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Adenovirus Mediated Homozygous Endometrial Epithelial *Pten* Deletion Results in Aggressive Endometrial Carcinoma

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Abstract

PTEN is the most frequently mutated gene in uterine endometrioid carcinoma (UEC) and its precursor complex atypical hyperplasia (CAH). Because the mutation frequency is similar in CAH and UEC, *PTEN* mutations are thought to occur relatively early in endometrial tumorigenesis. Previous work from our laboratory using the *Pten*^{+/-} mouse model has demonstrated somatic inactivation of the wild type allele of *Pten* in both CAH and UEC. In the present study, we injected adenoviruses expressing Cre into the uterine lumen of adult *Pten* floxed mice in an attempt to somatically delete both alleles of *Pten* specifically in the endometrium. Our results demonstrate that biallelic inactivation of *Pten* results in an increased incidence of carcinoma as compared to the *Pten*^{+/-} mouse model. In addition, the carcinomas were more aggressive with extension beyond the uterus into adjacent tissues and were associated with decreased expression of nuclear ERα as compared to associated CAH. Primary cultures of epithelial and stromal cells were prepared from uteri of *Pten* floxed mice and *Pten* was deleted *in vitro* using Cre expressing adenovirus. *Pten* deletion was evident in both the epithelial and stromal cells and the treatment of the primary cultures with estrogen had different effects on Akt activation as well as Cyclin D3 expression in the two purified components. This study demonstrates that somatic biallelic inactivation of *Pten* in endometrial epithelium *in vivo* results in an increased incidence and aggressiveness of endometrial carcinoma compared to mice carrying a germline deletion of one allele and provides an important *in vivo* and *in vitro* model system for understanding the genetic underpinnings of endometrial carcinoma.

Keywords

Pten; endometrial carcinoma; biallelic inactivation

Introduction

Endometrial cancer is the most common gynecological malignancy but despite its prevalence, the underlying molecular mechanisms resulting in the disease remain largely elusive. Most uterine endometrioid carcinomas (UEC) are indolent and arise in the setting of unopposed estrogen stimulation. Complex atypical hyperplasia (CAH) is the precursor of

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UEC and it has been reported that 25% CAH progress to UEC (Reviewed in [1]). However, the molecular changes that define the transition from CAH to UEC are unknown.

Molecular genetic analysis of primary human UEC samples has identified *PTEN* to be the most frequently mutated tumor suppressor gene, with mutations detected in 30–80% of cases [2–5]. Notably, a similar frequency of *PTEN* mutations occurs in CAH and UEC [6–8], suggesting that *PTEN* mutation is an early event in the development of endometrial cancer. PTEN is a dual –specificity phosphatase, capable of dephosphorylating both proteins and lipids. Its lipid phosphatase activity has been the most well characterized. Its major substrate is phosphatidylinositol-3,4,5-phosphate (PIP3), an important second messenger that is generated via phosphatidylinositol kinase (PI3K) as a result of growth factor signaling [9, 10]. PTEN activity converts PIP3 to phosphatidylinositol-4,5-phosphate (PIP2), indirectly inhibiting the action of PI3K. In normal cells, growth factor signaling activates PI3K resulting in activation of AKT by phosphorylation due to recruitment to the membrane by PIP3. AKT is a protein kinase that regulates a number of downstream pathways that impinge on cell proliferation, cell growth and apoptosis. Thus, by preventing AKT activation PTEN inhibits cell proliferation and growth [11]. Several downstream targets of AKT have been well characterized but the primary targets of this pathway in the context of endometrial cancer are not yet defined. In addition, other targets of PTEN (e.g., FAK) may play a role in the development of endometrial carcinoma.

The histologic appearance of the endometrial tumors arising in the *Pten*^{+/-} mouse model closely mimics that seen in humans [12, 13]. All *Pten*^{+/-} female mice develop CAH by 32 weeks of age and at approximately 52 weeks of age, 25% of the *Pten*^{+/-} female mice develop UEC. Although this frequency of progression is comparable to that in humans, it renders the model cost and time inefficient.

One striking feature of CAH and UEC in mice is the complete loss of Pten expression as demonstrated by immunostaining [14, 15]. DNA analysis of lesions microdissected from uterine sections (CAH or UEC) showed that approximately 70% of the lesions exhibit biallelic loss of *Pten* either due to intragenic mutations or loss of heterozygosity (LOH) of the wild type copy [14]. Using the *Pten*^{+/-}; *Mlh1*^{-/-} mouse model, we demonstrated that the frequency of inactivation (by intragenic mutation and LOH) was similar in CAH and carcinoma [15]. These observations suggest that biallelic inactivation of *Pten* occurs as a relatively early change in the progression of the disease in the mouse model. Absence of *Mlh1* on the background of *Pten* deficiency significantly accelerated the onset of endometrial disease as compared to *Pten*^{+/-} mice but the incidence of invasive disease remained the same. However, due to non-uterine disease the mice were sacrificed at a relatively young age. Of note, recent epidemiological studies show that loss of PTEN expression in CAH on endometrial sampling does not correlate with progression to carcinoma [16]. These findings suggest that while biallelic inactivation of *Pten* is an early event in endometrial tumorigenesis, in both humans and mice, it may not be sufficient for progression to invasive carcinoma.

Pten homozygous mice die during embryogenesis hence conditional knockout models are essential for studying the consequences of biallelic inactivation of *Pten* in the endometrium. *Pten* has been successfully deleted in a tissue specific manner using the Cre-Lox system in the prostate [17, 18], brain [19, 20], pancreas [21] and several other organs primarily because of the availability of well characterized tissue restricted promoters driving Cre expression. The endometrial pathology in mice with *Pten* deletion in the uterus obtained by crossing *Pten* floxed mice with those expressing Cre under the control of the progesterone receptor promoter (*PR*^{cre/+}) was recently described [22]. The female pups developed CAH as early as 10 days of age and by 1–2 months of age, all displayed extensive invasive disease

extending into the myometrium. Deletion of both copies in the uterus therefore significantly reduced the age of onset as well as accelerated the progression of the disease. However, in this model *Pten* is deleted by 10 days of age, suggesting that the inactivation occurs during embryogenesis. As such, this model does not accurately mimic the conditions under which sporadic UEC develops in humans.

In order to more closely recapitulate the situation in humans, we deleted *Pten* in the endometrium of adult female mice to determine the consequence of somatic biallelic *Pten* deletion on the development of endometrial hyperplasia and carcinoma.

Materials and Methods

Animals

Pten^{loxP/loxP} mice on a Balbc/129SvJ background and B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor/J} strains were obtained from the Jackson Laboratory. All animal experiments and surgeries were done in accordance to IACUC guidelines.

Injection of Adenovirus-Cre or Adenovirus-GFP into the uterine lumen

Adult, cycling female *Pten*^{loxP/loxP} mice were anaesthetized with isoflourane and the uteri exposed. The uterine horns were ligated with suture at the base to prevent leakage of virus solution out of the uterus into the vagina. The right horn was then injected with adenovirus expressing Cre (Adeno-*Cre*), while the left horn was injected with adenovirus expressing GFP (Adeno-*GFP*) in a total volume of 10 μ l (corresponding to approximately 10^8 pfu) using a 26 gauge needle attached to a Hamilton syringe. The virus solution was spiked with trypan blue to monitor the injection efficiency. The animals were euthanized 12–32 weeks after injection. The uteri were harvested, fixed in 10% formalin, paraffin embedded, sectioned and stained with Hematoxylin and Eosin. Additionally, we also injected uteri of B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor/J} mice (Jackson Laboratories), harboring the R26R-LacZ allele (R26R mice). The R26R mice allowed us to identify cell types infected with adenovirus by staining the uteri for LacZ. These mice were also used to determine the efficiency of injections with respect to virus delivery and Cre activity.

LacZ staining and Immunohistochemistry

Uteri from R26R mice were processed for LacZ staining. Staining was performed according to standard protocols and tissues were fixed in formalin, paraffin embedded and sectioned. Sections were de-paraffinized, hydrated and counterstained with Nuclear Fast Red (Sigma).

The uteri harvested from *Pten*^{loxP/loxP} mice were processed for Hematoxylin and Eosin (H&E) staining as well as immunohistochemical (IHC) analysis. For IHC, the slides were de-paraffinized and rehydrated, slides were subjected to antigen retrieval by boiling in the microwave in 10mM sodium citrate. The Vectastain ABC reagent (Vector Labs) was then used according to manufacturer's protocol. Antibodies used were anti-Pten (1:100), anti-phospho-Akt S473 (1:100) from Cell Signaling, Beverly, MA and anti-ER α (1:1000) from SantaCruz Biotechnologies.

Scoring of immunostained slides

Scoring of slides was done to quantify the expression of ER α in lesions and normal epithelium. The localization was determined to be either nuclear or cytoplasmic. Staining intensities were ranked from 1 to 4, corresponding to increasing intensity of the staining with 1 being the least and 4 the strongest. The percent positive cells were estimated by scoring 5 fields at 100 $\times$ magnification and ranked from 0 to 4 (0=0%, 1=1–25%, 2=26–50%,

3= 51–75%, 4=74–100%). Scores were expressed as a product of the intensity and percent ranks.

Primary uterine cell cultures and treatments

We used previously published protocols for preparation of primary cells with minor modifications [23]. For epithelial cells, uteri from *Pten^{loxP/loxP}* mice were dissected and the fat was trimmed. The horns were slit lengthwise and digested in a solution containing 0.25% trypsin and 2.5% pancreatin in Hank's balance salt solution (HBSS) for one hour at 4°C followed by another incubation for one hour at 22°C. The tissue pieces were transferred to ice cold HBSS and vortexed for 30 sec to release sheets of epithelial cells. After collecting the HBSS containing cells, the uteri were resuspended in HBSS and vortexed again. This procedure was repeated for a total of three times and all the collected epithelial cells were pooled. The cell suspension was filtered through a 20 µm nylon mesh, the epithelial sheets (retained on the filter) were collected and re-suspended in DMEM/F12 (1:1), 10% charcoal stripped FBS, 20 mM HEPES, 100 µg/ml streptomycin, 100U/ml penicillin and 2 mM L-glutamine. Cells were plated in 6-well dishes coated with 1:8 diluted Matrigel (Gibco) and cultured at 37°C in an incubator with 5% CO₂.

The remaining uterine tissue pieces were then used for preparing stromal cells. The tissues were washed and vortexed further 3–4 times in HBSS so that most of the remaining epithelial cells were removed. The fragments were digested in Trypsin-EDTA at 37°C for 30 minutes and the HBSS collected. The stromal cells were collected by centrifugation and the cell pellets were re-suspended in stromal cell culture medium (DMEM/F12 (1:1), 10% charcoal stripped FBS, 20 mM HEPES, 100 µg/ml streptomycin, 100U/ml penicillin. The cells were plated in 6-well dishes without matrigel and cultured at 37°C in an incubator with 5% CO₂.

To investigate the downstream signaling events occurring subsequent to *Pten* loss, the primary cultures were infected with adenoviruses encoding *Cre* recombinase (adeno-*Cre*) or GFP (adeno-*GFP*) (Vector Biolabs) after the cells were confluent (approximately 4 days after plating). Cells were infected with 10⁶ pfu/ml viruses expressing either GFP or *Cre*. After 48 hrs, the cells were harvested, lysed in RIPA buffer and analyzed by immunoblotting. The efficiency of infection was determined by monitoring GFP fluorescence. Additionally, cells were also treated with 7.9, 29.6 and 146 nM estradiol two days after adenovirus treatment. After 48 hours of estradiol treatment, cells were harvested for immunoblot analysis. Cells treated with vehicle were used as control. All cell cultures and treatments were performed three times and the results are summarized in the following sections.

Immunoblot analysis

For immunoblot analysis, cells were grown in a 6-well dish and lysed in 250µl per well of ice-cold 1X RIPA buffer with protease and phosphatase inhibitors. Cells were passed through a 27G needle and clarified by centrifugation at 13,000 rpm for 15 minutes at 4°C. All manipulations were carried out on ice. Lysates were resolved on 10% SDS-PAGE, transferred to PVDF membrane and blocked in 5% non-fat dried milk dissolved in 1X Tris buffered saline with 0.1% Tween-20 (TBS-T). Membranes were probed with *Pten*, phospho-Akt-Ser473 (Cell Signaling, Beverly, MA) and cyclin D3 (SantaCruz). All antibodies were used at a dilution of 1:1000 and membranes were incubated overnight at 4°C with primary antibody, washed liberally at room temperature in TBS-T and incubated for 1 hr with horseradish peroxidase-conjugated secondary antibody. After liberal washing in TBS-T, blots were incubated in Western Lightning Plus ECL (Perkin Elmer) reagent and visualized

by exposure to ECL film (Kodak). Quantitation was performed using the ImageJ software downloaded from the NIH website.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The fold regulation in cyclin D3 was analyzed using the unpaired t-test with $p < 0.05$.

Results and Discussion

Adenoviruses infect epithelium and stroma in-vivo

We initially tried to demonstrate efficacy of adenoviral infection and excision of *Pten* by immunostaining with GFP or *Pten* antibody on uterine sections of animals sacrificed at early time points (3 days). We were unable to detect expression of GFP or the deletion of *Pten* in these animals (data not shown). We therefore choose to use the B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J reporter mice (R26R mice) for injections. Uterine horns of mice were injected with adeno-Cre or medium without virus as control. Horns were harvested 4 days after injection and stained for LacZ expression. As seen in figure 1A, a small percentage of luminal epithelial and stromal cells of adeno-Cre injected mice showed strong staining for LacZ. Control mice completely lacked strongly positive LacZ cells (Fig. 1B). Furthermore, only 25% of the injected horns (3 out of 12) exhibited LacZ staining. This observation proved that we were able to infect the epithelial and stromal cells by this method of injection. The small percent of LacZ-positive cells likely explains why we were unable to detect GFP expression or *Pten* deletion in *Pten*^{loxP/loxP} mice. Despite infection of very few cells, aged out *Pten*^{loxP/loxP} mice developed CAH or carcinomas as described in the following sections.

Simultaneous loss of both copies of *Pten* increases the incidence and aggressiveness of endometrial carcinoma

In previous studies we found that by 16 weeks of age 70% of *Pten*^{+/-} mice had CAH and by 32 weeks of age 100% of the mice had developed endometrial lesions [14]. Therefore we chose to sacrifice *Pten*^{loxP/loxP} mice between 12 and 32 weeks after uterine injection of adenovirus. Analysis of H&E stained sections of the right uterine horns (adeno-Cre injected) revealed that out of a total of 24 mice, 12 (50%) exhibited disease, either CAH or invasive cancer or both (Table 1 and 2). The lesions were morphologically similar to those observed in *Pten*^{+/-} mice (Fig. 1, D&E) and lacked *Pten* expression as expected (Fig. 2C). They also displayed activation of Akt with localization to the membrane as visualized by immunostaining with p-Akt antibody (Fig. 2D). All of the left horns injected with Adeno-GFP demonstrated normal histology (Fig. 1C) and were positive for *Pten* expression (Fig. 2A). Using this approach, 50% of the mice had complete absence of disease. Since only 25% of the horns injected with virus from the R26R reporter mice showed LacZ-positive cells, we did not expect all of the mice to develop disease. Despite the technical challenges, 42% of *Pten*^{loxP/loxP} mice with lesions (5 out of 12) developed invasive carcinomas.

To date, only one report in the literature has used adenovirus-expressing Cre to specifically delete *Pten* in the uterus. The investigators were able to successfully ablate *Pten* expression but did not carry out the experiment over time to determine if the loss of *Pten* resulted in endometrial pathology [24]. They observed that the efficiency of infection was entirely dependant on the stage of the estrous cycle at the time of injection, with best results occurring during the metestrous and diestrous stages of the estrous cycle. We also determined the stage of estrous cycle of mice before adenoviral injections by vaginal smears. In our experiments we did not find a significant correlation between the stage of the

estrous cycle and the development of lesions. In fact, two of the mice with aggressive disease were injected during the estrous stage.

Out of the 12 mice with lesions, 5 (42%) exhibited invasive disease 2 of which had associated CAH and 7 (58%) developed CAH. One mouse (#101) demonstrated a single focus of adenocarcinoma *in situ* with associated invasive adenocarcinoma. All of the carcinomas invaded through the myometrium with extension into surrounding adipose tissue. Two tumors extended into the ovary and in one the tumor invaded the wall of the colon. We have previously reported a 25% incidence of invasive cancer in the *Pten* heterozygous mice but the mice never exhibited disease that extended beyond the uterus even at 52 weeks of age. Three of the carcinomas also exhibited mucinous metaplasia and one showed squamous metaplasia, features commonly seen in human UEC (Table 2). While performing intra-luminal injections, stromal cells were also infected with the virus (Fig. 1A) likely leading to *Pten* deletion. However, none of the uteri injected with adeno-Cre exhibited stromal tumors or areas of stroma with detectable loss of *Pten* expression.

Another mouse model describing deletion of a gene in adult endometrial epithelium leading to endometrial carcinoma is the *Lkb1*^{loxP/loxP} mouse model [25]. The investigators injected adenoviruses expressing Cre through the cervical OS to delete *Lkb1*. In this mouse model, 65% of the injected mice developed uterine tumors with invasion into the myometrium and one animal exhibited diffuse metastatic cells within the peritoneum. *Lkb1* deficient tumors were extremely well differentiated, lacking defects in cell polarity and activation of Akt. Although in the current study only 42% (5 out of 12) of *Pten*^{loxP/loxP} animals exhibited invasion, 4 animals developed adenocarcinomas that extended out into the ovary, adipose tissue and the colonic wall. Additionally in our model, unlike the *Lkb1*^{loxP/loxP} mouse model, we observed both CAH and carcinoma, recapitulating the entire spectrum of the disease in humans. Thus, these models have different phenotypes that will both be useful in interrogating human endometrial carcinoma.

Thus, uterine specific biallelic *Pten* deletion in adult mice increases the incidence of endometrial carcinoma as well as the extent of the disease. Although biallelic inactivation of *Pten* using a conditional knockout approach revealed early onset CAH and UEC this approach most likely resulted in inactivation of *Pten* during embryogenesis. This is the first report, to our knowledge, of the incidence of CAH and UEC in mice following the inactivation of *Pten* in adult endometrial epithelium *in vivo*. Additionally, biallelic loss of *Pten* in a few cells (as seen in the reporter R26R mice) was sufficient for development of CAH and/or UEC. Thus, our approach more closely mimics the timing of *Pten* inactivation in sporadic endometrial carcinoma in humans.

Reduced nuclear ER α expression in UEC versus CAH

Unopposed estrogen stimulation is a known factor involved in the pathogenesis of UEC. ER α is considered to be the main receptor for estrogen in the uterus and it is commonly expressed in well and moderately differentiated UEC in humans [26]. In order to determine the status of ER α in the adenovirus injected uteri the tissue sections were immunostained with a polyclonal ER α antibody, which is reactive to all isoforms of ER α . ER α immunostaining was quantified by scoring the slides as described in the previous sections (Table 3). Normal luminal and glandular epithelium exhibited very strong nuclear staining for ER α . In CAH and UEC, ER α was also localized to the nucleus (Fig. 3A). The intensity of staining in invasive carcinomas was reduced (3.8 ± 0.7) as compared to that in CAHs (8.7 ± 2.5 , Fig. 3B, arrow). Other investigators have also observed reduced nuclear ER α expression in high-grade lesions and carcinomas of the endometrium in mice [27]. In humans, well-differentiated UECs are generally positive for ER α while expression decreases

with increasing grade. Studies have shown that receptor positive carcinomas have significantly better disease-free survival as compared to the receptor negative tumors [28].

Isolation and short term cultures of primary epithelial and stromal cell cultures

To study downstream targets of *Pten*, we used *in vitro* cell culture models. Although several human endometrial cancer cell lines are available they are not ideal, as they have complex genetic backgrounds including mutations in genes like *KRAS*, *p53* and *PIK3CA* making it difficult to identify events regulated exclusively by PTEN. We chose to use primary epithelial and stromal cell cultures prepared from *Pten^{loxP/loxP}* uteri. *Pten* was deleted *in vitro* by treating cells with adenovirus expressing Cre.

Primary epithelial and stromal cells were isolated as described in the materials and methods section. The epithelial and stromal cells attached to the culture dishes and were 70–80% confluent in approximately 3–4 days. Before using these cells for further analysis, we established the relative purity of the cultures. Confluent cultures were lysed in RIPA and analyzed by SDS-PAGE analysis for expression of cytokeratin 8 (epithelial marker) and vimentin (stromal cell marker). As shown in figure 4A, cytokeratin 8 was expressed only in the epithelial cells while vimentin was detected only in the stromal cell lysates. This indicates that the preparations were relatively pure and free of significant cross contamination.

Next, we infected the epithelial and stromal cells with adeno-Cre or adeno-GFP. Efficiency of infection was monitored by GFP fluorescence. Both the epithelial and stromal cells were GFP-positive after 48 hours of infection (data not shown). These observations indicated that endometrial primary cultures were capable of being infected by adenovirus. To test loss of *Pten* after adenoviral infection, we examined the expression of *Pten* by immunoblot analysis. Adenoviral treatment significantly reduced the expression of *Pten* within 48 hrs after treatment in both the epithelial (Fig. 4B) and stromal cells (Fig. 4C). Activation of Akt by phosphorylation at Ser473 was also observed after *Pten* deletion in both the epithelial and stromal cells.

Differential Regulation of downstream signaling molecules in primary cultures

As discussed previously, many cases of UEC arise in the setting of unopposed estrogen stimulation and about 30–80% of cases (including the precursor lesion CAH) harbor mutations in *PTEN*. Oophorectomized *Pten^{+/-}* mice treated with slow release estradiol pellets developed more aggressive disease as compared to oophorectomized *Pten^{+/-}* mice treated with placebo pellets (unpublished results). *Previous studies have shown that activated Akt can phosphorylate the ERα receptor in a ligand independent manner* [29, 30]. Thus, it is likely that cross talk between estrogen and *Pten* signaling plays a role not only in the normal endometrium but also in endometrial tumorigenesis. However, the relationship between the two signaling pathways remains unclear. Although epithelial cells express estrogen receptor [31], it is believed that estrogen induced epithelial proliferation is driven by soluble paracrine factors produced by the stroma [32]. In short, it has been shown that under the regulation of estrogen, stromal cells secrete growth factors (e.g. IGF and EGF) that signal through their cognate cell surface receptors expressed on the epithelial cells resulting in epithelial cell proliferation. Additionally, induction of progesterone receptor in the epithelium is thought to be under the control of estrogen acting through stromal ERα receptor [32–35]. We therefore tested the effect of estrogen treatment on epithelial and stromal cells with and without *Pten* deletion.

The primary cultures were treated with varying concentrations of 17-β-estradiol 48 hrs after adenoviral infection. After 48 hours of estrogen treatment, the cells were harvested for

immunoblot analysis (Fig. 5). Treatment of epithelial cells expressing Pten with the highest estradiol dose (146nM) did not result in Akt activation, but clear activation was detected after deletion of *Pten* (Fig. 5A). Furthermore, treatment of epithelial cells with estradiol on the background of *Pten* deletion did not further activate Akt. In the stromal cells, activation of Akt was not only observed after *Pten* deletion, but it was also activated by estradiol in the presence of wild type *Pten* (Fig. 5B). In addition, treatment with increasing estradiol concentrations of stromal cells lacking expression of Pten led to further activation of Akt in a dose dependent manner. We have observed this pattern of Akt activation in at least three independent experiments.

These results underscore the differences in the response of endometrial epithelial and stromal cells to estradiol treatment. In the epithelium, regulation of the Akt pathway appears to be primarily under the control of Pten. This novel finding provides a possible explanation for the necessity of biallelic inactivation of Pten seen in CAH and carcinoma that has been observed in mouse models and the human disease. On the other hand, stromal cells do not appear to require Pten loss for activation of Akt. Thus, deletion of Pten in the stroma may not offer a significant growth advantage to the cells and therefore is not selected for. This is of note given that in the adenovirus injected mice, Cre was likely expressed in the stromal cells, but we were unable to detect lesions or Pten negative cells in the stromal compartment. In the *Pten*^{+/-} mouse model both the stromal and epithelial cells are heterozygous for Pten, but lesions with loss of Pten arise only from the epithelium [14, 15]. The surrounding stroma retains Pten expression and is histologically normal. These results suggest that despite relying on extensive cross talk in vivo, the two cell types respond differently to loss of Pten, and perhaps estrogen, thus in vitro experiments with the individual cell types may have ramifications for understanding these differences in vivo. In addition, we are in the process of interrogating these responses in stromal and epithelial in vitro co-culture systems.

We also analyzed regulation of cell cycle proteins, particularly cyclin D3 in the primary cultures. We initially focused on the D-type cyclins since several reports in literature suggest that early responses of epithelial cell proliferation induced by estradiol in rodents included up-regulation of cyclin D1 [36] and cyclin D3 mRNAs [37]. Moreover, immunostaining has shown translocation of cyclin D3 to the nuclear compartment in luminal epithelium during the estrous to metestrous transition. It has also been found that glandular and luminal epithelium use different cyclin D sub-types to transition into G1 stage after estrogen treatment [38].

Similar to activation of Akt, we observed a differential response of Cyclin D3 in the epithelial and stromal cells to estradiol treatment alone. In the stroma, estradiol treatment of cells with intact Pten expression results in the up-regulation of cyclin D3 by approximately 3 fold (Fig. 5A and 5C). In the epithelium this effect was more variable and did not reach statistical significance (Fig. 5 and 5B). Surprisingly, *Pten* deletion led to a marginally decreased cyclin D3 expression in both cell types. Estradiol treatment after Pten deletion however, did not change cyclin D3 levels in either cell type but Pten loss in the stroma altered the response to estradiol with respect to cyclin D3 in vitro. In our experiments, we did not observe any significant regulation of cyclin D1 (data not shown), even though it was expressed in both cell types.

Taken together, our studies demonstrate that biallelic inactivation of *Pten* in adult endometrial epithelium results in an increased incidence of endometrial carcinoma with a more aggressive behavior. The invasive tumors show a significant reduction in ER α , which may play a role in their more aggressive phenotype. The primary cell culture experiments suggest that in the epithelium deletion of Pten is required for the activation of Akt even in the presence of estradiol. In contrast the stromal cells showed activation of Akt in response

to estradiol in the presence of wild type Pten, however deletion of Pten further accentuated this response. In a recent study, investigators purified uterine epithelial cells from *Pten^{loxP/loxP}* mice and activated the PI3K pathway by deleting Pten using lentiviral-Cre *in vitro* [39]. The *Pten* deleted epithelial cells were combined with wild type neonatal stroma and implanted under the kidney capsule of SCID mice resulting in well-differentiated endometrial cancers. Although these studies corroborate our findings the model presented here offers the advantage of disease arising *in situ* and in the setting of both adult epithelial and stromal components. Our studies highlight the essential role of *Pten* in the development of CAH and UEC and offer a novel system in which to uncover the molecular alterations, and their important interactions with hormones, in endometrial carcinoma. In addition, we demonstrate the power of a genetically manipulated, physiologically relevant *in vitro* system for dissecting the critical pathways (genetic and hormonal) altered in the development of this common malignancy of women.

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Figure 1.

Adenoviral injections in uteri of R26R and *Pten^{loxP/loxP}* mice. (A) Adenoviruses infected stromal and epithelial cells as seen by LacZ positive blue cells in uteri of R26R mice. Control mice injected with medium only (B) were not positive for LacZ. Photomicrographs of H&E stained Adeno-Cre injected (D, E) horn and adeno-GFP injected (C) horn from *Pten^{loxP/loxP}* mice. The adeno-GFP injected horn showed normal histology while the adeno-Cre injected horns exhibited CAH (D) and invasion (E). Magnification 20X. Arrows in panel E indicate the invasion into the surrounding adipose tissue, as seen in the magnified (60X) image in the inset.

Figure 2.

Pten and p-Akt(S473) immunostaining of adeno-Cre and adeno-GFP injected horns of *Pten^{loxP/loxP}* mice. Pten expression was intact in Adeno-GFP horn (A) without activation of Akt (B). Areas of lesions (CAH and carcinomas/invasions, ♦) lost Pten expression (C) compared to adjacent normal glands (arrow) and activated Akt (D and high magnification inset) as shown by membrane localized, pAkt Ser-473 staining.

Figure 3. ER α Immunostaining. ER α localization is nuclear in CAH like lesions (A) while invasive lesions (B) exhibit reduced and cytoplasmic localization. Arrow points to the invasion into the myometrium, adjacent to a CAH like lesion. High magnification inset shows the reduced nuclear expression in the invasion.

Figure 4.

(A) Purity of endometrial epithelial and stromal primary cultures, as determined by immunoblot analysis using Vimentin and Cytokeratin 8 antibodies. (B) Adenovirus mediated deletion of Pten in primary epithelial cells and (C) stromal cells. Cells were infected with adenoviruses expressing Cre or GFP and harvested 48 hrs later. Loss of Pten was accompanied with activation of Akt by phosphorylation at Ser473. * indicates cytokeratin 8, arrow indicates vimentin

Figure 5.

Treatment of primary epithelial (A) and stromal (B) cells with Estradiol. Primary cells were prepared as described, treated with adenoviruses and subsequently with estradiol at the indicated concentrations for 48 hrs. Estradiol alone (adeno-GFP lane in (B)) activated Akt only in the stroma. Cyclin D3 also was regulated by estradiol alone only in the stroma. (C) Quantitation of cyclin D3 up-regulation in epithelium and stroma from 3 independent experiments. Cyclin D3 expression increased by 3-fold in the stroma (*: $p=0.0118$) after treatment with 146 nM estradiol. In the absence of Pten, estradiol did lead to cyclin D3 up-regulation. The fold regulation in the epithelial cells did not reach statistical significance when calculated from 3 independent experiments.

Table 1

Lesions in Adenovirus injected mice.

No. of Animals	Duration of treatment	No. with lesions (CAH and CA/Inv)	No. with CAH	No. with CA/Inv
24	4-8 M	12 (50%)	7 (58%)	5 (41%)

Table 2

Detailed histopathology of five animals with carcinoma/invasion

Animal	Age (months)	Time of treatment (months)	CAH	Carcinoma/Invasion
20	14	8		Invasive adenocarcinoma into the myometrium with extension into the ovary
54	12	4		Invasive adenocarcinoma extending into adipose tissue accompanied with mucinous metaplasia
59	12	8	Foci of CAH	Invasive adenocarcinoma extending into adjacent adipose tissue with mucinous metaplasia.
67	12	8	Multifocal CAH	Large invasive adenocarcinoma, extending through myometrium into adipose tissue, ovary and colon wall with mucinous and squamous metaplasia
101	11	3		Single focus of in situ carcinoma with associated invasive adenocarcinoma extending through the myometrium and into adipose tissue

Table 3Localization and scores for ER α immunostaining

	Nuclear ER α
	Score (mean $\pm$ SD)
CA with invasion	3.8 $\pm$ 0.7*
CAH	8.7 $\pm$ 2.5*
Luminal Epithelium	6.2 $\pm$ 2.25
Glandular Epithelium	11.1 $\pm$ 2.76

*
p value=0.007