Glandular epithelial AR inactivation enhances PTEN deletion-induced uterine pathology

Jaesung (Peter) Choi, Yu Zheng, David J Handelsman and Ulla Simanainen

ANZAC Research Institute, University of Sydney, Sydney, New South Wales, Australia

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

Abstract

Phosphatase and tensin homolog (PTEN) deletion induces uterine pathology, whereas androgen actions via androgen receptor (AR) support uterine growth and therefore may modify uterine cancer risk. We hypothesized that the androgen actions mediated via uterine glandular epithelial AR could modify PTEN deletion-induced uterine pathology. To test our hypothesis, we developed uterine glandular epithelium-specific PTEN and/or AR knockout mouse models comparing the uterine pathology among wild-type (WT), glandular epithelium-specific AR inactivation (ugeARKO), PTEN deletion (ugePTENKO), and the combined PTEN and AR knockout (ugePTENARKO) female mice. The double knockout restricted to glandular epithelium showed that AR inactivation enhanced PTEN deletion-induced uterine pathology with development of intraepithelial neoplasia by 20 weeks of age. In ugePTENARKO, 6/10 (60%) developed intraepithelial neoplasia, whereas 3/10 (30%) developed only glandular hyperplasia in ugePTENKO uterus. No uterine pathology was observed in WT ($n=8$) and ugeARKO ($n=7$) uteri. Uterine weight was significantly ($P=0.002$) increased in ugePTENARKO (374 ± 97 mg (mean $\pm$ s.e.)) compared with WT (97 ± 6 mg), ugeARKO (94 ± 12 mg), and ugePTENKO (205 ± 33 mg). Estrogen receptor alpha ($ER\alpha$) and P-AKT expression was modified by uterine pathology but did not differ between ugePTENKO and ugePTENARKO, suggesting that its expressions are not directly affected by androgens. However, progesterone receptor (PR) expression was reduced in ugePTENARKO compared to ugePTENKO uterus, suggesting that PR expression could be regulated by glandular epithelial AR inactivation. In conclusion, glandular epithelial AR inactivation (with persistent stromal AR action) enhanced PTEN deletion-induced uterine pathology possibly by downregulating PR expression in the uterus.

Key Words

- ▶ androgen receptor
- ▶ androgen
- ▶ uterine disorder
- ▶ PTEN
- ▶ mouse model

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Introduction

Uterine cancer is among the most frequent gynecological cancers. It is highly hormone dependent as it is promoted by estradiol (E_2) and inhibited by progesterone (P_4) exposure (Bender *et al.* 2011). However, the role of androgens in uterine cancer susceptibility, origins, and/or progression remains controversial. Androgens are 19-carbon steroid

hormones produced in women by ovarian and adrenal gland secretion of pro-androgen precursors together with extraglandular steroidogenic conversion. Androgens mediate their specific effects via activation of the androgen receptor (AR) expressed in target tissues such as the uterus (Somboonporn & Davis 2004, 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 roles in the female physiology have only recently been recognized (Walters *et al.* 2007). Previously, in females, androgens were only considered to be obligate precursors for conversion to estrogens by aromatase (Hillier *et al.* 1994).

The role of androgens in uterine growth is supported by experimental findings. The non-aromatizable androgen dihydrotestosterone (DHT) promotes uterine growth and differentiation of the rodent uterus (Schmidt & Katzenellenbogen 1979), whereas global AR inactivation in female mice resulted in thinner uterus with reduced total uterine area compared with wild-type (WT) controls (Walters *et al.* 2009). Recently, we have demonstrated that the effect of androgens (testosterone or DHT) on uterine growth is mediated via AR (Choi *et al.* 2015b). However, in contrast to promoting effects, an *in vitro* study suggested that androgens, acting via AR, may inhibit uterine growth as androstenedione (an aromatizable pro-androgen) inhibited proliferation of human endometrial cells, effects that were reversed by administration of the steroidal antiandrogen and progestin, cyproterone acetate (Tuckerman *et al.* 2000). These findings suggest that androgens may have cell-specific roles in the uterus, as demonstrated for prostate (Heinlein & Chang 2004, Simanainen *et al.* 2007).

AR is also strongly expressed in various uterine cancer types, suggesting a role of AR-mediated androgen action in the origins and progression of uterine cancer (Ito *et al.* 2002). This is supported by clinical studies, showing that increased tissue androgen sensitivity due to the presence of polymorphic shorter CAG repeat lengths in exon 1 of the AR was associated with increased uterine cancer risk (McGrath *et al.* 2006). Similarly, in our recent study using global phosphatase and tensin homolog (PTEN) deletion-induced experimental uterine cancer, the severity of cancer progression was reduced when combined with global AR inactivation. (Choi *et al.* 2015a). This finding further suggests that AR-mediated androgen actions enhance the development of PTEN deletion-induced uterine cancer.

Pten is a tumor suppressor gene located on chromosome 10 (Dahia 2000). Physiologically, PTEN functions as a phosphatase that inhibits growth factor signaling transduced through PI3K by inhibiting phosphorylation of AKT (Li *et al.* 1998). *Pten* is mutated or deleted in a wide range of human cancers, including uterine cancers (Dahia 2000). *Pten* mutations are observed in 30–80% of type 1 endometrial carcinomas

(EMCs) and in 20–70% of complex atypical hyperplasia, a premalignant stage of EMC (Tashiro *et al.* 1997, Levine *et al.* 1998, Lee *et al.* 2012). As uterine cancers commonly arise from the uterine endometrial glands (Newbold *et al.* 1990), we therefore investigated the impact of AR-mediated androgen action on PTEN deletion-induced uterine cancer in our novel uterine cancer mouse model, where deletions are restricted to uterine glandular epithelium (Choi *et al.* 2015b). Accordingly, we hypothesized that the inactivation of glandular epithelial AR would similarly decrease PTEN deletion-induced uterine pathogenesis as we have shown that androgen action via AR promotes PTEN deletion-induced uterine cancers (Choi *et al.* 2015a).

Materials and methods

Mice

Three separate founder mouse lines have been used to generate the mice used in these experiments WT, ugeARKO, ugePTENKO, and ugePTENARKO female mice: Tg (Pbsn-cre) 20Fwan mice (denoted PBSN-Cre; Jin *et al.* 2003, Choi *et al.* 2015b) – Cre expressed selectively in prostate epithelial cells and uterine glandular epithelial cells (kindly donated by Dr Fen Wang, Center for Cancer Biology and Nutrition, Houston, TX, USA); Ar^{tm1Jdz} (denoted ARflox; Notini *et al.* 2005); and Pten^{tm1Hwu} mice (denoted PTENflox; Lesche *et al.* 2002). All founder mouse lines were backcrossed to the FVB/N background for at least six generations (ARflox originally C57Bl background). The ugeARKO and ugePTENKO females were generated by crossing the PBSN-Cre and ARflox or PTENflox lines, respectively, with Cre-negative females used as WT controls as previously described for males (Jin *et al.* 2003). All mice were housed under standard conditions (19–22°C, 12h light: 12h darkness cycle) in cages with *ad libitum* access to water and food.

Experimental design and tissue collection

Female mice were sacrificed by cardiac exsanguination under ketamine/xylazine anesthesia at the median age of 20 weeks (range 18.4–21.7 weeks) at diestrus stage. Body weight was recorded, and ovary and uterus were dissected and weighed. Organs were either fixed (4% paraformaldehyde at 4°C overnight) for histological processing or snap frozen in liquid nitrogen for mRNA extraction (uterus). All experiments and procedures were approved by the Sydney Local Health District Animal

Ethics Committee (Sydney, Australia) within NHMRC guidelines for animal experimentation.

Histology and immunohistochemistry

A mid-section (between the fimbrial end and the cervical end) from a fixed uterine horn was embedded in paraffin, sectioned at 5 µm. Sections were either stained with hematoxylin and eosin (H&E) for histopathological analyses or used for immunohistochemistry. Using light microscopy, different areas of uterine section were marked (total area, myometrium, endometrium, and lumen), and each area was calculated using CASTGRID software (Olympus). Measurements were repeated on three different uterine sections for each sample, and measurements were averaged.

Immunostaining was performed as previously described in (Gao et al. 2014). In brief, sections were microwaved with 10mM citric acid, pH 6 for antigen retrieval for 12min. Pierce SuperBlock (Thermo Scientific; containing 0.5% bovine serum albumin) was used as a blocking solution, and estrogen receptor alpha (ERα) (SC-542, Santa Cruz Biotechnology; 1:200 dilution), progesterone receptor (PR) (SC-538, Santa Cruz Biotechnology; 1:100 dilution), AKT (11E7, Cell Signaling Technology; 1:50 dilution), and P-AKT (D9E, Cell Signaling Technology; 1:50 dilution) were used as primary antibodies. Primary antibody was detected with biotinylated anti-rabbit secondary antibody (Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA) as per manufacturers' instructions. 3,3'-diaminobenzidine (DAB; Invitrogen) was used as a peroxidase substrate for detection. Tissues were counterstained with hematoxylin, dehydrated, left air dry, and cover slipped for microscopy. Immunostaining was compared between the genotype and different histopathology classifications, and representative images were shown. Immunopositivity and immunointensity of ERα and PR were quantified by the semiquantitative H-score method as previously described (Choi et al. 2015a).

Uterine disorder classification

All uteri from experimental mice were classified into three categories based on histological characteristics: normal glands (Fig. 1A), hyperplastic glands (Fig. 1B), and intraepithelial neoplastic glands (Fig. 1C). Normal gland had widely separated tubular ductular glands embedded in a cellular stroma (Fig. 1A). Hyperplastic gland had marked increase in the number of irregularly disorganized glands lined by enlarged glandular epithelial cells (Fig. 1B).

Intraepithelial neoplastic gland was characterized by high-grade atypia, which exhibited intraglandular epithelial cell proliferation that was papillary or cribriform in appearance as previously reported (Stambolic et al. 2000) (Fig. 1C).

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

RNA was extracted from whole uterus using TRIzol reagent (Sigma-Aldrich) as per manufacturers' instructions. Residual genomic DNA was removed by ribonuclease-free DNase I (0.5 IU/µg RNA; Invitrogen). cDNA was reverse-transcribed from 2βg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR (qPCR) was performed using a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) and SensiMix SYBR kit (Bioline, Alexandria, Australia) according to manufacturers' instructions. To measure ERα gene expression as well as expression of ERα-dependent genes (*G6PDH* and *Complement C3*), primer sequences were used as previously described (Choi et al. 2015a). To measure PR gene expression together with expression of PR-dependent genes (*Indian hedgehog* (*Ihh*) and *interleukin 13 receptor α2* (*Il13ra2*)) and *Cyclooxygenase* (*Cox*)-1 and -2 mRNA expression, the following target transcript primer sequences were used: PR-forward (5'-GCTTTCCTGCACAGCAGTAC-3'), PR-reverse (5'-CGAAGAGCCAAGCACCAGTA-3'), *Ihh*-forward (5'-GCTT CGACTGGGTGTATTACG-3'), *Ihh*-reverse (5'-TCTCCTGGC TTTACAGCTGAC-3'), *Il13ra2*-forward (5'-TGTGGAAAGGA GGACAAAGAG-3'), *Il13ra2*-reverse (5'-CAAAGAAGAAATGAA ACACAAGCA-3'), *Cox-1*-forward (5'-ACCTACGTCTACGCC AAAGG-3'), *Cox-1*-reverse (5'-GTGGTTTCCAACCAAGAT CA-3'), *Cox-2*-forward (5'-CCGTGCTGCTCTGTCTTAAC-3') and *Cox-2*-reverse (5'-TTGGGAACCCCTCTTTGTTC-3'). Amplicon products were assessed by melting curve analysis (Rotor-Gene 6000 software), and all reaction efficiencies were equivalent (95–100%). Target genes were normalized to expression of internal housekeeping genes, *rpl19*, *cyclophilin*, and *18s rRNA*, as previously described (Simanainen et al. 2015, Choi et al. 2015a).

Statistical analysis

Statistics were performed by two-way ANOVA and one-way ANOVA with LSD method for *post hoc* test using SPSS software (SPSS, Inc.), unless stated otherwise. *P*-values less than or equal to 0.05 were considered statistically significant.

Figure 1

Histological categorization of uterine phenotype and uterine weight. Representative photos of H&E staining of three different uterine histological categories: (A) normal glands; (B) hyperplastic uterine glands; (C) intraepithelial neoplastic glands (scale bar = 100 μ m). The table shows histological categorization of uterine abnormalities in WT, ugeARKO, ugePTENKO, and ugePTENARKO females (^a, significantly different to WT, ugeARKO, and ugePTENKO, $P < 0.05$; Pearson chi-square). (D) Uterus weights in WT, ugeARKO, ugePTENKO, and ugePTENARKO females at termination (^a, significantly different to WT, ugeARKO, and ugePTENKO, $P < 0.05$; one-way ANOVA, mean $\pm$ S.E.M; $n \geq 9$). Representative gross anatomy images of ugePTENKO (E) and ugePTENARKO (F) uteri. Body weight (G) and ovary weight (H) were compared among WT, ugeARKO, ugePTENKO, and ugePTENARKO (mean $\pm$ S.E.M; $n \geq 9$). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0039>.

Results

Glandular epithelial PTEN deletion-induced uterine pathology that was enhanced by glandular AR inactivation

Uteri were classified into three progressive histological categories of carcinogenesis using H&E staining: uteri with normal glands (Fig. 1A), hyperplastic glands (Fig. 1B), and intraepithelial neoplasia (Fig. 1C). All uteri of WT and ugeARKO females were categorized as normal featuring a clear distinction between myometrium and endometrium layers as well as regularly shaped endometrial glands. As previously reported, glandular epithelial PTEN deletion caused formation of hyperplastic glands (Choi et al. 2015b). In this study, abnormal uteri were found solely in ugePTENKO and ugePTENARKO females (table in Fig. 1), where glandular epithelial hyperplasia was present in 30% of both the ugePTENKO and ugePTENARKO uteri. In addition, intraepithelial neoplasia characterized by

cellular atypia was present in 60% of ugePTENARKO but not in any ugePTENKO uteri (table in Fig. 1). Intraepithelial neoplasia incidence was significantly influenced by both AR inactivation ($P = 0.008$; Fisher's exact test) and PTEN deletion ($P = 0.027$); interaction of PTEN deletion and AR inactivation was significant ($P = 0.02$; Cochran's test).

The uterine pathology in ugePTENKO and ugePTENARKO was also reflected in increased uterine weight (Fig. 1D). The uterine weights were significantly ($P = 0.003$; two-way ANOVA) increased by PTEN deletion but was not affected by AR inactivation ($P = 0.172$) or any interaction between PTEN deletion and AR inactivation. As a result, the weights of ugePTENARKO uteri were significantly increased compared to other genotypes, whereas uterine weights did not differ between WT and ugeARKO uteri (Fig. 1D). Macroscopic anatomies of ugePTENKO (Fig. 1E) and ugePTENARKO (Fig. 1F) uteri are shown. Body weights (Fig. 1G) and ovarian weights (Fig. 1H) were not affected by genotype.

Figure 2

Morphological analysis of the uterus. Total (A), endometrial (B), and myometrial (C) uterine areas (mm²) were measured in WT, ugeARKO, ugePTENKO, and ugePTENARKO females. Endometrial-to-myometrial ratio (E/M) was calculated by dividing the area of the endometrium by the area of the myometrium (D) (^a, significantly different to WT, ugeARKO, and ugePTENKO, $P < 0.05$; one-way ANOVA, mean $\pm$ S.E.M.; $n \geq 5$).

Simultaneous glandular epithelial AR inactivation and PTEN deletion increased endometrial area but did not affect myometrial area

As the uterine weight was increased in ugePTENARKO uterus, we investigated whether different layers of the uterus were affected (Fig. 2A, B, C and D). The total uterine area was significantly ($P = 0.029$; two-way ANOVA) increased by AR inactivation but was not affected by PTEN deletion ($P = 0.098$). The endometrial area was significantly increased by both AR inactivation ($P = 0.01$) and PTEN deletion ($P = 0.015$). There was a significant interaction between AR inactivation and PTEN deletion ($P = 0.025$). As a result, the total uterine (Fig. 2A) and endometrial (Fig. 2B) cross-sectional areas were increased in ugePTENARKO uterus compared to other genotypes. However, the myometrial areas were not significantly affected by either AR inactivation ($P = 0.085$) or PTEN deletion ($P = 0.486$) (Fig. 2C). These changes resulted in increased endometrial-to-myometrial ratio in ugePTENARKO females (1.4 ± 0.1) compared to WT (0.7 ± 0.1), ARKO (0.8 ± 0.2), and PTENKO (0.9 ± 0.1), which indicates uterine abnormality (De Bosschere et al. 2002) in ugePTENARKO uterus (Fig. 2D). The endometrial-to-myometrial ratios were significantly increased by both AR inactivation ($P = 0.036$) and PTEN deletion ($P = 0.009$); interaction of AR inactivation and PTEN deletion was not significant.

P-AKT expression was increased as uterine pathology progressed

As PTEN regulates the AKT signaling pathway, to investigate if glandular epithelial AR inactivation influenced total AKT and P-AKT expression, we compared

AKT immunopositivity in ugePTENKO and ugePTENARKO uteri. In all uteri, AKT was present throughout with similar expression in ugePTENKO and ugePTENARKO compared within the same histopathological categories (normal and hyperplastic) (Fig. 3A, B, C, D and E). However, AKT immunopositivity was stronger in the epithelia with intraepithelial neoplasia compared to normal or hyperplastic epithelium (Fig. 3E).

P-AKT is the active form of AKT which promotes cell growth. Similar to total-AKT, P-AKT immunopositivity was stronger in intraepithelial neoplasia (Fig. 3J) compared to normal (Fig. 3F and G) or hyperplastic (Fig. 3H and I) glandular epithelial cells. P-AKT expression appeared similar between ugePTENKO and ugePTENARKO within normal (Fig. 3F and G) and hyperplastic (Fig. 3H and I) glands.

ER α expression was reduced in uterine stroma and myometrium with intraepithelial neoplastic glands compared to the uteri with hyperplastic glands

As uterine pathology is highly hormone dependent and promoted by E₂ (Bender et al. 2011), we investigated ER α expression by immunohistochemistry. ER α expression was quantified according to percentage immunopositive (Fig. 4A), whereas the intensity of staining (Fig. 4B) was quantified using the H-score method. Samples analysis was stratified according to their histopathology as different uterine pathology stage is expected to affect ER α expression (Choi et al. 2015a).

ER α immunopositivity and immunointensity were not significantly different between histologically normal WT (Fig. 4C) and ugeARKO (Fig. 4D) uteri, noting that ER α was relatively weakly expressed in all cells types

Figure 3

Representative AKT and P-AKT immunohistochemistry in ugePTENKO and ugePTENARKO. AKT expression was compared between normal ugePTENKO (A) and ugePTENARKO (B), ugePTENKO (C) and ugePTENARKO (D) with hyperplastic glands, and intraepithelial neoplastic glands (E). P-AKT expression was compared between normal ugePTENKO (F) and ugePTENARKO (G), ugePTENKO (H) and ugePTENARKO (I) with hyperplastic glands, and intraepithelial neoplastic glands (J). Scale bar = 100 μ m for A, B, E, G, and J; 200 μ m for C, D, H, and I; $n \geq 4$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0039>.

(epithelium, stroma, and myometrium). However, glandular epithelial PTEN deletion significantly increased ER α immunopositivity ($P \leq 0.028$) and immunointensity ($P \leq 0.028$) throughout the uterus in histologically normal appearing ugePTENKO (Fig. 4E). The histologically normal ugePTENARKO (Fig. 4F) uterus had a similar ER α expression as ugePTENKO (normal); however, immunopositivity and immunointensity could not be quantified as there was only one case of histologically normal ugePTENARKO.

In the uterus containing hyperplastic glands (ugePTENKO and ugePTENARKO) (Fig. 4G and H), ER α immunopositivity ($P \leq 0.028$) and immunointensity ($P \leq 0.028$) were significantly increased compared to histologically normal WT and ugeARKO uteri (Fig. 4C and D) regardless of cell type. However, compared with histologically normal ugePTENKO and ugePTENARKO uteri (Fig. 4E and F) there were no significant changes. The ER α immunopositivity was similar in ugePTENKO (Fig. 4G) and ugePTENARKO (Fig. 4H) uteri with hyperplastic glands.

The intraepithelial neoplastic glands were only detected in ugePTENARKO females (Fig. 4I and J). In these samples, the ER α immunopositivity ($P \leq 0.032$) and immunointensity ($P \leq 0.032$) in glandular epithelial cells as well as immunointensity in stromal cells ($P \leq 0.037$) were significantly increased compared to histologically normal uterus in WT and ugeARKO females. However, in intraepithelial neoplastic glands detected in ugePTENARKO females, the ER α immunopositivity and immunointensity in the stroma and myometrium were significantly ($P = 0.037$) decreased compared to ugePTENKO (normal), ugePTENKO (hyperplasia), and ugePTENARKO (hyperplasia). No significant changes were observed in luminal and glandular epithelial ER α expression.

Collectively, these results suggest that the ER α expression is dependent on the histopathological stage as the glandular epithelial ER α expression was increased in the uterus with intraepithelial neoplastic glands compared to histologically normal uterus, whereas stromal and myometrial ER α expression was decreased compared to

A	Histopathology	Glandular epithelial	Luminal epithelial	Stroma	Myometrium
WT	Normal	56.3 ± 5.9	67.3 ± 7.8	49.6 ± 2.9	29.4 ± 5.3
ugeARKO	Normal	60 ± 1.1	61.8 ± 1.3	40.6 ± 6.4	24.3 ± 4.9
ugePTENKO	Normal	100 ± 0 ^a	100 ± 0 ^a	100 ± 0 ^a	100 ± 0 ^a
	Hyperplasia	100 ± 0 ^a	100 ± 0 ^a	100 ± 0 ^a	100 ± 0 ^a
ugePTENARKO	Normal	-	-	-	-
	Hyperplasia	100 ± 0 ^a	100 ± 0 ^a	100 ± 0 ^a	100 ± 0 ^a
	Intraepithelial neoplasia	95 ± 5 ^a	82.3 ± 18	51.5 ± 11 ^b	41.2 ± 15 ^b

B	Histopathology	Glandular epithelial	Luminal epithelial	Stroma	Myometrium
WT	Normal	0.21 ± 0.02	0.24 ± 0.02	0.18 ± 0.01	0.1 ± 0.02
ugeARKO	Normal	0.22 ± 0	0.22 ± 0.01	0.13 ± 0.02	0.08 ± 0.02
ugePTENKO	Normal	1.0 ± 0 ^a	1.0 ± 0 ^a	1.0 ± 0 ^a	1.0 ± 0 ^a
	Hyperplasia	1.0 ± 0 ^a	1.0 ± 0 ^a	1.0 ± 0 ^a	1.0 ± 0 ^a
ugePTENARKO	Normal	-	-	-	-
	Hyperplasia	1.0 ± 0 ^a	1.0 ± 0 ^a	1.0 ± 0 ^a	1.0 ± 0 ^a
	Intraepithelial neoplasia	0.89 ± 0.11 ^a	0.74 ± 0.26	0.29 ± 0.08 ^{ab}	0.17 ± 0.05 ^b

Figure 4

ERα immunohistochemistry on the uterus at 20 weeks old. Percentage of ERα-positive cells (A) and intensity (B) in different compartments of the uterus (^a, significantly different to WT and ugeARKO; ^b, significantly different to ugePTENKO (normal), ugePTENKO (hyperplasia), and ugePTENARKO (hyperplasia) ($P \leq 0.037$; nonparametric test, mean ± s.e.m.; $n \geq 3$). Representative images of ERα immunohistochemistry in WT (C), ugeARKO (D), histologically normal ugePTENKO (E) and ugePTENARKO (F), ugePTENKO (G) and ugePTENARKO (H) with hyperplastic glands, and ugePTENARKO with intraepithelial neoplastic glands (I and J). Box in (I) is magnified in (J). Scale bar = 100 μm for C, D, G, H, and J; 200 μm for E, F, and I; $n \geq 4$. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0039>.

hyperplastic uterus. In addition, glandular epithelial PTEN deletion upregulated ERα expression, whereas glandular epithelial AR inactivation may not significantly affect ERα expression in the uterus.

Glandular epithelial AR inactivation decreases glandular and luminal epithelial, stromal, and myometrial PR expression

As P_4 action protects against uterine cancer (Bender et al. 2011), expression of PR was also investigated by immunohistochemistry. Similar to ERα, PR expression was also quantified into immunopositivity (Fig. 5A) and immunointensity (Fig. 5B) using H-score method and further categorized based on histopathology.

PR was abundantly expressed in all cell types (epithelial, stromal, and smooth muscle cells) of WT uterus (Fig. 5C). PR immunopositivity ($P \leq 0.021$) and immunointensity ($P \leq 0.021$) in ugeARKO uterus (Fig. 5D) were significantly reduced compared to WT in all cell types, suggesting that glandular epithelial AR inactivation downregulates PR expression in the uterus. However, in histologically normal uterus of ugePTENKO females (Fig. 5E), PR immunopositivity ($P \leq 0.028$) and immunointensity ($P \leq 0.028$) were significantly increased in the stroma and myometrium compared to WT and ugeARKO (Fig. 5C and D). The PR expression in glandular epithelial and luminal epithelial cells was not affected by PTEN deletion. PR expression in ugePTENARKO (normal) uterus (Fig. 5F) appeared to be similar to ugePTENKO

(normal), but PR expression was not quantified as there was only one case of histologically normal ugePTENARKO.

In the uterus with hyperplastic glands (both ugePTENKO and ugePTENARKO) (Fig. 5G and H), PR was strongly expressed throughout the uterus, and PR immunopositivity ($P \leq 0.028$) and immunointensity ($P \leq 0.028$) were significantly increased compared to WT and ugeARKO uteri. PR immunopositivity was similar between ugePTENKO (hyperplasia) and ugePTENARKO (hyperplasia).

However, when the uterine pathology progressed to intraepithelial neoplasia (only in ugePTENARKO), PR expression was very weak throughout the uterus (Fig. 5I and J). PR immunopositivity ($P = 0.037$) and immunointensity ($P = 0.037$) were significantly reduced in all cell types compared to hyperplastic uterus. Furthermore,

stromal and myometrial PR expression were significantly ($P \leq 0.05$) reduced compared to WT and ugeARKO uteri as well. Hence, simultaneous glandular epithelial AR inactivation along with PTEN deletion significantly reduced PR expression in the uterus.

***ERα* and PR and expression of their dependent genes were not significantly affected by genotype**

RT-PCR was performed to determine the expression of *ERα* and PR and their dependent genes in the uterus. No significant changes were observed in *ERα* gene expression as well as in the expression of *ERα*-dependent genes, *G6PDH* and *Complement C3* (Fig. 6A, B and C). Although PR and its dependent genes, *Ihh* and *Il13ra2*, appeared to be increased by PTEN deletion (ugePTENKO),

A	Histopathology	Glandular epithelial	Luminal epithelial	Stroma	Myometrium
WT	Normal	64.8 ± 5.8	76.3 ± 8.0	50.7 ± 0.6	35.7 ± 2.0
ugeARKO	Normal	37.0 ± 1.7 ^a	41.75 ± 4.8 ^a	17.1 ± 2.9 ^a	11.95 ± 1.6 ^a
ugePTENKO	Normal	71.7 ± 28	76.7 ± 23	100 ± 0 ^{ab}	100 ± 0 ^{ab}
	Hyperplasia	100 ± 0 ^{ab}	100 ± 0 ^{ab}	100 ± 0 ^{ab}	100 ± 0 ^{ab}
ugePTENARKO	Normal	-	-	-	-
	Hyperplasia	100 ± 0 ^{ab}	100 ± 0 ^{ab}	100 ± 0 ^{ab}	100 ± 0 ^{ab}
	Intraepithelial neoplasia	63.3 ± 15 ^d	39.3 ± 23 ^d	8.47 ± 2.3 ^{abcd}	3.53 ± 2.2 ^{abcd}

B	Histopathology	Glandular epithelial	Luminal epithelial	Stroma	Myometrium
WT	Normal	0.32 ± 0.04	0.4 ± 0.06	0.22 ± 0.01	0.15 ± 0.01
ugeARKO	Normal	0.13 ± 0.01 ^a	0.15 ± 0.02 ^a	0.06 ± 0.01 ^a	0.04 ± 0.01 ^a
ugePTENKO	Normal	0.68 ± 0.32	0.7 ± 0.3	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}
	Hyperplasia	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}
ugePTENARKO	Normal	-	-	-	-
	Hyperplasia	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}	1.0 ± 0 ^{ab}
	Intraepithelial neoplasia	0.28 ± 0.1 ^d	0.18 ± 0.12 ^d	0.03 ± 0.01 ^{abcd}	0.01 ± 0.01 ^{abcd}

Figure 5

PR immunohistochemistry on the uterus at 20 weeks old. Percentage of PR-positive cells (A) and intensity (B) in different compartments of the uterus (^a, significantly different to WT; ^b, significantly different to ugeARKO; ^c, significantly different to ugePTENKO (normal); ^d, significantly different to ugePTENKO (hyperplasia) and ugePTENARKO (hyperplasia) ($P \leq 0.05$; nonparametric test, mean ± S.E.M; $n \geq 3$). Representative images of PR immunohistochemistry in WT (C), ugeARKO (D), histologically normal ugePTENKO (E) and ugePTENARKO (F), ugePTENKO (G) and ugePTENARKO (H) with hyperplastic glands, and ugePTENARKO with intraepithelial neoplastic glands (I and J). Box in (I) is magnified in (J). Scale bar = 100 μm for C, D, and J; 200 μm for E, F, G, H, and I; $n \geq 4$). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-16-0039>.

which was reversed by simultaneous AR inactivation (ugePTENARKO), the changes were not statistically significant (Fig. 6D, E and F).

Cox-2 gene expression was significantly increased in ugePTENARKO uterus

As modification of COX signaling is reported in PTEN knockout-induced uterine cancers in which *Cox-2* expression was increased in early stages of endometrial cancers in mouse (Daikoku et al. 2008), we quantified the *Cox-1* and *Cox-2* mRNA expression to determine if the expression was modified by PTEN and/or AR knockout. *Cox-2* mRNA expression significantly increased by both AR inactivation ($P=0.006$; two-way ANOVA) and PTEN deletion ($P=0.013$). As a result, *Cox-2* mRNA expression was increased in ugePTENARKO compared to WT and ugePTENKO uteri, whereas *Cox-2* expression was similar among WT, ugeARKO, and ugePTENKO uteri (Fig. 7A). *Cox-1* mRNA expression was not affected by either AR inactivation ($P=0.76$) or PTEN deletion ($P=0.241$) (Fig. 7B).

Discussion

We have recently demonstrated that androgen actions via AR in the epithelial glands of the uterine endometrium

can support uterine growth (Choi et al. 2015b). As androgens may also have roles in uterine pathology (Gibson et al. 2014) and AR is expressed in many uterine cancers notably of epithelial cells (Ito et al. 2002), we investigated the role of AR-mediated androgen action confined to uterine glandular epithelium in experimental, PTEN deletion-induced uterine pathology. Our study has demonstrated that glandular epithelial AR inactivation significantly enhanced and accelerated the progression of PTEN deletion-induced uterine pathology. We provide evidence that this may be due to a downregulation of uterine PR expression, which normally protects against uterine cancer development.

A clinical study suggested that increased androgen sensitivity (McGrath et al. 2006) and increased androgen levels (Nagamani et al. 1986, Smyczek-Gargya & Geppert 1992) in women were associated with a higher risk of uterine cancer, suggesting that androgen action may enhance or promote uterine cancer development. These clinical observations were supported by our recent study in which androgens acting via AR promoted PTEN deletion (global)-induced experimental uterine cancer (Choi et al. 2015a). However, due to systemic effects of increased androgen sensitivity/levels or AR inactivation, the uterine-specific role of AR-mediated androgen actions could not be established. Therefore,

Figure 6
mRNA expression of *ERα* and *PR* and their dependent genes. *ERα* (A) and *ERα*-dependent genes (B and C) and *PR* (D) and *PR*-dependent genes (E and F) were measured in the uterus at 20 weeks of age. Gene expressions were relative to 18s rRNA housekeeping gene.

Figure 7

Uterine *Cox-1* and *Cox-2* gene expression at 20 weeks. *Cox-2* (A) and *Cox-1* (B) mRNA expression was compared among experimental groups, and gene expression was standardized using *rpl19* and *cyclophilin* (*, significantly different to WT and ugePTENKO; $P < 0.03$; nonparametric test, mean $\pm$ S.E.M; $n \geq 5$).

we explored the specific role of AR-mediated androgen actions occurring simultaneously with PTEN knockout to induce glandular cell proliferation. Unexpectedly, contrary to our hypothesis and the clinical studies as well as our global AR inactivation study (Choi et al. 2015a), this study suggests that androgens acting via glandular epithelial AR decelerated the progression of experimental, PTEN deletion-induced uterine pathology. Alternatively, this may be due to persistent stromal AR actions in the absence of the balancing act of glandular epithelial AR. Similar regulatory balance between the stromal and epithelial ARs has been previously suggested for prostate (Simanainen et al. 2007, Wen et al. 2015).

The major differences between our previous (Choi et al. 2015a) and current knockout mouse models are outlined in Table 1. Previously, AR and/or PTEN were knocked out in all cells of the mouse, including all uterine cells (glandular epithelium, luminal epithelium, stroma, and myometrium) as well as other hormone-dependent organs such as ovaries and pituitary. In the current uterine gland-specific model, the AR and/or PTEN were knocked out selectively in the uterine glandular epithelial cells (Choi et al. 2015b), whereas both the AR and the PTEN are still functional in all other cells of the uterus and other nontarget organs (Choi et al. 2015b).

Heterozygous PTEN deletion in the uterus induced glandular hyperplasia (pre-malignant precursor to endometrial cancer) in the uterus (Risinger et al. 1997, Stambolic et al. 2000). Furthermore, conditional homozygous PTEN deletion in the uterus or in uterine epithelial cells resulted in EMC (Daikoku et al. 2008, Memarzadeh et al. 2010, Mirantes et al. 2013, Tirodkar et al. 2014, Choi et al. 2015b). In our current study, simultaneous glandular epithelial AR inactivation along with PTEN deletion significantly accelerated the severity of uterine pathology from hyperplastic glands (detected in both ugePTENKO and ugePTENARKO uteri) to intraepithelial neoplastic glands with atypia (only in ugePTENARKO uterus). The increased uterine pathology in ugePTENARKO females was also reflected in increased uterine weights and in increased endometrial area of the uterus. These findings suggested that the AR-mediated androgen actions in glandular epithelial cells decelerated the progression of PTEN deletion-induced uterine pathology.

In line with our findings, an *in vitro* study suggested growth-preventing effect of androgens via AR in endometrial cells in which androstenedione (an aromatizable pro-androgen) inhibited the proliferation of human endometrial cells (Tuckerman et al. 2000). The

Table 1 Comparison of glandular epithelial and global knockout mouse models.

	Glandular epithelial specific knockout model (ugePTENARKO)	Global knockout model (PTENARKO) (Choi et al. 2015a)
Uterus		
Glandular epithelial AR and PTEN	Non-functional	Non-functional
Luminal epithelial AR and PTEN	Functional	Non-functional
Stromal AR and PTEN	Functional	Non-functional
Myometrial AR and PTEN	Functional	Non-functional
Other organs (i.e., ovary, pituitary)		
AR & PTEN	Functional	Non-functional
Uterine pathology	↑	↓
Mechanistic observation	↓ PR expression	↓ ERα expression
Mechanistic explanation	↓ PR-mediated inhibition of uterine pathogenesis	↓ ERα-mediated promotion of uterine pathogenesis

effect was reversed by cyproterone acetate (antiandrogen) demonstrating AR-mediated effects. Together with the results of this study, it is proposed that cell-specific effects of androgens acting via AR in the uterus warrants further investigation to more clearly define the mechanism of the cell-specific role of AR, including the relationship of AR activity in nonglandular uterine cells and a possible paracrine interaction of AR-expressing cells in the uterus.

In this study, P-AKT was only detected in glandular epithelial cells of the uterus following complete deletion of PTEN, but not in luminal epithelial cells, stromal and myometrial cells of ugePTENKO, or WT glandular epithelial cells in which PTEN was present. Very low P-AKT levels in uterine cells expressing PTEN and upregulation following PTEN deletion are supported by previous studies (Stambolic et al. 1998, 2000). P-AKT expression between ugePTENKO and ugePTENARKO glandular epithelial cells within the same histopathological categories appeared to be similar. However, P-AKT expression was increased in intraepithelial neoplastic glands, which were only present in ugePTENARKO. Therefore, in contrast to breast and prostate cancer cells (Wang et al. 2011), the AR inactivation in uterine glandular cells did not appear to affect AKT signaling pathway. However, as intraepithelial neoplastic glands were only present in ugePTENARKO, further investigation (i.e., cell culture) is warranted to determine the direct role of glandular epithelial AR in AKT pathway.

A previous study suggested that PTEN deletion increased ER α expression in mouse uterus (Lian et al. 2006). Similarly, androgens may regulate ER α expression as shown by testosterone-induced suppression of ER α expression in mammary tissue (Zhou et al. 2000). Alternatively, AR could interact with PTEN to indirectly modify ER α expression as seen in prostate and breast cancer cells (Wang et al. 2011). Therefore, we explored whether glandular epithelial AR inactivation modified ER α expression in PTEN-deleted uterus. Increased ER α expression was detected throughout the uterus upon PTEN deletion. However, in our study, glandular epithelial AR inactivation in the uterus did not modify ER α expression. Also, ER α expression was similar between histologically normal and hyperplastic uteri in ugePTENKO females. However, when hyperplastic glands progressed to intraepithelial neoplastic glands (only in ugePTENARKO), ER α expression in the glandular and luminal epithelial cells did not change, whereas ER α expression in the stroma and myometrium decreased compared with hyperplastic glands (ugePTENKO and

ugePTENARKO). Therefore, although altered ER α expression in the uterus may not explain accelerated uterine pathology in ugePTENARKO uterus, further studies are warranted in intraepithelial neoplastic glands.

Progestin actions mediated via PR (Kurita et al. 1998, Mulac-Jericevic et al. 2000) protect against uterine cancer (Martin et al. 1973, Persson 1996). Therefore, we also investigated PR expression in the uterus of our experimental female mice. The WT uterus showed weak expression PR in glandular and luminal epithelial cells, stroma, and myometrium. Glandular epithelial AR inactivation in the ugeARKO uterus further diminished PR expression throughout the ugeARKO uterus compared to WT, suggesting that glandular epithelial AR actions maintain PR expression. Second, in glandular epithelial PTEN-deleted uterus (ugePTENKO and ugePTENARKO) with hyperplastic glands, increased PR expression was detected throughout the uterus. The finding supports previous findings in endometrial regeneration model, in which increased PR expression was reported following epithelial PTEN deletion (Memarzadeh et al. 2010). Furthermore, this study demonstrates that a uterus bearing intraepithelial neoplastic glands has further diminished PR expression in all cells compared with a uterus with hyperplastic glands. Our finding was supported by the observations that PR expression decreased as uterine pathology advances to endometrial cancers (Ehrlich et al. 1981, Arnett-Mansfield et al. 2001, Kim et al. 2010). In a previous study, 90% of hyperplastic uteri in women had moderate-to-strong PR expression, although the PR expression was very weak throughout the uterus, when hyperplastic glands progressed to intraepithelial neoplasia (Arnett-Mansfield et al. 2001). Due to progression of pathology only in ugePTENARKO, this study could not directly confirm whether altered PR expression is due to the histopathological change or glandular AR inactivation. However, as the pathology in ugePTENKO uterus did not progress to intraepithelial neoplastic glands, it is unlikely that progression to intraepithelial neoplasia is due to further diminished uterine PR expression alone, even if it is due to glandular AR inactivation.

Despite the PR immunohistochemistry results, PR gene expression or expression of PR-dependent genes, *Ihh* and *Il13ra2*, expressions were not significantly changed. A probable explanation for this is that gene expression was quantified using RNA extracted from the whole uterus, whereas AR and PTEN deletions are only in glandular epithelial cells constituting only a very small fraction of all uterine cells. Hence, the significant changes could

have been diluted and lost. Nevertheless, we did observed similar, though not statistically significant, pattern in which *PR*, *Ihh*, and *Il13ra2* gene expressions were increased by PTEN deletion (ugePTENKO) compared to WT and ugeARKO, which was reversed by simultaneous AR inactivation (ugePTENARKO). This could also be due to the high variability in PTEN knockout-induced pathology, which varies in the rate of progression between individuals, as shown in histopathology/immunostaining. Therefore, gene expressions should be analyzed by cell types (i.e., glandular epithelial cells) as well as within the comparable histopathological stages with more sample size.

As the uterus is highly a hormone-dependent organ, changes in hormone profile could regulate its pathogenesis. However, as we did not observe any changes in steroid hormone levels in our global knockout study (Choi et al. 2015a) in which both PTEN and AR were inactivated (including in ovaries as well), we did not expect any significant hormonal changes in this study in which the PTEN and/or AR inactivation is restricted to only the glandular epithelial cells in the uterus (Choi et al. 2015b).

Cox enzymes are associated with development of many cancers (Mazhar et al. 2005) and their expression is regulated by androgens (Yazawa et al. 2013, Simanainen et al. 2015) and AKT (Leng et al. 2003, St-Germain et al. 2004). Therefore, we measured uterine *Cox-1* and *Cox-2* mRNA expressions. *Cox-2* is important in progression of uterine hyperplasia to carcinoma, whereas *Cox-2* decreases apoptosis and increases angiogenesis with its levels associated with invasiveness (Boruban et al. 2008). We found that *Cox-2* expression was not significantly affected by glandular epithelial AR or PTEN mutations alone but was significantly increased by simultaneous AR and PTEN mutations in ugePTENARKO uterus. Increased *Cox-2* expression could be due to increased P-AKT expression in intraepithelial neoplastic glands found in ugePTENARKO uterus as previous studies in human endometrial cancer cells (St-Germain et al. 2004) and hepatocellular carcinoma cells (Leng et al. 2003) reported positive correlation between P-AKT and *Cox-2* expression. Furthermore, previous clinical studies have reported overexpression of uterine *Cox-2* in patients with EMC (Erkanli et al. 2007, Nasir et al. 2007). However, *Cox-1* that is involved in cell signaling and maintains tissue homeostasis was not affected by uterine-specific PTEN or AR knockout in our study. *Cox-1* has been shown to be expressed in most tissues, and its expression is not altered by cytokines and growth factors (Erkanli et al.

2007). However, further analysis of *Cox* gene expression (i.e., immunohistochemistry) in the uterus is required to localize its expressions in different uterine cell types to further clarify a possible role of *Cox* pathway in the present murine uterine carcinogenesis model.

In conclusion, our study has demonstrated that the glandular epithelial PTEN deletion is likely to cause uterine gland pathology by increasing P-AKT and ER α expression in glandular epithelial cells, resulting in increased uterine *Cox-2* expression. Furthermore, glandular epithelial AR inactivation enhanced PTEN inactivation-induced uterine gland pathology possibly by decreasing PR expression, thus diminishing the protective role of P₄ in uterine pathogenesis. This suggests a different mechanism similar to our previous global knockout study (Choi et al. 2015a), in which global AR inactivation reduced uterine pathology incidence by decreasing ER α compared with glandular epithelial AR inactivation increased uterine pathology by reducing PR (Table 1). Overall, our study suggests more localized uterine cell-specific effect of AR in uterine carcinogenesis, which warrants further investigations on the role of AR in different uterine cells as well as its paracrine–cell interaction. These cell-specific AR interactions as well as regulation of other steroid receptors or AKT pathways could be clarified using cell-specific conditional knockout models, recombination models, or cell culture studies.

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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