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 α 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

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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

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 ER α expression in EC cells (Lian *et al.* 2006) and activates ER α 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 PTENflox (Lesche *et al.* 2002)) lines with FBV/N genetic background; and *Ar*^{tm1Jdz} (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*^{flox/flox}) is crossed with a female from the ARflox mouse (*Ar*^{flox/wt}) to generate *Pten*^{flox/wt}*Ar*^{flox/wt} females as well as *Pten*^{flox/wt}*Ar*^{flox} and *Pten*^{flox/wt}*Ar*^{wt} males. In step 2, a *Pten*^{flox/wt}*Ar*^{flox/wt} female is crossed with a SOX2-Cre male mouse (*Sox*^{cre/cre}) to produce *Pten*^{wt/wt}*Ar*^{cut/wt}*Cre*^{+/-} and *Pten*^{wt/wt}*Ar*^{wt/wt}*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.

Figure 1

Breeding protocol and genotyping PCR on uterus cDNA. (A) White boxes represent male mice and black boxes represent female mice and grey boxes represent experimental groups. PTEN^{flox} mice have AR^{wt/wt} and Cre^{-/-} alleles, whereas AR^{flox} mice have PTEN^{wt/wt} and Cre^{-/-} alleles. Sox2cre

Genotype confirmation

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* produces stop codon; hence, no exons are produced after exon 4, meaning no bands will be produced 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,

mice have AR^{wt/wt} and PTEN^{wt/wt} alleles. (B) PTEN PCR showing PTEN deletion. (C) PTEN PCR showing WT PTEN. (D) ARCT RT-PCR showing WT AR and exon 3 excised AR. (E) PTEN western blot showing PTEN reduction in PTENKO and PTENARKO.

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 *ER α* , *lactoferrin*, complement component 3 (*C3*), and glucose-6-phosphate dehydrogenase (*G6PDH*), the following primers were used: *ER α* -forward (5'-ATGAAAGGCGGCATACGGAAG-3') and *ER α* -reverse (5'-CACCCATTTCATTTCGGCCTTC-3'), *lactoferrin*-forward (5'-CTAACCAGACCAGATCCTGCA-3') and *lactoferrin*-reverse (5'-CCTTCTCAGCCAGACACCTT-3'), *C3*-forward (5'-AACTGCTGGCCTCTGGAGTA-3') and *C3*-reverse (5'-GCATGATTCTCGAGTTGT-3'), and *G6PDH*-forward (5'-GCCTGGCATGTTCTTTAACC-3') and *G6PDH*-reverse (5'-CAATCTTGTGCAGCAGTGGT-3'). Target genes were normalized to internal housekeeping gene *18s* rRNA. *B-actin* and hydroxymethylbilane synthase (*Hmbs*) 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'-AACTTTCGATGGTAGTCGCCG-3') and *18s* rRNA-reverse (5'-AAACGGCTACCACATCCAAGG-3'),

B-actin-forward (5'-AGCCATGTACGTAGCCATCC-3') and *B-actin*-reverse (5'-GGAACCGCTCGTTGCCAATA-3'), and *Hmbs*-forward (5'-GGCAATGCGGCTGCAA-3') and *Hmbs*-reverse (5'-GGGTACCCACGCGAATCAC-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 μ m. 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 α (sc-542, Santa Cruz Biotechnology, 1:200 dilution), progesterone receptor (PR; sc-538, Santa Cruz Biotechnology, 1:100), 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 immunopositivity and immunointensity of ER α were assessed by the *H* score as previously described ([McNamara et al. 2013](#)). Different compartments of uterus 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, 1:500), and B-actin (ab8229, Abcam, Cambridge, UK, 1:3000).

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 $\pm$ 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 μ l 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 μ m 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

Figure 2

Uterus morphological classification at the median age of 45 weeks. (A, B and C) Representative images of macroscopic uterine category: normal uterus (A) and abnormal uterus (B and C). (D and E) Absolute uterine weight (D) and standardized uterine weight (E). ^aSignificantly different to WT, ^bsignificantly different to ARKO, and ^csignificantly different to PTENARKO ($P < 0.05$; one-way ANOVA); mean $\pm$ s.e.m.

(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 μ m). (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).

uterus as these mice were heterozygous for *Pten* deletion. In addition, the presence of native *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 *Ar* products (Fig. 1D). Exon 5 excision in the *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 *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

	No. of mice	Age (weeks), mean $\pm$ s.d.	n (%) ^a of abnormal uterus
WT	32	43 $\pm$ 10	0 (0)
ARKO	14	46 $\pm$ 9	0 (0)
PTENKO	52	46 $\pm$ 8	24 (46) ^b
PTENARKO	24	43 $\pm$ 7	5 (21)

^aPercentages shown are calculated over total number of mice.

^bSignificantly different to PTENARKO.

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). ^aSignificantly different to WT, ARKO, and PTENARKO ($P<0.05$; one-way ANOVA); mean $\pm$ 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 μ m).

Figure 4

Uterine weight and histology (H&E) at 20 weeks of age upon ovariectomy and testosterone treatment. (A) Uterine weight (relative to intact).

^aSignificantly different to OVX group and PTENARKO (OVX+testosterone) ($P < 0.05$; one-way ANOVA); mean $\pm$ s.e.m. (B, C, D and E) Representative

photos of H&E stained of PTENKO (OVX) (B), PTENKO (OVX+testosterone) (C), PTENARKO (OVX) (D), and PTENARKO (OVX+testosterone) (E) (scale bar=200 μ m for image B, D, and E and 400 μ m for image C).

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 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 ER α expression in PTENKO females was inhibited by simultaneous AR inactivation in PTENARKO females at 20 weeks

As ER α 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 ER α . ER α 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 B-actin as a housekeeper. ^aSignificantly different

to PTENKO ($P=0.043$; one-way ANOVA); mean $\pm$ s.e.m.; $n \geq 5$. (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 μ m). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0203>.

glandular epithelial cells, luminal epithelial cells, endometrial stroma, and myometrium. ER α positivity in each cell type was not significantly affected by genotype. However, ER α intensity in glandular epithelial cells was significantly decreased in ARKO ($P=0.015$; one-way ANOVA) and PTENARKO ($P=0.026$) uterus compared to WT. The ER α intensity in endometrial stromal cells was significantly increased in the PTENKO uterus compared to WT ($P=0.036$), ARKO ($P=0.004$), and PTENKARKO

($P=0.002$). A representative image of each genotype is shown in Fig. 6C, D, E and F.

Real time RT-PCR was performed to determine ER α gene expression. ER α 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 ER α target genes; G6PDH expression was

Figure 6

Expression of ER α and ER α dependent genes in the uterus of 20-week-old females. (A and B) Percentage of ER α positive cells (A) and intensity (B) in glandular epithelial cells, luminal epithelial cells, stroma, and myometrium in experimental groups. ^aSignificantly different to WT, ARKO, and PTENARKO (one-way ANOVA); mean $\pm$ s.e.m.; $n=4$. (C, D, E and F) Representative images of ER α immunohistochemistry on WT (C), ARKO (D), PTENKO (E), and PTENARKO (F) (scale bar = 100 μ m).

(G, H, I and J) Relative expression (relative to 18S rRNA housekeeping gene analyzed by real-time RT-PCR) of ER α mRNA (G) and ER α -dependent genes (H, I and J) in the uterus at 20 weeks of age. ^aSignificantly different to WT ($P=0.019$; t -test); mean $\pm$ s.e.m.; $n \geq 6$. (K) Representative image of ER α immunohistochemistry on ovariectomized and testosterone-treated PTENKO uterus (scale bar = 100 μ m). 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 Era-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 ER α 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 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.

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. ^aSignificantly different to WT, ^bsignificantly different to ARKO, and ^csignificantly different to PTENARKO ($P<0.05$; one-way ANOVA); mean $\pm$ s.e.m.; WT, $n=32$; ARKO, $n=7$; PTENKO, $n=43$; and PTENARKO, $n=23$. Ovarian P₄ (C) and E₂ (D) levels (mean $\pm$ 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) ^aSignificantly different to WT, ^bsignificantly different to ARKO, and ^csignificantly different to PTENARKO ($P<0.05$; nonparametric test); mean $\pm$ s.e.m.; $n=4$. PR immunohistochemistry on PTENKO (G), PTENARKO (H), and PTENKO (OVX + testosterone) (I) at 20 weeks old (scale bar = 100 μ m). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0203>.

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 ER α expression. Furthermore, it is important to note that our uterine cancer model resembles Cowden syndrome, the germline mutation of

Figure 8

Body weight, absolute organ weights, and standardized organ weights of female mice at the median age of 45 weeks. ^aSignificantly different to WT, ^bsignificantly different to ARKO, and ^csignificantly different to PTENARKO ($P < 0.05$; one-way ANOVA); mean $\pm$ S.E.M.; WT, $n = 32$; ARKO, $n > 7$; PTENKO, $n > 43$; and PTENARKO, $n > 23$.

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

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

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.* 2011), being associated with an increased EC risk (McGrath *et al.* 2006). Furthermore, there is also evidence that 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 testosterone 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). Diminished PTEN expression in the PTENKO (OVX + testosterone) uterus resulted in an increased P-AKT expression in glandular and luminal epithelial cells, supporting previous reports of negative correlation between PTEN and P-AKT in the uterus (Stambolic *et al.* 1998, 2000). Hence, further investigations are warranted to examine AR and PTEN interaction in the origins of uterine cancers.

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

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 ER α 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 ER α 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 ER α expression, consistent with the reduced ER α immunointensity in endometrial stroma and ER α mRNA expression. There could be two possible mechanisms for how global AR inactivation reversed increased ER α induced by PTEN deletion. First, AR directly modifies ER α expression in the uterus. Second, AR could interact with PTEN to indirectly modify ER α expression. Supporting the first mechanism, testosterone was shown to suppress ER α 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 ER α 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 ER α 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 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 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 ER α expression and thereby estrogen sensitivity. However, further investigation is required to understand how PTEN and AR modify ER α 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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