Androgen actions via androgen receptor promote PTEN inactivation induced uterine cancer

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Abstract

Haploinsufficient inactivating phosphatase and tensin homolog (Pten) mutations cause Cowden syndrome, an autosomal dominant risk genotype for hormone dependent reproductive cancers. As androgen actions mediated via the androgen receptor (AR) supports uterine growth and may modify uterine cancer risk, we hypothesized that a functional AR may increase PTEN inactivation induced uterine cancer. To test the hypothesis, we compared the PTEN knockout (PTENKO) induced uterine pathology in heterozygous PTENKO and combined heterozygous PTEN and complete AR knockout (PTENARKO) female mice. PTENKO induced uterine pathology was significantly reduced by AR inactivation with severe macroscopic uterine pathology present in 21% of PTENARKO vs 46% of PTENKO at a median age of 45 weeks. This could be due to reduced stroma ER\(_\alpha\) expression in PTENARKO compared to PTENKO uterus, while AR inactivation did not modify PTEN or P-AKT levels. Unexpectedly, while progesterone (P\(_4\)) is assumed protective in uterine cancers, serum P\(_4\) was significantly higher in PTENKO females compared to WT, ARKO, and PTENARKO females consistent with more corpora lutea in PTENKO ovaries. Serum testosterone and ovarian estradiol were similar between all females. Hence, our results demonstrated AR inactivation mediated protection against PTENKO induced uterine pathology and suggests a potential role for antiandrogens in uterine cancer prevention and treatment.

Key Words

- androgens
- androgen receptor
- PTEN
- uterine cancer
- mouse

Introduction

Endometrial cancer (EC) is a frequent gynecological cancer. It is highly hormone dependent being promoted by estradiol (E\(_2\)) and inhibited by progesterone (P\(_4\)) (Bender et al. 2011), while the role of androgens remains controversial. Androgens are 19-carbon steroid hormones produced in females mainly by ovarian and adrenal gland secretion together with extraglandular conversion. Androgens mediate their effects via the androgen receptor (AR) expressed in target tissues such as the uterus of women (Somboonporn et al. 2004) and rodents (Walters et al. 2010). AR is a member of the nuclear receptor superfamily and encoded by an X chromosomal gene (Quigley et al. 1995). The biological effects of androgens in male physiology are well characterized, but the physiological roles in the female other than as precursors for conversion to estrogens by aromatase (Hillier et al. 1994) have only
recently been recognized as critical for optimal female reproduction (Walters et al. 2007).

The possible role for androgens in uterine growth is supported by in vivo experimental findings. The non-aromatizable androgen, dihydrotestosterone (DHT), promotes growth and differentiation of the rodent uterus (Schmidt & Katzenellenbogen 1979), whereas AR inactivation in mice resulted in a thinner uterus with a reduced total uterine area compared to WT females (Walters et al. 2009). However, in contrast, androstenedione (an aromatizable proandrogen) inhibited proliferation of human endometrial cells in vitro, an effect that was reversed by an antiandrogen (cyproterone acetate) suggesting these effects were mediated via AR actions in endometrial cells (Tuckerman et al. 2000). Androgens may also have a role in the development and/or progression of uterine carcinogenesis as increased androgen activity due to shorter CAG repeat in AR was related to an increased EC risk in women (McGrath et al. 2006). In addition, AR is strongly expressed in human endometrial disorders including different types of ECs (Ito et al. 2002), further supporting a role of androgens acting via AR in uterine cancer.

Phosphatase and tensin homolog (Pten) is a tumor suppressor gene located on chromosome 10 (Dahia 2000). Pten is mutated or deleted in a wide range of human cancers including breast and ECs (Dahia 2000). PTEN functions as a phosphatase and inhibits the growth factor signals transduced through PI3K by inhibiting AKT phosphorylation (Li et al. 1998). PTEN mutations are observed in 30–80% of type 1 ECs and in 20–70% of complex atypical hyperplasia, a premalignant stage of EC (Tashiro et al. 1997, Levine et al. 1998, Lee et al. 2012). Global homozygous PTEN inactivation is embryo lethal and the mice die between gestation days 6.5 and 9.5 (Suzuki et al. 1998). Global heterozygous Pten knockout (PTENKO) in mice resembles Cowden syndrome in patients and it causes EC, and therefore, PTENKO females are used as EC models (Stambolic et al. 2000). AR and PTEN interact in breast and prostate cancers (Wang et al. 2011) and they cross-regulate by reciprocal feedback (Carver et al. 2011, Mulholland et al. 2011). Furthermore, PTENKO increases ERα expression in EC cells (Lian et al. 2006) and activates ERα dependent pathways in mouse EC (Vilgelm et al. 2006).

The goal of this study was to investigate the role of AR-mediated androgen actions in PTEN inactivation induced experimental uterine cancer. To achieve our goal, we have generated and characterized global homozygous AR knockout (ARKO) females with or without global heterozygous PTEN inactivation using the Cre/LoxP system to determine the effects of AR inactivation on PTEN inactivation induced uterine cancer susceptibility and the possible mechanisms involved.

Materials and methods

Mice

All of the mice were housed under standard conditions (19–22 °C, 12 h light:12 h darkness cycle) in cages with access to water and food ad libitum. For the generation of the WT (Cre negative littermate females with functional AR and PTEN denoted as WT), ARKO, PTENKO, and PTENARKO (combined heterozygous PTEN deletion and homozygous AR inactivation) models, three founding lines have been used: Tg(Sox2-cre)1Amc (denoted SOX2-Cre (Hayashi et al. 2002)) kindly donated by Dr L Rob (Walter and Eliza Hall Institute) and Prof. A McMahon (Harvard University); Pten<sup>tm1Hwu</sup> mice (denoted PTENflox (Lesche et al. 2002) lines with FBV/N genetic background; and Ar<sup>tm1Jdz</sup> (denoted ARflox (Notini et al. 2005a)) line backcrossed to the FVB/N background for at least six generations (originally C57Bl background). SOX2-Cre promoter is a universal deleter that is expressed from embryonic day 6.5 (Hayashi et al. 2002). To generate the experimental mice, the following breeding steps were used in a Cre/LoxP recombination strategy (Fig. 1A). First, a male mouse from the PTENflox mouse (Pten<sup>flox/flox</sup>) is crossed with a female from the ARflox mouse (Ar<sup>flox/wt</sup>) to generate Pten<sup>flox/wt</sup>Ar<sup>flox/wt</sup> females as well as Pten<sup>flox/lox</sup>Ar<sup>flox/wt</sup> females and Pten<sup>flox/lox</sup>Ar<sup>flox/lox</sup> males. In step 2, a Pten<sup>flox/wt</sup>Ar<sup>flox/lox</sup> female is crossed with a SOX2-Cre male mouse (Sox2<sup>cre/cre</sup>) to produce Pten<sup>wt/wt</sup>Ar<sup>cut/wt</sup>Cre<sup>+/−</sup> and Pten<sup>wt/wt</sup>Ar<sup>cut/wt</sup>Cre<sup>+/−</sup> females in which the exon 3 of the floxed AR allele is deleted by Cre, creating AR cut allele.

Experimental design and tissue collection

Mice were sacrificed by cardiac exsanguination under ketamine/xylazine anesthesia at the mean age of 20 or 45 weeks. Body weight and reproductive organs (ovary and uterus), adrenal glands, spleen, and liver were dissected and weighed. Organs were either fixed (4% paraformaldehyde at 4 °C overnight) for histological processing or snap frozen in liquid nitrogen for steroid analysis or mRNA extraction. Serum was separated and stored at −20 °C. All of the procedures were approved by the Sydney South West Area Health Service Animal Welfare Committee within the National Health and Medical Research Council (NHMRC) guidelines for animal experimentation.
mice: Endocrine-Related Cancer

The excision of exon 5 in PTENKO and exon 3 in ARKO were confirmed at genomic DNA and mRNA levels. The genomic DNA was released using proteinase K digestion of uterine tissues. The excision of exon 5 of Pten was confirmed using the same primers as for genotyping (Byun et al. 2011). The excision of AR exon 3 was confirmed as described (Notini et al. 2005b). In addition, the excision of exon 3 of Ar in the uterus was confirmed by RT-PCR as previously described (Simanainen et al. 2007). Two product sizes were obtained: 288 bp for intact Ar and 171 bp for Cre-mediated exon 3 excised Ar. Exon 5 deletions in Pten was not confirmed by RT-PCR using RNA because exon 5 deletion in Pten was not confirmed. Two product sizes were obtained: 650 bp for intact Pten and 300 bp for Cre mediated exon 5 excised Pten. β-actin was used as a loading control.

Ovariectomy and implants

To determine the role of androgens in the uterine pathology in the controlled hormonal environment, PTENKO and PTENARKO mice were ovariectomized (OVX) under anesthesia at 5 weeks of age and implanted with 1 cm silastic implants (inner diameter = 1.47 mm and outer diameter = 1.95 mm; Dow-Corning, Sydney, NSW, Australia) either containing ~10 mg crystalline testosterone or no steroid placed subdermally for 15 weeks and subsequently collected at 20 weeks of age.

RNA extraction, DNase treatment, cDNA synthesis, and quantitative real-time RT-PCR

RNA was extracted from the whole uterus from 20-week-old female mice. These procedures were performed as previously described (Simanainen et al. 2009). To measure mRNA expression of Erα, lactoferrin, complement component 3 (C3), and glucose-6-phosphate dehydrogenase (G6PDH), the following primers were used: Erα-forward (5′-ATGAAAGCGGCATACGGAAATG-3′) and Erα-reverse (5′-CACCATTTCTTGCAGCCGACT-3′), lactoferrin-forward (5′-CTAACGAGACCCAGATCGAT-3′) and lactoferrin-reverse (5′-CTTCTACGCGACCCCCT-3′), C3-forward (5′-AATGTGCTGGCTCTTGGAGTA-3′) and C3-reverse (5′-GATATTCTCTGTCTCGTGT-3′), and G6PDH-forward (5′-GCTGCGTCAGTTCTTTAACC-3′) and G6PDH-reverse (5′-CAATCTTTGCGACGCAGTGT-3′). Target genes were normalized to internal housekeeping gene 18s rRNA. β-actin and hydroxymethylbilane synthase (Hmb s) were tested as housekeeping genes but 18s rRNA was used because its expression was most consistent among the experimental groups. The following primers were used: 18s rRNA-forward (5′-ACATTTGTGAGTGTGAG-3′) and 18s rRNA-reverse (5′-AACGCTACCACATCTCAAGG-3′),...
**Endocrine-Related Cancer**

The presence of unusual growths (Fig. 2C). Abnormal uteri had positivity and immunointensity of ER (sc-528, Santa Cruz Biotechnology, 1:50). The immunostaining was performed as previously described in Gao *et al.* (2014). The antibodies used were ERz (sc-542, Santa Cruz Biotechnology, 1:200 dilution), progesterone receptor (PR; sc-538, Santa Cruz Biotechnology, 1:100), PTEN (138G6, Cell Signaling, 1:500), AKT (11E7, Cell Signaling, 1:50), P-AKT (D9E, Cell Signaling, Danvers, MA, USA, 1:50), and p27 (sc-528, Santa Cruz Biotechnology, 1:50). The immunopositivity and immunointensity of ERz were assessed by the H score as previously described (McNamara *et al.* 2013). Different compartments of uterine were assessed separately: glandular epithelial cells, luminal epithelial cells, stroma, and myometrium. In brief, the H score was obtained by assessing immunointensity (scales of 0–3) and prevalence in 100 cells over five different areas in stroma and myometrium and prevalence in 20 cells over five different areas in glandular and luminal epithelial cells. All slides were counted twice to assess inter-observer variability.

**Protein extraction and western blot**

Proteins were extracted from the whole uterus from 20-week-old female mice and western blot was performed to measure PTEN, AKT, P-AKT, and p27 levels as previously described (Vignarajan *et al.* 2014). The antibodies used were PTEN (138G6, Cell Signaling, 1:500), AKT (sc-8312, Santa Cruz Biotechnology, 1:500), P-AKT (sc-7985, Santa Cruz Biotechnology, 1:300), p27 (sc-528, Santa Cruz Biotechnology, 1:50), and B-actin (ab8229, Abcam, Cambridge, UK, 1:3000).

**Hormone assays**

Testosterone, E2, and P4 levels were measured in extracts of 100 μl mouse serum and ovarian homogenates by liquid chromatography tandem mass spectrometry (LC-MS/MS; Harwood & Handelsman 2009) as modified for mouse serum and ovary (McNamara *et al.* 2010). The lowest limits of quantification (defined as detectable with a coefficient of variation <20%) were 25 pg/ml for testosterone, 2.5 pg/ml for E2, and 50 pg/ml for P4.

**Corpus lutea count**

Ovaries were fixed and processed into glycol methacrylate resin (Technovit 7100; Heraeus Kulzer, Chatswood, NSW, Australia) and serially sectioned at 20 μm and stained with periodic acid–Schiff. Corpus luteum (CL) was identified by morphological properties consistent with luteinized follicles and by being present in several serial sections. CL were counted on each ovary using an Olympus microscope with Stereo Investigator Software (MicroBright Field, Williston, VT, USA; Walters *et al.* 2009).

**Uterine cancer classification**

All uteri from experimental mice were classified into two categories based on macroscopic characteristics: normal or abnormal. Normal uteri (Fig. 2A) had a uterine weight <300 mg and did not exhibit any macroscopic abnormalities such as extensive vascularization (Fig. 2B) or the presence of unusual growths (Fig. 2C). Abnormal uteri had a uterine weight ≥300 mg (upper range of WT uterine weight mean ±s.d.) resulting in enlarged uterine horns with any of the macroscopic abnormal features. Histopathological analysis of the macroscopic abnormal uteri was performed by a pathologist (Dr G Watson).

**Statistical analysis**

Statistics were performed by one-way ANOVA and two-way ANOVA with the least significant difference method for post hoc test using SPSS Software (SPSS, Inc.) unless stated otherwise. P values smaller than 0.05 were considered statistically significant.

**Results**

**Confirmation of SOX2-Cre mediated excision of DNA between loxP sites of Ar and Pten**

PCR was used to confirm SOX2-Cre mediated excision of exon 3 of Ar and exon 5 of Pten in the uterus. The exon 5 deleted Pten PCR products (primers designed to amplify only the smaller exon 5 deleted product) were detected only in the uterus of PTENKO and PTENARKO mice (Fig. 1B), while only the native Pten product was detected in WT and ARKO uterus (Fig. 1C). However, the native Pten product was also detected in the PTENKO and PTENARKO
uterus as these mice were heterozygous for \( \text{Pten} \) deletion. In addition, the presence of native \( \text{Ar} \) (larger product containing exon 3) was confirmed in the WT and PTENKO female uterus at a cDNA level (RT-PCR), while the ARKO and PTENARKO uterus showed exclusively the smaller exon 3 deleted \( \text{Ar} \) products (Fig. 1D). Exon 5 excision in the \( \text{Pten} \) gene was not confirmed at the mRNA level because exon 5 excision produces stop codon and early termination of the transcription of mRNA.

Western blot was used to compare PTEN protein levels between the WT, ARKO, PTENKO, and PTENARKO uterus (Fig. 1E). While the PTEN levels were variable between individual uteri, the levels appeared to be decreased in the PTENKO and PTENARKO uterus (note PTENKO is heterozygous, thereby retaining a normal \( \text{Pten} \) allele) compared to the WT and ARKO uterus.

**AR inactivation modifies PTEN deletion induced uterine pathology**

All of the uteri of WT and ARKO females at the mean age of 43 and 46 weeks respectively were macroscopically normal. In PTENKO females, mean age of 46 weeks, 46% (24/52) had a macroscopic abnormal uterus compared to only 21% (5/24) in PTENARKO females, mean age of 43 weeks (Table 1). PTEN deletion significantly increased \( P<0.001 \); log-linear model) uterine cancer incidence, whereas AR inactivation significantly decreased \( P=0.0012 \) the PTEN induced uterine cancer incidence; interaction between PTEN deletion and AR inactivation was not statistically significant.

Comparable to pathology, uterine weights at 45 weeks was increased in PTENKO and PTENARKO compared to

**Table 1** Macroscopic categorization of uterine abnormalities

<table>
<thead>
<tr>
<th></th>
<th>No. of mice</th>
<th>Age (weeks), mean ± s.d.</th>
<th>( n (%) )* of abnormal uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>32</td>
<td>43 ± 10</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>ARKO</strong></td>
<td>14</td>
<td>46 ± 9</td>
<td>0 (0)</td>
</tr>
<tr>
<td><strong>PTENKO</strong></td>
<td>52</td>
<td>46 ± 8</td>
<td>24 (46)*</td>
</tr>
<tr>
<td><strong>PTENARKO</strong></td>
<td>24</td>
<td>43 ± 7</td>
<td>5 (21)</td>
</tr>
</tbody>
</table>

*aPercentages shown are calculated over total number of mice.
*bSignificantly different to PTENARKO.*

(F, G, H and I) Representative images of normal uterus of WT (F), ARKO (G), PTENKO (H), and PTENARKO (I) at 45 weeks (scale bar = 400 µm). (J and K) Representative images of major uterine histopathologies in PTENKO and PTENARKO: high-grade carcinoma with dedifferentiated morphology (J) and low-grade (focal) adenocarcinoma (K).
WT and ARKO (Fig. 2D). The uterine weights were significantly increased \((P=0.004;\) two-way ANOVA) by PTEN deletion but were not affected by AR inactivation \((P=0.077);\) interaction of PTEN deletion and AR inactivation was not significant \((P=0.091).\) Similar results were observed when uterine weights were standardized against the body weight (Fig. 2E). In addition, the PTENKO uterus was significantly heavier than the PTENARKO uterus. Uterine weights were similar between ARKO and WT females (Fig. 2D). Uteri that were categorized as normal in each genotype did not exhibit any abnormal histology and appeared similar (Fig. 2F, G, H and I).

Histopathological analysis of the macroscopic abnormal uteri showed different types of histopathologies. In PTENKO, 58\% (14/24) were high-grade (poorly differentiated) carcinoma, (Fig. 2J), 29\% (7/24) were low-grade adenocarcinoma (Fig. 2K) and the remaining 13\% (3/24) were endometritis, hematoma and an indeterminate tumor. In PTENARKO, only high-grade carcinoma (3/5, 60\%) and low-grade adenocarcinoma (2/5, 40\%) were observed. There was no statistically significant difference between PTENKO and PTENARKO in the distribution of histological grade among high-grade carcinoma and low-grade adenocarcinoma.

Similarly to uterine weights at 45 weeks, uterine weights at 20 weeks were significantly increased \((P=0.008;\) two-way ANOVA) by PTEN deletion but were not affected by AR inactivation \((P=0.065);\) interaction of PTEN deletion and AR inactivation was significant \((P=0.009),\) with smaller uteri in PTENARKO than in PTENKO females (Fig. 3A). Histologically, no uterine cancers were detected at 20 weeks (Fig. 3B, C, D and E), although PTENKO uteri were histologically abnormal with enlarged and disorganized endometrial glands not present in uteri from other genotypes (Fig. 3D).

AR-mediated androgen actions are required for testosterone-induced uterine regrowth

OVX and testosterone treatment were performed on PTENKO and PTENARKO females to investigate the direct role of testosterone in the uterus (Fig. 4A). OVX prevented uterine growth in PTENKO and PTENARKO females with the uterine weights in OVX groups being about 15\% of the intact female uterine weights at 20 weeks of age without any difference between the groups. In PTENKO females, testosterone treatments following OVX (OVX + testosterone) fully restored the uterine weight to the intact level at 20 weeks of age. However, no uterine growth was detected following OVX and testosterone treatment in PTENARKO females and the uterus weight remained similar to OVX-only females.

**Figure 3**
Uterine weight and histology (H&E) at 20 weeks of age. (A) Uterine weight (mg). *Significantly different to WT, ARKO, and PTENARKO \(P<0.05;\) one-way ANOVA); mean ± s.e.m.; \(n \geq 8.\) (B, C, D and E) Representative photos of H&E stained WT (B), ARKO (C), PTENKO (D), and PTENARKO (E) at 20 weeks (scale bar = 400 \(\mu m).\)
Histologically, the uteri of PTENKO (OVX), PTENARKO (OVX), and PTENARKO (OVX + testosterone) appeared similar (Fig. 4B, D and E).

AR inactivation did not significantly affect PTEN, P-AKT, and p27 levels in the heterozygous PTEN-deleted uterus

As it has been suggested that AR can regulate PTEN expression and, hence, modify the AKT pathway (Wang et al. 2011), we compared the PTEN, AKT, and P-AKT levels in the PTENKO and PTENARKO uterus. While no significant differences were found in the PTEN protein expression (Fig. 5A) between the PTENKO (native Ar) and PTENARKO (inactive Ar) uterus, the AKT levels (Fig. 5B) were significantly increased ($P<0.02$; one-way ANOVA) in the PTENARKO uterus compared to PTENKO. However, the P-AKT (active form of AKT) levels (Fig. 5C) were not affected by the AR inactivation in the PTENKO uterus. As p27 is a key target of the growth regulatory activity exerted by the AKT pathway (Liang et al. 2002) and is deregulated in multiple cancers including the endometrioid uterus, the p27 protein, while reduced in PTENARKO (Fig. 5D), was not significantly different ($P=0.082$) between the PTENKO and PTENARKO uterus. Immunohistochemically, no differences were observed in PTEN (Fig. 5E and F) and P-AKT (Fig. 5H and I) expression in the PTENKO and PTENARKO uterus in supporting the western blot results.

Whereas, all of the cells of the PTENKO were immuno-positive for p27, whereas in the PTENARKO uterus, glandular epithelial cells were immunonegative supporting the lower protein levels observed by western blots (Fig. 5K and L).

To further examine the effect of androgens on PTEN, P-AKT, and p27 expression, we compared the levels between the PTENKO and PTENARKO (OVX + testosterone) uterus. The OVX + testosterone-treated PTENARKO uterus was not included as the uteri were underdeveloped (Fig. 4). PTEN expression was decreased in all cells on OVX and testosterone treatment compared to intact PTENKO (Fig. 5G), whereas P-AKT expression was increased in glandular and luminal epithelial cells (Fig. 5J). p27 expression was increased in all cells on OVX and testosterone treatment in the PTENKO uterus compared to the intact PTENKO (Fig. 5M).

Increased uterine ERα expression in PTENKO females was inhibited by simultaneous AR inactivation in PTENARKO females at 20 weeks

As ERα expression is significantly increased in PTENKO uterus (Lian et al. 2006, Vilgelm et al. 2006), we examined if AR inactivation could influence this PTENKO induced increase in ERα. ERα positivity (Fig. 6A) and intensity (Fig. 6B) were quantified according to different cell types:
Figure 5
p27, AKT, P-AKT, and PTEN western blot and immunohistochemistry on PTEN deleted uterus at 20 weeks. Representative western blots for each protein are shown in the figure with the name of the protein labeled on the left and the protein band (kDa) shown on the right. (A, B, C and D) Quantification of western blot bands by measuring the intensity of each band standardized using β-actin as a housekeeper. *Significantly different to WT (P=0.043; one-way ANOVA); mean ± S.E.M.; n ≥ 5. (E, F, G, H, I, J, K, L and M) Representative images of immunohistochemistry of PTEN, P-AKT, and p27 in PTENKO, PTENARKO, and PTENKO (OVX + testosterone) uterus at 20 weeks (scale bar = 100 μm). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0203.

Figure 6
Expression of ERα and ERβ dependent genes in the uterus of 20-week-old females. (A and B) Percentage of ERα positive cells (A) and intensity (B) in glandular epithelial cells, luminal epithelial cells, endometrial stroma, and myometrium in experimental groups. *Significantly different to WT and †significantly different to WT, ARKO, and PTENARKO (one-way ANOVA); mean ± S.E.M.; n = 4. (C, D, E and F) Representative images of ERα immunohistochemistry on WT (C), ARKO (D), PTENKO (E), and PTENARKO (F) (scale bar = 100 μm). (G, H, I and J) Relative expression (relative to 18s rRNA housekeeping gene analyzed by real-time RT-PCR) of ERα mRNA (G) and Era-dependent genes (H, I and J) in the uterus at 20 weeks of age. *Significantly different to WT (P=0.019; t-test); mean ± S.E.M.; n ≥ 6. (K) Representative image of ERα immunohistochemistry on ovariectomized and testosterone-treated PTENKO uterus (scale bar= 100 μm). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0203.
PTENKO in females increased serum P4

As uterine cancer is highly hormone dependent (Martin et al. 1973a,b, Persson 1996), circulating sex steroid levels were investigated to determine if PTEN deletion and/or AR inactivation modified exposure to key reproductive hormones. Serum testosterone was not affected by genotype (Fig. 7A), whereas serum P4 (Fig. 7B) was significantly increased (P=0.003; two-way ANOVA) by PTEN deletion and significantly decreased (P=0.008) by AR inactivation; interaction of PTEN deletion and AR inactivation was significant (P=0.026), resulting in serum P4 being significantly increased in PTENKO compared to other groups.

In addition, the ovarian E2 content was analyzed, as serum E2 in intact female mice is usually below the detection limit of LC–MS/MS (2.5 pg E2/ml; McNamara et al. 2010). The ovarian E2 content was similar between the genotypes (Fig. 7C). Ovarian P4 (Fig. 7D) was also increased in PTENKO when compared to other genotypes; however, it was statistically non-significant due to the high variability in PTENKO.

Furthermore, as high levels of P4 are produced by the CL in the ovaries (Stocco et al. 2007) and PTEN is known to affect ovarian CL numbers (Fan et al. 2008), we quantified ovarian CL content in WT, ARKO, PTENKO, and PTENARKO females. The CL number was significantly reduced (P=0.006; two-way ANOVA) by AR inactivation and increased by PTEN deletion (P=0.08); the significance of AR inactivation and PTEN deletion interaction was 0.053 (Fig. 7E and F), suggesting that increased P4 levels could be due to an increased CL number and that the increase in CL due to PTEN deletion was reversed by AR inactivation.

PR immunohistochemistry was performed on the PTENKO (Fig. 7G) and PTENARKO (Fig. 7H) uterus at 20 weeks old (scale bar = 100 μm). A full colour version of this figure is available at http://dx.doi.org/10.1530/ERC-15-0203.

Figure 7
Steroid hormone levels and CL count at 45 weeks and PR expression in PTENKO and PTENARKO uterus at 20 weeks. Serum testosterone (A) and P4 (B) levels. *Significantly different to WT, †significantly different to ARKO, and ‡significantly different to PTENARKO (P<0.05; one-way ANOVA), mean ± s.e.m.; WT, n=32; ARKO, n=7; PTENKO, n=43; and PTENARKO, n=23. Ovarian P4 (C) and E2 (D) levels (mean ± s.e.m.; n=4). (E and F) Total number of corpus luteum (CL) in ovaries of WT, ARKO, PTENKO, and PTENARKO females with representative image. (F) *Significantly different to WT, †significantly different to ARKO, and ‡significantly different to PTENARKO (P<0.05; nonparametric test); mean ± s.e.m.; n=4. PR immunohistochemistry on PTENKO (G), PTENARKO (H), and PTENKO (OVX + testosterone) (I) at 20 weeks old (scale bar = 100 μm)
Multiple organs were affected by PTEN deletion and AR inactivation in females

Body weight was significantly increased ($P<0.001$; two-way ANOVA) by PTEN deletion but AR inactivation did not affect body weight; interaction between PTEN deletion and AR inactivation was significant ($P=0.001$) (Fig. 8). As a result, other organ weights were evaluated with and without an adjustment for body weight.

Ovary and adrenal gland weights were significantly ($P<0.02$) increased by PTEN deletion but were not affected by AR inactivation; there was no significant interaction between PTEN deletion and AR inactivation (Fig. 8). However, when adjusted for body weight, ovary weights were not affected by PTEN deletion, whereas the adrenal gland remained similar (Fig. 8). Absolute liver and spleen weights were not affected by AR inactivation and PTEN deletion; however, the interaction between AR inactivation and PTEN deletion was significant ($P<0.05$) for both organs (Fig. 8). When adjusted for body weight only, the interaction between AR inactivation and PTEN deletion was significant (0.043) in the spleen but not in the liver (Fig. 8).

Other incidental abnormalities

Global heterozygous PTENKO also induced abnormalities other than uterine pathology in female mice (Table 2). Mammary gland abnormalities were the most frequent with 54% of the PTENKO and PTENARKO females bearing abnormal mammary glands (palpable tumors or alveolar-ized mammary glands with milk production (Li et al. 2002)) compared with none detected in WT and ARKO. Histologically, the palpable tumors within the mammary glands were lymphomas (17% vs 21% in PTENKO vs PTENARKO) and fibroadenomas (23% vs 30%). In addition, some females had benign mammary abnormalities comprising alveolarized mammary glands with milk production (13% vs 4%; Table 2).

Very low frequencies of macroscopic abnormalities were also observed in the adrenal glands, liver, lung, ovaries, pancreas, and spleen (Table 2). Among these, the most frequent abnormality was the appearance of unusual black spots on the organ surface.

Discussion

Direct AR-mediated androgen actions on female reproductive physiology have been established (Walters et al. 2010) leading to suggestions that they may have an impact on the physiology and pathology of the uterus and breast (Somboonporn et al. 2004, Walters et al. 2010). As AR is expressed in the uterus of mice (Walters et al. 2010) and humans (Somboonporn et al. 2004), we explored the role of androgens acting via the AR in experimental, PTENKO induced murine uterine cancer. Our findings demonstrate that androgens acting via AR have a significant role in the uterine carcinogenesis, although it remains to be determined whether this is manifesting directly in the uterus or indirectly via systemic hormonal effects. We demonstrate that global AR inactivation significantly reduces the frequency of uterine cancers induced by PTEN deletion. Our findings also suggest that this preventive effect may be mediated by the elimination of a heterozygous PTENKO induced increase in uterine ERα expression. Furthermore, it is important to note that our uterine cancer model resembles Cowden syndrome, the germline mutation of PTEN.
one allele of PTEN resulting in hereditary cancer predisposition (Liaw et al. 1997). Hence, the prevention of uterine cancers by interrupting androgen action in our model may not only represent an effective simulation of the ECs in Cowden syndrome but also point to the potential role of antiandrogens in its treatment.

This study demonstrates that androgens acting via AR decrease uterine cancer incidence in a PTENKO experimental uterine cancer model. The heterozygous PTENKO induced uterine cancers as previously reported (Risinger et al. 1997, Stambolic et al. 2000). However, these changes in the PTENKO uterus were significantly reduced by AR inactivation in PTENARKO females. Our findings also suggest that the AR inactivation slows the carcinogenic process, as at 20 weeks of age PTENKO females showed only premalignant uterine hyperplasia, a premalignant precursor to EC. While not experimentally tested before, an influence of androgens on uterine cancer development is consistent with a previous clinical study showing an excess of a shorter CAG repeat in the AR gene, which increases tissue androgen sensitivity (Simanainen et al. 1997). Hence, the prevention of polycystic ovary syndrome (PCOS) patients with elevated plasma androgens (Nagamani et al. 1986) have an increased risk of uterine cancer (Smyczek-Gargya & Geppert 1992).

Also, our study has shown AR-mediated uterine regrowth upon ovariectomy and testosteron treatment.

In breast and prostate cancers the AR is suggested to regulate the PTEN levels (Wang et al. 2011), and therefore, we determined if the PTEN expression was modified by AR inactivation in the PTENARKO uterus and could therefore modify the risk of uterine cancer. However, we were unable to detect a significant effect of AR inactivation on PTEN gene expression in the uterus. However, the total AKT levels were significantly increased in the PTENARKO uterus when compared to PTENKO, whereas the active phosphorylated AKT levels were unaffected. These findings suggest that the reduced uterine cancer incidence in PTENARKO females may not be due to the modification of the AKT signaling pathway. Yet, in prostate cancer cells, AR-mediated protection from PTEN induced apoptosis is independent of AKT pathway activation (Li et al. 2001). In addition, our study showed diminished PTEN expression in the PTEN (OVX + testosterone) uterus compared to the intact suggesting that testosterone alone, in the absence of other steroid hormones, negatively regulate PTEN expression in the uterus. This was supported in a prostate cancer cell study, in which DHT via AR inhibited PTEN transcription and translation (Wang et al. 2011).

Table 2 Abnormalities found in 45-week-old experimental female mice. Percentages shown are calculated over total number of mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of mice</th>
<th>Palpable</th>
<th>Lymphoma</th>
<th>Fibroadenoma</th>
<th>Alveolarization/milk production</th>
<th>Liver</th>
<th>Ovary</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>32</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ARKO</td>
<td>11</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PTENKO</td>
<td>52</td>
<td>9 (17%)</td>
<td>12 (23%)</td>
<td>7 (13%)</td>
<td>6 (12%)</td>
<td>2 (4%)</td>
<td>3 (6%)</td>
<td>NA</td>
</tr>
<tr>
<td>PTENARKO</td>
<td>24</td>
<td>5 (21%)</td>
<td>7 (30%)</td>
<td>1 (4%)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Interestingly, we found that the expression of the tumor suppressor p27 appeared to be reduced ($P=0.082$) in the PTENARKO uterus when compared to PTENKO. This could be due to a reduced p27 expression in glandular epithelial cells of the PTENARKO uterus. This was unexpected as the p27 levels are usually negatively correlated with malignancy with the level of p27 being reduced in $\sim50\%$ of human cancers (Sgambato et al. 2000, Slingerland & Pagano 2000). However, our finding suggesting a positive correlation between p27 expression and uterine cancer was supported by a clinical studies where p27 expression was increased in patients with endometrioid adenocarcinomas (Watanabe et al. 2002) and advanced grading EC (Nycum et al. 2001). However, contrary to our findings, other clinical studies reported a negative correlation between p27 expression and uterine cancers (An et al. 2002). Hence, our study is the first experimental evidence suggesting the positive correlation between p27 and uterine cancer. The increased p27 could be
due to a compensatory mechanism, as a tumor suppressor gene combating uterine cancer rather than causing uterine cancer, which warrants further investigations.

Furthermore, we have shown an increased p27 expression throughout the OVX + testosterone PTENKO uterus compared to the PTENKO uterus suggesting testosterone alone, in absence of other steroid hormones, can upregulate p27 expression in the uterus. Yet, in prostate cancer and epithelial cells, a negative correlation was observed between androgens and p27 expression (Waltregny et al. 2001, Fang et al. 2012). The most widely accepted mechanism of increased risk for uterine cancer in PCOS patients is that of excessive unopposed estrogen exposure (Gambrell et al. 1983) as these women have persistent circulating E2 levels; however, as they are frequently anovulatory, they lack the usual P4 opposition against excessive estrogen action (Lobo et al. 1981). While we were unable to measure serum E2 concentrations as they were below sensitivity of our LC–MS/MS methodology (limit of quantitation 2.5 pg/ml), ovarian E2 content was not significantly different between the genotypes suggesting the circulating E2 levels were probably similar for each genotype. Furthermore, serum testosterone levels did not differ according to genotype. However, as testosterone could be aromatized within the uterus (Huhtinen et al. 2012), the uterine content of E2 should be analyzed in the future. In addition, while an exclusively direct role of androgens has not been confirmed, AR expression is increased in the endometrium of women with PCOS, suggesting the direct AR-mediated androgen actions in uterine carcinogenesis (Apparao et al. 2002). AR is also strongly expressed in various endometrial disorders including different types of ECs (Ito et al. 2002), further supporting a role of androgens acting via AR. These findings raise the hypothesis that antiandrogens may have an unrecognized role in the prevention and early stages of uterine carcinogenesis or in treatment.

Interestingly, the increased uterine estrogen sensitivity due to the PTEN inactivation in PTENKO females corresponded to an increasing expression of not only ERα but also a variety of estrogen sensitive target genes (lactoferrin, G6PDH, and complement C3) in the PTENKO uterus. However, further analysis is warranted to determine if the increase in estrogen-regulated genes in the PTENKO uterus is due to increased estrogen sensitivity or increased epithelial cell numbers. This finding extends to an in vivo model and previous in vitro study in which PTEN inactivation increased ERα expression (Lian et al. 2006). Similarly, the loss of Pten and subsequent AKT activation in the uterus was shown to activate ERA-dependent pathways in mouse EC (Vilgelm et al. 2006). Furthermore, our findings suggest that the reduced uterine pathology following AR inactivation in PTENARKO females could be due to a reduced ERα expression, consistent with the reduced ERα immunointensity in endometrial stroma and ERα mRNA expression. There could be two possible mechanisms for how global AR inactivation reversed increased ERα induced by PTEN deletion. First, AR directly modifies ERα expression in the uterus. Second, AR could interact with PTEN to indirectly modify ERα expression. Supporting the first mechanism, testosterone was shown to suppress ERα expression in mammary tissue (Zhou et al. 2000). However, our study has shown that testosterone treatment in the OVX PTENKO female caused a marked increase in ERα expression in the PTENKO uterus in all of the cells. The second mechanism is supported by findings in both the prostate and breast cancers (Wang et al. 2011), whereby AR-mediated actions modify PTEN (noting our model still has one functional PTEN allele) and results in alterations in ERα expression (Vilgelm et al. 2006). Either or both of these mechanisms could represent the molecular pathway involved in the impact of AR-mediated androgen action on PTEN inactivation induced uterine cancer. Clearly, further mechanistic studies are warranted to elucidate the role of AR-mediated androgen action at various stages of uterine carcinogenesis.

Our study is also informative about the role of P4 action in uterine carcinogenesis. Whereas progestins are known to protect against uterine cancer (Martin et al. 1973a, Persson 1996), we observed unexpectedly (given that P4 protects against uterine cancer) that serum P4 was significantly increased in PTENKO females compared to other genotypes. However, in our study the PTENKO uterus did not seem to benefit from the increased serum P4 levels although PR was expressed in all of the cells of the uterus, consistent with a previous finding that heterozygous PTEN inactivation induced uterine cancer was unresponsive to progestin treatments (Fyles et al. 2008). Nevertheless, our finding of increased serum P4 is consistent with a previous study showing increased CL numbers in the ovaries of the granulosa cell specific PTEN inactivation in mice (Fan et al. 2008), a finding that might explain increased serum P4 as CLs characteristically secrete P4. Conversely, complete AR inactivation in female mice results in reduced CL numbers compared with WT (Walters et al. 2007). Therefore, we analyzed CL numbers in our experimental females and observed that CL numbers were significantly increased in the ovaries of PTENKO females and this increase was also prevented by simultaneous AR inactivation in PTENARKO females.
While there were no obvious differences in PR immunopositivity between the PTENKO and PTENARKO uterus, we showed that testosterone treatment in OVX PTENKO females increased PR immunopositivity throughout the PTENKO uterus. However, this could be due to the supraphysiological levels of testosterone produced by our testosterone implant, which may not reflect normal physiological effects of testosterone in intact mouse.

Among the many non-uterine abnormalities found in our PTEN inactivated mice, mammary tumors were the most common. A majority (54%) of PTENKO and PTENARKO females displayed abnormal mammary glands that contained mainly lymphomas and fibroadenomas (Stambolic et al. 2000) as well as a benign pathology of inappropriate mammary alveolarization and milk production in virgin mice (Conneely et al. 2003). In mammary pathology, however, there were no significant differences between PTENKO and PTENARKO females. Previously, the role of androgens in breast cancer has been controversial but a recent study has suggested an inhibitory role of androgens in breast cancer (Simanainen et al. 2012). However, the present study did not show a protective role of androgen actions via AR on PTENKO induced breast cancer incidence compared to PTENARKO. This suggests a different mechanism such as involving the increased serum P4 level in PTENKO but not in PTENARKO females. While controversial, there has been a suggestion that P4 may promote breast tissue proliferation and could thereby increase the risk in PTENKO to the same level as in PTENARKO (Russo et al. 2000). In addition, the different mechanisms of inducing experimental breast cancers (i.e., PTEN inactivation vs overactive ERBB2 signaling vs chemically induced) could explain the findings. Finally, while overactive ERBB2 and chemically induced cancers are mainly carcinomas, the PTENKO induced fibroadenomas. Mammary glands with alveolarization could be due to the increased P4 levels, as P4 is known to cause alveolarization and milk production (Conneely et al. 2003). A previous study also observed increased mammary gland alveolarization in heterozygous PTENKO females, but P4 levels were not determined (Guigon et al. 2011). We also showed that less PTENARKO females appeared to have alveolized mammary glands compared to PTENKO, further supporting the reduced P4 levels in PTENARKO compared to PTENKO females.

As we have used global PTEN and/or AR inactivation in our experimental mice, we found changes in organ weights other than the uterus such as the adrenal glands, breast, liver, lung, ovaries, pancreas, and spleen. These findings were supported by previous studies using PTEN inactivated mouse models. PTEN inactivation causes hyperplastic adrenal glands, an enlarged liver, epithelial hyperplasia in the lung, altered ovarian physiology, and an increased spleen weight (Knobbe et al. 2008). From our study, the role of androgens in these organs remain speculative, however, androgens may play an organ-specific role of inducing or inhibiting pathogenesis, which requires further investigations.

In conclusion, our study has demonstrated that the global AR inactivation reduced PTEN inactivation induced uterine carcinogenesis by decreasing stroma ERα expression and thereby estrogen sensitivity. However, further investigation is required to understand how PTEN and AR modify ERα expression in the uterus. Most importantly, our study provides first in vivo evidence that androgen actions via AR play a significant role in the uterine cancer development and raises the hypothesis that antiandrogen therapy may have a role in the prevention and early stages of uterine carcinogenesis or its treatment.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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