably, many myometrial cells in *Pten^{Amhr2(d/d)}* uteri showed histological characteristics of adipocytes (n=9), and staining with Sudan Black B and Oil O Red showed accumulation of neutral lipids in those cells (Table 1, Fig. 2B & C). This phenotype was not evident in mice in which uterine *Pten* was deleted by *PR-Cre* (*Pten^{Pr(d/d)}*) even at 150 days of age (n=4; Fig. 2D). To assess whether myometrial adipogenesis was progressive, we examined lipid accumulation by Sudan Black B staining in uteri of *Pten^{Amhr2(d/d)}* at the age of 30 and 60 days. Notably, *Amhr2* expression is observed in the myometrium as early as 14 days of age (6). While no positive staining for lipid accumulation was noted in the myometrium of *Pten^{Amhr2(d/d)}* females at 30 days of age (n=6, Fig 3A), foci of positive staining were evident in myometrium of *Pten^{Amhr2(d/d)}* females by 60 days of age (n=5, Fig 3B). The accumulation of lipid droplets was more restricted in uteri at the age of 60 days than seen at 150 days. These results suggest that myometrial adipogenesis started between 30 to 60 days of age in *Pten^{Amhr2(d/d)}* females.

To further confirm that myometrial cells transformed into adipocytes, we analyzed the expression levels of *Adfp* (adipose differentiation-related protein), *Lpl* (lipoprotein lipase), and *Fabp4* (fatty acid binding protein 4), known markers of adipogenesis by quantitative RT-PCR (16–18). Expression levels of *Lpl* and *Fabp4* were significantly increased in *Pten^{Amhr2(d/d)}* uteri (Fig. 4A). In contrast, levels of *Adfp* were similar between *Pten^{Amhr2(d/d)}* and *Pten^{f/f}* uteri, indicating that *Adfp* did not participate at the formation of intracellular lipid droplets (Fig. 4A). These data indicate that myometrial *Pten* deletion transforms myometrial cells into adipocytes.

Conditional deletion of *Pten* in the uterine stroma and myometrium activates Akt and upregulates SREBP1 and PPAR γ expression

Akt has been shown to upregulate lipogenesis in adipose and muscle tissues (reviewed in (19)) and there is also evidence that activation of Akt increases the level of a transcription factor SREBP1 (sterol regulatory element binding protein 1) in osteosarcoma cells (20). SREBP1 activates another transcription factor, PPAR γ , and induces adipose differentiation in 3T3-L1 cells (7–10). Since pAKT levels are upregulated in *Pten^{Pr(d/d)}* uteri with the development of EMC (3), we also examined the levels of pAKT as well as the status of SREBP1 and PPAR γ in *Pten^{Amhr2(d/d)}* uteri.

We found that the levels of pAKT and SREBP1 are upregulated in *Pten^{Amhr2(d/d)}* uteri compared to *Pten^{f/f}* uteri as evident from Western blot analysis (Fig. 4B). In addition, PPAR γ immunostaining showed an increase in myometrial cells with distinct nuclear

localization of PPAR γ in *Pten*^{Amhr2(d/d)} mice compared to *Pten*^{f/f} mice (Fig. 4C). Notably, PPAR γ nuclear staining was mainly observed in the longitudinal muscle layer, where adipogenesis occurred after *Pten* deletion. These results suggest that the transformation from myometrial cells to adipocytes by *Pten* deletion is mediated through the activation of the pAKT-SREBP1-PPAR γ pathway (Fig. 4D).

Discussion

The observation that *Pten*^{Pr(d/d)} mice with conditional deletion of *Pten* in all major uterine cell types and *Pten*^{Amhr2(d/d)} mice with conditional deletion of *Pten* in the uterine stroma and myometrium produce different phenotypes is very interesting. These results establish a new paradigm that deletion of *Pten* in the stroma/myometrial compartment is not sufficient to drive the development of EMC even by the age of 150 days, although the influence of the stroma in the initiation and development of EMC cannot be ruled out, since foci of CAH were seen. Addressing this issue would require deleting *Pten* specifically in the uterine epithelium. Unfortunately, our attempts to delete epithelial *Pten* using *Wnt7a-Cre* (provided by Richard Behringer) met with failure; *Pten*^{Wnt7a(d/d)} mice died around 10 days of age (data not shown). It was recently reported that epithelial-specific deletion of *Lkb1* in the uterus using *Spr2f-Cre* produces EMC, suggesting that EMC primarily originates from epithelial cells (21). However, *Spr2f-Cre* mice cannot be used in studying PTEN's roles in EMC, since deletion of *Pten* by *Spr2f-Cre* induces brain cancer due to Cre expression in the cerebellum and limits the life span of those mice (personal communication, Diego Castrillon, UT Southwestern Medical Center) (21). In addition, a recent report shows that only the epithelial absence of *Pten* can induce EMC in an *in vivo* endometrial regeneration system (22). Collectively, our results and the results of others provide evidence that EMC is of epithelial origin.

Another significant finding of our present study is the transformation of myometrial cells into adipocytes in *Pten*^{Amhr2(d/d)} mice. This transformation, however, does not occur in mice with *Pten* conditionally deleted in all major uterine cell types (epithelium, stroma, and myometrium) using *PR-Cre*. PTEN's role in adipogenesis has previously been reported in other systems. Chondrocyte-specific deletion of *Pten* leads to the formation of adipose tissues and lipomas along the spine (23,24), hepatocyte-specific deletion of *Pten* leads to steatohepatitis with hepatocellular carcinomas, and patients with *Pten* mutations often develop vascular anomalies with ectopic accumulations of fat (25). However, myocytes do not transform into adipocytes when *Pten* is conditionally deleted in skeletal muscles and cardiomyocytes in mice (26,27). These results may suggest that the transformation occurs only in smooth muscles. In the myometrium, PTEN's role in adipogenesis is specific to the longitudinal muscle layer; the reason for this specificity remains unknown.

In this study, we provide evidence that *Pten* deletion in the uterine stroma and myometrium activates the pAKT-SREBP1-PPAR γ pathway to trigger myometrial adipogenesis (Fig. 4D). Increased levels of SREBP1 and PPAR γ were also observed in mice with hepatocyte-specific *Pten* deletion, resulting in steatohepatitis (24). However, defining the exact mechanism and the role of stroma and smooth muscle in this process would require further investigation. There is evidence that conditional deletion of β -catenin using *Amhr2-Cre* mice switches myogenesis to adipogenesis in the uterine myometrium of adult mice. Since *Amhr2* (also known as Mullerian inhibiting substance type receptor) is expressed in the Mullerian duct mesenchyme before postnatal differentiation of the uterus into distinct tissue types, it was suggested that deletion of β -catenin in the mesenchymal tissue resulted in the switching of myogenesis to adipogenesis in the adult myometrium (28). This perhaps explains the transformation of myocytes to adipocytes in mice conditionally deleted of *Pten* by Cre expression driven by the *Amhr2* promoter as opposed to Cre driven by PR whose

expression becomes prominent from postnatal day 10. It is interesting to note that impairment of both canonical Wnt signaling and PTEN signaling have similar effects in shifting myogenesis to adipogenesis, although deficiency of these two pathways has opposing effects on tumorigenesis. Further investigation is warranted to better understand this complex process.

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Figure 1. *Pten* expression and conditional deletion models in the mouse uterus

A. *In situ* hybridization of *Pten* in the wild-type uterus. ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium. Bar, 400 μ m. **B.** The conditional gene recombination by Cre induced by the *Amhr2* promoter (upper panel) or *PR* promoter (lower panel) was visualized by LacZ staining. Tissues were harvested at the age of 60 days. Note the absence of recombination by *Amhr2-Cre* in the epithelium (arrows). F, follicle; CL, corpus luteum. Bar, 400 μ m.

Figure 2. The myometrium from *Pten*^{Amhr2(d/d)} mice shows conversion of myocytes to adipocytes at the age of 150 days

A. H & E (upper panel), CK8 (middle panel), and αSMA (lower panel) staining. CK8 and αSMA are typical markers for the epithelium and myometrium, respectively. Arrows indicate adipocytes. le, luminal epithelium; ge, glandular epithelium; myo, myometrium. Bar, 400 μm. **B.** Sudan Black B staining of fat droplets. Bar, 400 μm. **C.** Oil O Red staining of fat droplets. Bar, 400 μm. **D.** H & E staining of 150 days old *Pten*^{f/f} and *Pten*^{Pr(d/d)} mice uteri. Note the transformation of the *Pten*^{Pr(d/d)} epithelium into cancer. Bar, 400 μm.

Figure 3. The myometrial switching to adipogenesis in *Pten^{Amhr2(d/d)}* mice occurs as early as 60 days of age

Sudan Black B staining of lipid droplets at the age of 30 days (**A**) and 60 days (**B**). Arrows indicate adipocytes with lipid accumulation. le, luminal epithelium; ge, glandular epithelium; myo, myometrium. Bar, 400 μm.

Figure 4. Myometrial differentiation to adipocytes in *Pten^{Amhr2(d/d)}* uteri examined at the age of 150 days

A. Quantitative RT-PCR of adipose tissue markers. Values were normalized to 18S rRNA and are representative of 3 mice per group. **B.** Western blot analysis of pAKT and SREBP1 (upper panel) and their relative levels assessed by Scion Image (lower panel). Relative levels of pAKT and SREBP1 were normalized to AKT and ACTIN levels (loading controls), respectively. **C.** PPAR γ Immunohistochemistry. Brown deposits in nuclei indicate positive immunostaining for PPAR γ . Lower (left panel) and higher (right panel) magnifications are shown. cm, circular muscle; lm, longitudinal muscle. Bar; 100 μ m. **D.** A proposed model showing a potential pathway for transforming myometrial cells to adipocytes with the loss of *Pten*.

Table 1

Conditional deletion of uterine *Pten* by *Amhr2-Cre* switches myocytes to adipocytes.

Age (days)	Genotype	No. of mice examined	Adipogenesis (%)
30	<i>Pten^{fl/f}</i>	6	0
	<i>Pten^{Amhr2(d/d)}</i>	6	0
60	<i>Pten^{fl/f}</i>	4	0
	<i>Pten^{Amhr2(d/d)}</i>	5	100
150	<i>Pten^{fl/f}</i>	4	0
	<i>Pten^{Amhr2(d/d)}</i>	9	100