Androgen actions via androgen receptor promote PTEN inactivation induced uterine cancer

Jaesung (Peter) Choi, Reena Desai, Yu Zheng, Mu Yao, Qihan Dong, Geoff Watson, David J Handelsman and Ulla Simanainen

ANZAC Research Institute, University of Sydney, Sydney, New South Wales 2139, Australia
1Discipline of Endocrinology, Central Clinical School, Bosch Institute, Charles Perkins Centre, Royal Prince Alfred Hospital, University of Sydney, Sydney, New South Wales 2050, Australia
2Department of Anatomical Pathology, Royal Prince Alfred Hospital, Sydney, New South Wales 2050, Australia

Correspondence should be addressed to D J Handelsman
Email djh@anzac.edu.au

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 ERα expression in PTENARKO compared to PTENKO uterus, while AR inactivation did not modify PTEN or P-AKT levels. Unexpectedly, while progesterone (P4) is assumed protective in uterine cancers, serum P4 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 (E2) and inhibited by progesterone (P4) (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
Endocrine-Related Cancer

complex atypical hyperplasia, a premalignant stage of EC (PTENKO) in mice resembles Cowden syndrome in (Suzuki and the mice die between gestation days 6.5 and 9.5. Global homozygous PTEN inactivation is embryo lethal (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 ERx expression in EC cells (Lian et al. 2006) and activates ERx 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^tm1Hwu mice (denoted PTENfloxflox (Lesche et al. 2002)) lines with FVB/N genetic background; and Ar^tm1Jdz (denoted ARfloxflox (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 PTENfloxflox mouse (Pten^floxflox/floxflox) is crossed with a female from the ARfloxflox mice (Pten^floxflox/floxflox and Ar^floxflox/floxflox females as well as Pten^floxflox/floxflox Ar^floxflox/floxflox and Pten^floxflox/floxflox Ar^floxflox/floxflox males). In step 2, a Pten^floxflox/floxflox Ar^floxflox/floxflox female is crossed with a SOX2-Cre male mouse (Sox2^cre/cre) to produce Pten^floxflox/floxflox Ar^floxflox/floxflox Cre^+/− and Pten^floxflox/floxflox Ar^floxflox/floxflox Cre^+/− 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.
The excision of exon 5 of PTEN 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 were not confirmed by RT-PCR using RNA because exon 5 deletion in Pten produces stop codon; hence, no exons are produced after exon 4, meaning no bands will be produces at cDNA level. 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 Erecht, lactoferrin, complement component 3 (C3), and glucose-6-phosphate dehydrogenase (G6PDH), the following primers were used: Erecht-forward (5’-ATGAAAGGCGCATACGGAAAG-3’), lactoferrin-forward (5’-CTAACAGACAGATCTGCA-3’), lactoferrin-reverse (5’-CCTTCTAGCCACACCTC-3’), C3-forward (5’-AICTTGCTGGCTCTGGAGTA-3’) and C3-reverse (5’-GATATTCTGCAGGTTGTG-3’), and G6PDH-forward (5’-GCCCTGCGATTTCTTTAACC-3’) and G6PDH-reverse (5’-CAATCTTGTCGACAGTGTT-3’). Target genes were normalized to internal housekeeping gene 18s rRNA. β-actin and hydroxymethylbilane synthase (Hmbz) 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’-AATTTGAGTGAGTGCCCG-3’) and 18s rRNA-reverse (5’-AAGGCTACACCATCCAG-3’),
B-actin-forward (5'-AGCCATGTACGGCTGCAATCC-3') and B-actin-reverse (5'-GGAAACGCTGCTTGGCCTAATA-3'), and Hmbs-forward (5'-GGCAATGCGGCTGCAA-3') and Hmbs-reverse (5'-GGGTACCGCAATCAC-3').

Histology and immunohistochemistry

A midsection (between the fimbrial end and cervical end) from a fixed uterine horn was embedded in paraffin and sectioned at 5 μm. Sections were either stained with hematoxylin and eosin (H&E) for histopathological analyses or used for immunohistochemistry. Immunostaining was performed as previously described in Gao et al. (2014). The antibodies used were ER (sc-542, Santa Cruz Biotechnology, 1:200 dilution), progesterone receptor (PR; sc-538, Santa Cruz Biotechnology, 1:50), AKT (11E7, Cell Signaling, Danvers, MA, USA, 1:50), and p27 (sc-528, Santa Cruz Biotechnology, 1:50). The immunoreactivity and immunointensity of ERz were assessed by the H score as previously described (McNamara et al. 2013). Different compartments of uterine sections 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, 1:50), 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 immunoreactivity and immunointensity of ERz were assessed by the H score as previously described (McNamara et al. 2013). Different compartments of uterine sections 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.

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).

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).

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 \textit{Pten} deletion. In addition, the presence of native \textit{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 \textit{Ar} products (Fig. 1D). Exon 5 excision in the \textit{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 \textit{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 \((P<0.001; \text{log-linear model})\) 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 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 \((P<0.001; \text{log-linear model})\) 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.

### 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>WT</td>
<td>32</td>
<td>43 ± 10</td>
<td>0 (0)</td>
</tr>
<tr>
<td>ARKO</td>
<td>14</td>
<td>46 ± 9</td>
<td>0 (0)</td>
</tr>
<tr>
<td>PTENKO</td>
<td>52</td>
<td>46 ± 8</td>
<td>24 (46)(^b)</td>
</tr>
<tr>
<td>PTENARKO</td>
<td>24</td>
<td>43 ± 7</td>
<td>5 (21)</td>
</tr>
</tbody>
</table>

\(^a\)Percentages shown are calculated over total number of mice.  
\(^b\)Significantly different to PTENARKO.
WT and ARKO (Fig. 2D). The uterine weights were significantly increased \((P=0.004;\text{ two-way ANOVA})\) 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 \((P=0.008;\text{ two-way ANOVA})\) 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 uterine weight remained similar to OVX-only females.
Histologically, the uteri of PTENKO (OVX), PTENARKO (OVX), and PTENARKO (OVX + testosterone) appeared similar (Fig. 4B, D and E).

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 immunopositive 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 PTENKO (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:
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, 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).

Expression of ERα significantly decreased in ARKO (P = 0.015; one-way ANOVA) and PTENARKO (P = 0.026) uterus compared to WT. The ERα intensity in endometrial stromal cells was significantly increased in the PTENKO uterus compared to WT (P = 0.036), ARKO (P = 0.004), and PTENKAROKO (P = 0.002). A representative image of each genotype is shown in Fig. 6C, D, E and F.

Real time RT-PCR was performed to determine ERα gene expression. ERα mRNA expression was increased (P = 0.064; one-way ANOVA) in the PTENKO uterus compared to WT, but this effect was prevented by simultaneous AR inactivation in the PTENARKO uterus (Fig. 6G). Congruent findings were observed in the expression of ERα target genes; G6PDH expression was significantly increased in the PTENKO (P = 0.043; one-way ANOVA); mean ± s.e.m.; n ≥ 5. (E, F, G, H, I, 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.
significantly increased ($P=0.019$; $t$-test) in the PTENKO uterus compared to WT, and again this effect was prevented by simultaneous AR inactivation in the PTENARKO uterus (Fig. 6H). Although not statistically significant, other Erα-dependent genes, lactoferrin ($P=0.064$; $t$-test; Fig. 6I) and complement C3 ($P=0.097$; $t$-test; Fig. 6J) were also increased in the PTENKO uterus compared to WT and similarly this effect was prevented by simultaneous AR inactivation in the PTENARKO uterus.

Furthermore, when compared to the intact PTENKO uterus, the OVX + testosterone-treated PTENKO uterus had a marked increase in Erα expression immunopositivity (Fig. 6K), which was observed in all of the cell types.

**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 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 and both the PTENKO and PTENARKO uterus expressed PR without obvious differences. However, compared to the intact PTENKO uterus, the OVX + testosterone-treated PTENKO uterus had a marked increase in PR immunopositivity (Fig. 7I). This increase was observed in all cell types.
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 alveolarized 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...
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 is consistent with a previous clinical study showing an increased risk of uterine cancer (McGrath et al. 2011), being associated with an increased EC risk (McGrath et al. 1986) have an increased risk of uterine cancer. The increased p27 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 ~50% of human cancers (Sgambato et al. 2000, Slingerland & Pagano 2000). 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

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 ~50% 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

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**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 Lymphoma</th>
<th>Palpable Fibroadenoma</th>
<th>Alveolarization/ milk production Liver</th>
<th>Alveolarization/ milk production Ovary</th>
<th>Alveolarization/ milk production 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>
</tr>
<tr>
<td>ARKO</td>
<td>11</td>
<td>9 (17%)</td>
<td>12 (23%)</td>
<td>7 (13%)</td>
<td>6 (12%)</td>
<td>3 (6%)</td>
</tr>
<tr>
<td>PTENKO</td>
<td>52</td>
<td>5 (21%)</td>
<td>7 (30%)</td>
<td>1 (4%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PTENARKO</td>
<td>24</td>
<td>9 (17%)</td>
<td>12 (23%)</td>
<td>7 (13%)</td>
<td>6 (12%)</td>
<td>3 (6%)</td>
</tr>
</tbody>
</table>
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 immuno
positivity 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 pro-
duction 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 fibroadeno-
mas. 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
alveolarized 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
cspeculative, 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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