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# The role of androgens acting via androgen receptor in uterine development and PTEN deletion induced uterine cancer

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## Summary

The androgen receptor (AR) is widely expressed in human and rodent uterus during development, adulthood as well as in different uterine diseases, indicating an important role of AR-mediated androgen actions in uterine development, function and pathology. However, the specific role(s) and mechanisms remain controversial. Hence, the aim of this thesis was to investigate the role of androgen actions mediated via AR in uterine development, function and PTEN inactivation induced uterine pathology.

In the first experiment, the uterine glandular epithelial specific AR knockout mouse model (ugeARKO) was generated to investigate the role of AR in uterine development and function. Uterine development and function was compared between ugeARKO, global AR knockout (ARKO) and wild-type (WT) females. The uterine development and fertility appeared normal in ugeARKO females despite the uterine gland specific inactivation suggesting AR mediated endogenous androgen actions in uterine gland epithelium are not critical for normal uterine development and function. Following ovariectomy, the uterus regressed to about 15% of intact control uterine weights in all genotypes. However, after ovariectomy and androgen (testosterone or dihydrotestosterone, DHT) treatment, full regrowth from ovariectomy level was detected in WT uterus, only partial regrowth in ugeARKO and no regrowth in ARKO females. These findings suggest that androgens acting via AR specifically within uterus can promote full uterine regrowth in the absence of estrogens (Chapter 3).

In the second experiment, the role of AR mediated androgen actions in PTEN inactivation induced uterine pathology was investigated. Uterine cancer incidence was compared between WT, ARKO, heterozygous PTEN knockout (PTENKO) and combined heterozygous PTEN and complete AR knockout (PTENARKO) female mice. PTENKO-induced uterine cancer incidence was significantly reduced by simultaneous AR inactivation, indicating androgen actions via AR either systemically or locally within uterus modifies PTEN knockout induced uterine cancer incidence. This could be due to reduced estrogen receptor alpha (ERα) expression in PTENARKO compared to PTENKO uterus. AR inactivation did not modify PTEN or P-AKT levels (Chapter 4).

In the third experiment, the role of glandular epithelial AR in PTEN inactivation induced uterine pathology was determined. We compared development and progression of uterine pathology between WT, ugeARKO, uterine glandular epithelial specific PTEN knockout mouse model (ugePTENKO) and uterine glandular epithelial specific AR and PTEN knockout mouse model (ugePTENARKO). The AR inactivation enhanced PTEN knockout-induced uterine pathology with development of intraepithelial neoplasia in ugePTENARKO uterus but not in ugePTENKO. This could be due to reduced progesterone receptor (PR) expression in ugePTENARKO uterus compared to ugePTENKO suggesting PR expression could be regulated by glandular epithelial AR (Chapter 5). These findings together with Chapter 4 indicate that indicating that androgen actions via uterine AR

can modify PTENKO-induced uterine pathology, but the role may differ depending on site of androgen actions

In summary, this thesis has resulted in a number of significant findings on the role of AR-mediated androgens actions in uterine development, function and uterine pathology. I have generated and validated novel uterine glandular epithelial cell specific knockout mouse models that could be utilized in future research exploring uterine development and pathology. The findings support a significant role for androgens via AR inducing uterine growth that could be mediated locally via AR present in glandular epithelial cells. Furthermore, I have shown that AR modifies PTEN inactivation induced uterine pathology and the androgen actions can be mediated locally via uterine cell specific, AR mediated androgen actions modifying other hormone receptors such as ERα and PR.

# Declaration

I, **Jaesung (Peter) Choi,** declare that this thesis is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy (Medicine).

I declare that this thesis is my own work and effort and that has not been submitted for any other award. Where other sources of information have been used, they have been duly acknowledged.

Signature: 

Initial submission date: 31 July 2015

Signature: 

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# Publications and presentations

## 1. Publications:

**i)** Bin Zhao, **Jaesung (Peter) Choi**, Maria Jaehne, Yan Ru (Ellen) Gao, Reena Desai, Jan Tuckermann, Hong Zhou, David J. Handelsman, and Ulla Simanainen. Glucocorticoid receptor in prostate epithelia is not required for corticosteroid-induced epithelial hyperproliferation in the mouse prostate. *The Prostate* 74:1068-1078 (**2014**)

**ii)** **Jaesung Choi,** Basil Psarommatis**,** Yan Ru Gao**,** Yu Zheng**,** David J Handelsman and Ulla Simanainen. The role of androgens in experimental rodent mammary carcinogenesis. *Breast Cancer Research* 16:483 (**2014**)

**iii)** **Jaesung (Peter) Choi**, Reena Desai, Yu Zheng, Mu Yao, Qihan Dong, Geoff Watson, David Handelsman, and Ulla Simanainen. Androgen actions via androgen receptor promote PTEN inactivation induced uterine cancer. *Endocrine-Related Cancers*. 22(5):687-710 (**2015**).

**iv)** **Jaesung (Peter) Choi**, Yu Zheng, Kate Skulte, David Handelsman, and Ulla Simanainen. Development and characterization of uterine glandular epithelium specific AR knockout mouse model. *Biology of Reproduction*. 93(5):120 (**2015**).

## 2. Manuscripts in review:

**i)** **Jaesung (Peter) Choi**, Yu Zheng, David Handelsman and Ulla Simanainen. Glandular epithelial AR inactivation accelerates PTEN inactivation-induced uterine pathology by cell specific modification of PR. **Manuscript under consideration for publication.**

**ii)** **Jaesung (Peter) Choi**, Yan Ru Gao, Francia Garces Suarez, David J Handelsman, Ulla Simanainen. The Role of Androgens in Mammary Gland Development and Function. **Review manuscript under consideration for publication.**

## 3. Manuscripts in preparation:

**i)** **Jaesung (Peter) Choi**, Yu Zheng, David J Handelsman, Ulla Simanainen. The Role of Androgens in Rodent Uterine Development and Experimental Carcinogenesis. **Review manuscript in preparation.**

## 4. Invited Presentations:

i) **13th Sydney University Reproduction Forum (SURF), Sydney, Australia (November, 2013).** "Role of androgen actions via AR in PTEN inactivation induced uterine pathology"

ii) **Australian Rotary Health PhD Scholar's dinner, Sydney, Australia (May, 2012).**

iii) **Australian Rotary Health PhD Scholar's dinner, Sydney, Australia (May, 2014).**

## 5. Conference Presentations:

i) **Gordon research seminar and conference: Hormone-Dependent Cancers, Sunday River, Newry, ME, USA (August, 2015)-Poster** "Glandular epithelial AR inactivation accelerates PTEN inactivation-induced uterine pathology by cell specific modification of PR expression"- **Jaesung (Peter) Choi[1]**, Yu Zheng[2], David Handelsman[1] and Ulla Simanainen[1]

ii) **Postgraduate Cancer Research Symposium, Uni. of Sydney (November, 2014)-Oral** "Endometrial gland specific androgen actions regulate PTEN inactivation induced uterine disorders"- **Choi J[1]**, Zheng Y[2], Handelsman DJ[1], Simanainen U[1]

iii) **Endocrine Society of Australian Annual Conference, Melbourne, Australia (August, 2014)- "ESA-Novartis Junior Scientist Award" session-Oral** "Glandular epithelial AR regulates androgen mediated androgen effects on the uterine growth"- **Choi J[1]***, Kate Skulte[1], Zheng Y[2], Handelsman DJ[1], Simanainen U[1]

iv) **16th International Congress of Endocrinology & the Endocrine Society's 96th Annual Meeting & Expo (ICE/ENDO 2014), Chicago, USA (June 2014)-Poster** "Androgen receptor inactivation reduces PTEN inactivation induced endometrial cancer by decreasing estrogen sensitivity"- **Choi J[1*]**, Desai R[1], Zheng Y[2], Handelsman DJ[1], Simanainen U[1]

v) **15th EMBL PhD Symposium: Competition in Biology. The Race for Survival from Molecules to Systems, Heidelberg, Germany (November 2013)-Poster** "Uterine gland specific androgen actions regulate PTEN inactivation induced uterine pathology"- **Choi J***, Zheng Y, Handelsman DJ, Simanainen U

vi) **Endocrine Society of Australian Annual Conference, Sydney, Australia (August, 2013)- "Emerging Investigators" session-Oral** "Uterine gland specific androgen actions regulate PTEN inactivation induced uterine pathology"- **Choi J***, Zheng Y, Handelsman DJ, Simanainen U

vii) **Gordon research conferences: Hormone-Dependent Cancers, Bryant University, Smithfield, RI, USA (July-Aug, 2013)-Poster** "Androgen actions via androgen receptor promote PTEN inactivation induced uterine growth and pathology"- **Choi J***, Desai R, Zheng Y, Handelsman DJ, Simanainen U

viii) **Postgraduate Cancer Research Symposium, Uni. of Sydney (November, 2012)-Oral** "Androgen actions via AR reduce PTEN inactivation induced uterine pathology in mice"- **Choi J***, Desai R, Zheng Y, Lindsay L, Handelsman DJ, Simanainen U

ix) **International Congress on Hormonal Steroids and Hormones & Cancer, Kanazawa, Japan (November 2012)-Poster** "AR inactivation reduces uterine pathology induced by PTEN tumor suppressor inactivation"- **Jae Sung Choi**, Reena Desai, Yu Zheng, Laura Lindsay, David Handelsman, Ulla Simanainen*

x) **Endocrine Society of Australian Annual Conference, Gold Coast, Australia (August, 2012)- "ESA-Novartis Junior Scientist Award" session-Oral** "Role of androgens via AR in PTEN inactivation induced uterine pathology"- **Choi J***, Desai R, Zheng Y, Lindsay L, Handelsman DJ, Simanainen U

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# Abbreviations

| | |
|---|---|
| **AR** | Androgen receptor |
| **ARE** | Androgen responsive element |
| **ARKO** | Complete Androgen receptor knockout |
| **C3** | Complement component 3 |
| **cAIS** | Complete androgen insensitivity syndrome |
| **cDNA** | Complementary DNA |
| **CL** | Corpus luteum or corpus lutea |
| **CS** | Cowden Syndrome |
| **CYP** | Cytochrome P450 |
| **DAB** | 3,3'-Diaminobenzidine |
| **DBD** | DNA-binding domain |
| **DES** | Diethylstilbestrol |
| **DHEA** | Dehydroepiandrosterone |
| **DHT** | 5α-dihydrotestosterone |
| **DHT-B** | Dihydrotestosterone-benzonate |
| **E1** | Estrone |
| **E2** | Estradiol |
| **EMC** | Endometrial cancer |
| **ER** | Estrogen receptor |
| **ERα** | Estrogen receptor α |
| **ERαKO** | ERα knockout |
| **ERβ** | Estrogen receptor β |
| **ERβKO** | ERβ knockout |
| **FFPE** | Formalin fixed paraffin embedded |

| | |
|---|---|
| **G6PDH** | Glucose-6-phosphate dehydrogenase |
| **gDNA** | Genomic DNA |
| **GR** | Glucocorticoid receptor |
| **IGF-1** | Insulin-like growth factor-1 |
| **IL-6** | Interleukin 6 |
| **LBD** | Ligand-binding domain |
| **LC-MS/MS** | Liquid chromatography tandem mass spectrometry |
| **MPU** | Molecular physiology unit |
| **NLS** | Nuclear localization signal |
| **NTD** | N-terminal domain |
| **OVX** | Ovariectomy |
| **P4** | Progesterone |
| **P-Akt** | Phosphorylated Akt |
| **PBS** | Phosphate buffered saline |
| **PBSN** | Probasin |
| **PCNA** | Proliferating cell nuclear antigen |
| **PCOS** | Polycystic ovarian syndrome |
| **PDK** | Phosphotidylinositol-dependent kinase |
| **PFA** | Paraformaldehyde |
| **$PIP_2$** | Phosphatidylinositol 4, 5-bisphosphate |
| **$PIP_3$** | Phosphotidylinositol-3, 4, 5-triphosphate |
| **PI3K** | Phosphotidylinosital-3-kinase |
| **PR** | Progesterone receptor |
| **PRKO** | PR knockout |
| **PTEN** | Phosphatase and tensin homolog |

| | |
|---|---|
| **PTENKO** | Heterozygous PTEN knockout |
| **PTENARKO** | Heterozygous PTEN and complete AR knockout |
| **PTP** | Protein tyrosine phosphatase |
| **R26R** | Rosa 26 reporter mouse |
| **Rpl19** | Mouse ribosomal protein L19 |
| **SPF** | Specific pathogen free |
| **ugeARKO** | Uterine glandular epithelial specific AR knockout |
| **ugePTENKO** | Uterine glandular epithelial specific PTEN knockout |
| **ugePTENARKO** | Uterine glandular epithelial specific PTEN and AR knockout |
| **WT** | Wild-type |
| **X-gal** | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

# Chapter 1: Literature review

The uterus is a major reproductive sex organ in female mammals including humans and rodents. Both the development and carcinogenesis of the uterus are highly dependent on ovarian hormones including estradiol (E2) and progesterone (P4). However, recent studies have suggested that androgens acting via androgen receptor (AR) could also play a vital role in the human and rodent uterus (Somboonporn and Davis, 2004, Walters et al., 2010, Beral et al., 2005) .

## 1.1 Anatomy and histology of the mouse uterus

Mice have a bicornuate uterus consisting of two lateral horns (cornua) that join distally into a single body (corpus) (Figure 1.1). The mouse uterus is suspended from the dorsal body wall by the mesometria, which are heavy broad ligaments carrying blood and lymphatic vessels as well as abundant nerves. The body of mouse uteri consists of cranial/fundal and caudal/cervical segments. The mouse cranial segment is divided into two cavities by a median septum. The caudal segment or cervix consists of a single cavity that protrudes into the vaginal opening. In mice, the walls of the cervix and the vagina are continuous both dorsally and ventrally but not laterally. The lateral vaginal walls extend superiorly and form deep fornices on either side (Treuting and Dintzis, 2012).

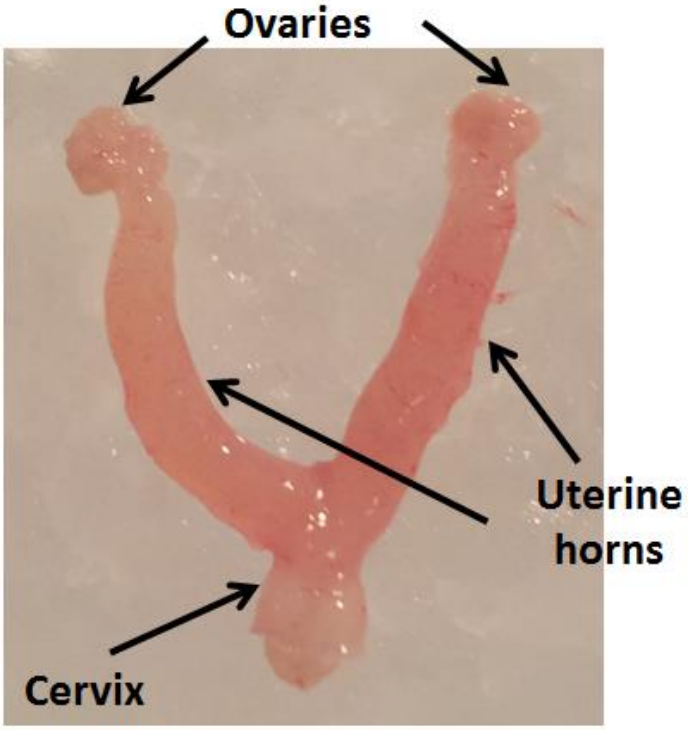



**Figure 1.1. Mouse uterus at 5 weeks of age.** Mouse uterus has two uterine horns which are connected by cervix. Ovaries are attached to each end of uterine horns via oviducts.

Histologically, mouse uterus comprises two main tissue compartments: endometrium and myometrium (Figure 1.2). Endometrium is the inner layer which mainly consists of stromal (loosely arranged reticular connective tissue with many small polyhedral cells) and epithelial cells and also contains endometrial glands, vessels and nerves. In the non-pregnant mouse uterus, the invaginating endometrium is arranged in elevated transverse folds supplied by vessels and nerves. The mucosal epithelium consists of simple columnar cells that extend into branched tubular glands within the endometrial stroma. The endometrium typically contains only 10-20 glands in a cross-section of the uterine wall and does not contain the tightly coiled and slightly branched glands

characteristic of endometria in humans (Branham et al., 1985a, Branham et al., 1985b). Myometrium is the outer layer of the uterus which mainly consists of smooth muscle fibers separated by loose, highly vascularized connective tissue, the stratum vasculosum (Treuting and Dintzis, 2012). The myometrium is composed of an inner circular layer and outer longitudinal layer of smooth muscle fibres separated by loose connective tissue, the stratum vasculosum. The outer serosa is continuous with the mesometrium.

Endometrial glands in the uterus are lined by epithelial cells (Figure 1.2). They synthesize, secrete and transport proteins and related substances that nourish the developing embryo (Bazer, 1975, Roberts and Szego, 1953, Simmen and Simmen, 1990) and these secretions are vital for the survival and development of embryos (Gray et al., 2001b). Therefore, successful morphogenesis of the endometrial glands is critical to the gestational capacity of the uterus. Furthermore, disrupted endometrial gland formation in adult mice can result in permanent infertility (Jeong et al., 2010, Franco et al., 2010). There is also evidence that endometrial glands play a role in uterine cancer as a previous study has shown that uterine cancers arise from the endometrial glands (Newbold et al., 1990).

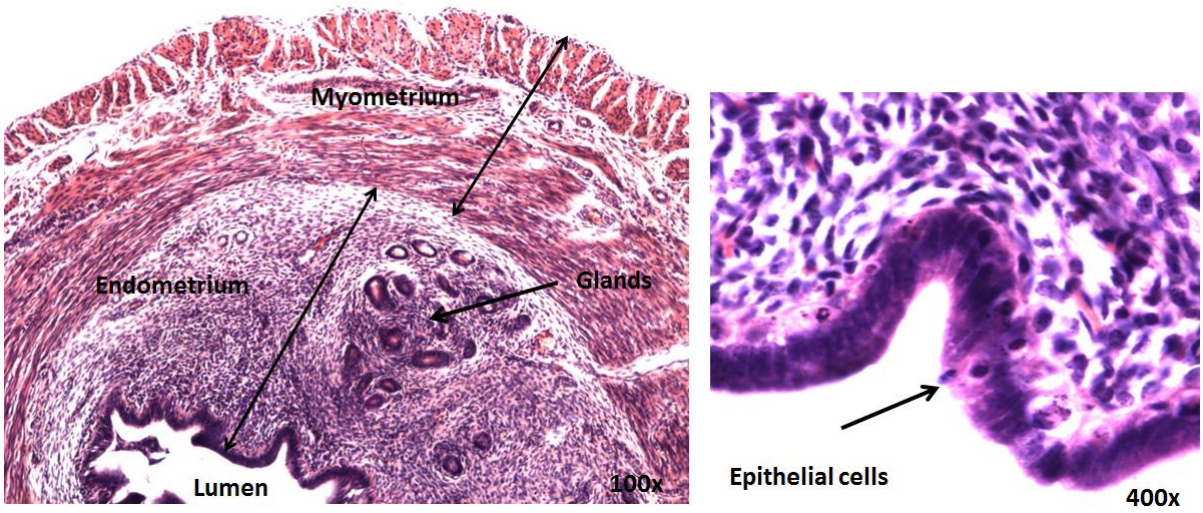



**Figure 1.2. Morphology of mouse uterus.** *Left:* mouse uterus consists of myometrium (outer layer) and endometrium (inner layer). Endometrium has endometrial glands and lumen. *Right:* endometrial glands and lumen is lined by epithelial cells.

## 1.2 Mouse as an experimental model

The mouse has been widely used to study the hormonal influence on development and tumorigenesis of different organs including uterus. Recent development of transgenic and knockout mouse models has emerged as a powerful tool to experimentally study uterine development and cancer. The greatest benefits of the mouse as an experimental model include easy availability, low maintenance cost, practicality to perform large group studies and availability of technology to genetically modify mice. Despite the great advantages, disadvantages exist as well. For developmental studies, rodent uterus develops after birth initially involving differentiation of the mesenchyme into endometrial stroma and myometrium, whereas the uterine mesenchyme in humans is already at birth radially patterned into endometrial stroma and myometrium (Gray et al., 2001a). For uterine disorder studies, mice do not shed their endometrial tissue like humans and non-human primates and therefore do not spontaneously develop certain uterine disorders (i.e. uterine cancer and endometriosis) (Grummer, 2006).

The female genital system in both the female mouse and women is very dynamic with morphologic appearance powerfully influenced by hormones during estrous/menstrual cycle, pregnancy and ovarian senescence. In particular, although the gross anatomy differs between the two species, the histologic appearance and basic functions are similar. Although both humans and mice are mammals with uteri, there are several structural and functional differences (Table 1.1).

**Table 1.1. Uterine anatomy of mouse and human. Adapted from Comparative anatomy and histology by Treuting and Dintziz 2012.**

| | Mouse | Human |
|---|---|---|
| Uterine type | Duplex | Simplex |
| Endometrium | Mucosa simple columnar epithelium that extends to branched tubular glands in myometrium | Composed of glands and specialized stroma |
| Myometrium | Inner circular and outer longitudinal smooth muscle layers | Single muscular layer |
| Cycle | Estrous cycle | Menstrual cycle |
| Cancer | Hormone dependent | Hormone dependent |

## 1.3 Hormone dependency of the uterine development

In prior to introducing hormone dependency of the uterus, terminology of estrogen and progestogen will be briefly explained. Firstly, estrogen refers to a group of female sex hormones which includes hormones such as estrone (E1) and E2. E2 is the most abundant estrogen in premenopausal women and it is also the most potent estrogen whereas E1 is the most abundant estrogen in postmenopausal women. Secondly, progestogen refers to a group of steroid hormones that binds to progesterone receptor (PR) such as P4 and progestins. P4 is the major progestogen produced in women whereas progestin is a synthetic progestogen.

Uterus is a highly hormone dependent organ where its growth is promoted by estrogens and inhibited by progestins. However, the role of ovarian hormones varies depending on the developmental stage of the uterus.

In 1973, Jost *et al* established the concept that prenatal uterine development in mammals is an ovary-independent process (Jost et al., 1973) as ovariectomy at birth did not affect uterine development. Similarly, early postnatal events in rodent uterine development and endometrial gland development are both ovary-independent (Clark and Gorski, 1970) and adrenal-independent processes (Ogasawara et al., 1983, Branham and Sheehan, 1995). In contrast, in rodents, from postnatal day 10 to 14, uterine growth becomes ovary-dependent as ovaries develop and start producing estrogens (Branham and Sheehan, 1995). The change is thought to be mediated by the secretion of systemic estrogens beginning at this age (Dohler and Wuttke, 1975).

Early studies have shown the estrogen-induced growth of the rodent uterus (Roberts and Szego, 1953, Mueller et al., 1958, Mueller, 1971). In 1937, it was demonstrated that the mitotic activity first became evident in the uterine epithelial cells of ovariectomised mouse about 13.5 hours after the subcutaneous administration of estrone (Allen, 1937). The mitotic activity resulted in hyperplasia and hypertrophy of uterine muscular components (Worthington and Allen, 1939). After treatment with E2, uterine weight gradually increased with time with substantial increase in the dry weight and cell proliferation (Astwood, 1938, Cole, 1950, Roberts and Szego, 1953). Furthermore, estrogens stimulate proliferation of both epithelial and stromal cells in neonatal mice and this proliferative activity appears to be confined to the epithelial compartment in the adult mouse (Martin et al., 1973b, Quarmby and Korach, 1984). Mice with low levels of estrogens due to the absence of aromatase enzyme (Cyp19a1), have underdeveloped external genitalia and uteri at nine weeks of age further supporting the growth promoting role of estrogens in uterus (Fisher et al., 1998).

The uterus undergoes continuous cycle of proliferation and differentiation in response to the rise and fall of E2 and P4 during the estrous cycle (Hall, 1969, Marcus, 1974) resulting in the morphological changes of the mouse endometrium depending on the stage of the estrous cycle. Estrogens act via estrogen receptors (ER)s of which there are two types: ERα and ERβ. In the rat uterus, overall patterns of ERα and ERβ expression were very similar with both receptors present in the luminal epithelium, glandular epithelium and stroma (Saunders et al., 1997, Wang et al., 1999). However, ERβ mRNA and protein levels are much lower than those of ERα (Kuiper et al., 1997, Wang et al., 1999,

Saunders et al., 1997, Matsuzaki et al., 1999). Uterine ERα expression is regulated by other steroid hormones such as estrogens, P4 and androgens (Rochefort et al., 1972, Wang et al., 1999, Bergman et al., 1992, Wang et al., 2000).

The growth promoting role of estrogens through ER has been shown using ER knockout mouse models. Complete ERα knockout mice (ERαKO) have hypoplastic uteri that contain all characteristic cell types but in reduced proportions (Lubahn et al., 1993), which suggest that ERα-mediated actions are not essential for organogenic development and differentiation of the uterine histogenesis (Hewitt et al., 2010), but essential for normal peripubertal uterine growth and development in mice (Lubahn et al., 1993). Hence, this also suggests that estrogen action via ERα is required for normal peripubertal development and androgens alone cannot support normal peripubertal uterine development. On the other hand, complete ERβ knockout mice (ERβKO) mice have no defects in female reproductive tract differentiation or fertility (Krege et al., 1998), suggesting that estrogen action via ERβ is not essential for normal uterine growth and development in mice.

In contrast, P4 is known to inhibit estrogen-induced hyperplasia of the luminal and glandular epithelial compartments (Martin et al., 1973a). As a result, P4 is often administered clinically to remove the hyperplastic endometrial effects of unopposed estrogen given for postmenopausal hormonal replacement therapy (Persson, 1996). Despite growth inhibiting role of P4, functional differentiation of the uterus after puberty requires P4 (Spencer et al., 2004). This is supported by findings that treatment of neonatal mice with P4 after birth for 5 days delayed formation of uterine glands before puberty (at 3 weeks) but increased

gland formation after puberty (at 8 weeks) (Stewart et al., 2011). P4 acts via progesterone receptor (PR) which is expressed throughout uterus including luminal and glandular epithelium, endometrial stroma and myometrium (Kurita et al., 2000). In mouse uterus, PR is regulated by E2 via ER (Kurita et al., 2000, Tibbetts et al., 1998, Murakami et al., 1990).

The role of androgens in uterine development will be discussed in a separate section 1.5 as it is of the major interest of this thesis.

## 1.4 Androgens and AR

### 1.4.1 Androgens

Androgens are a group of 19-carbon steroid hormones produced mainly in the testes and to lesser extent ovaries, adrenal glands and placenta but also in peripheral tissues by conversion of precursors. In males, androgens are essential for the induction of male secondary sexual characteristics. While estrogens (18-carbon steroid hormones) are the main female hormones, there are higher concentrations of androgens in circulation than estrogens. Androgens circulate from nanomolar to micromolar range, in contrast to the picomolar range of circulating estrogens because of the two order of magnitude potency difference (Somboonporn and Davis, 2004). The major androgens are testosterone and its 5α-reduced more potent metabolite 5α-dihydrotestosterone (DHT). Androgens mediate their effects via the AR present in target tissues (Somboonporn and Davis, 2004). Using global AR knockout (ARKO) female mouse model generated by Cre/loxP system (natural breeding is not possible as explained in section 1.4.3) it was recently confirmed that androgens can also act directly via AR throughout the female body and exhibit important physiological effects (Walters and Simanainen, 2010). In addition, androgens are indirectly important in females as testosterone is the precursor hormone for E2 biosynthesis in the ovaries and other tissues (Simpson et al., 2000).

In addition to the roles of the androgens in sexual function and fertility, androgens also have important roles in prostate disorders, cardiovascular risk and body composition, bone metabolism, testicular cancer, psychiatric status, neurodegenerative disorders. How androgens action in these roles and its complexity in its mode of action have recently been reviewed in depth (Tirabassi et al., 2015)

### 1.4.2 Androgen biosynthesis

In women androgens are biosynthesized in the ovary and in the adrenal gland, but in mice the adrenal androgen production is minimal (van Weerden et al., 1992) (Figure 1.3). The biosynthesis of androgens is modulated by two cytochrome P450 (CYP) enzymes. Cholesterol side-chain cleavage enzyme (P450Scc) catalyzes cholesterol side-chain cleavage and P450 C17 catalyzes 17-hydroxylation and 17-20 bond cleavage (17/20 lyase), which is required for the production of dehydroepiandrosterone (DHEA) and androstenedione from pregnenolone and P4, respectively. DHEA and androstenedione are proandrogens because they need to be converted into testosterone to exhibit androgenic effects (Somboonporn and Davis, 2004). Circulating testosterone can be converted to DHT by 5α-reductase (type 1 and 2) and to E2 by the aromatase enzyme. Hence, the regulation of 5α-reductase and aromatase enzyme controls DHT or E2 levels.

While 5α-reductase type 1 is expressed in mouse uterus (Mahendroo et al., 1997), clinical studies have shown contradictory results on aromatase activity in human uterus. Endometrium is unable to convert androstenedione to estrone

by aromatase activity (Baxendale et al., 1981) whereas another study showed aromatization of testosterone in human endometrium (Tseng et al., 1982), demonstrating differences possibly due to species in uterine aromatase activity. More recent studies have suggested that normal uterine tissues do not have aromatase activity, whereas pathological uterine tissues display aberrant aromatase activity (Noble et al., 1997, Bulun et al., 1994).

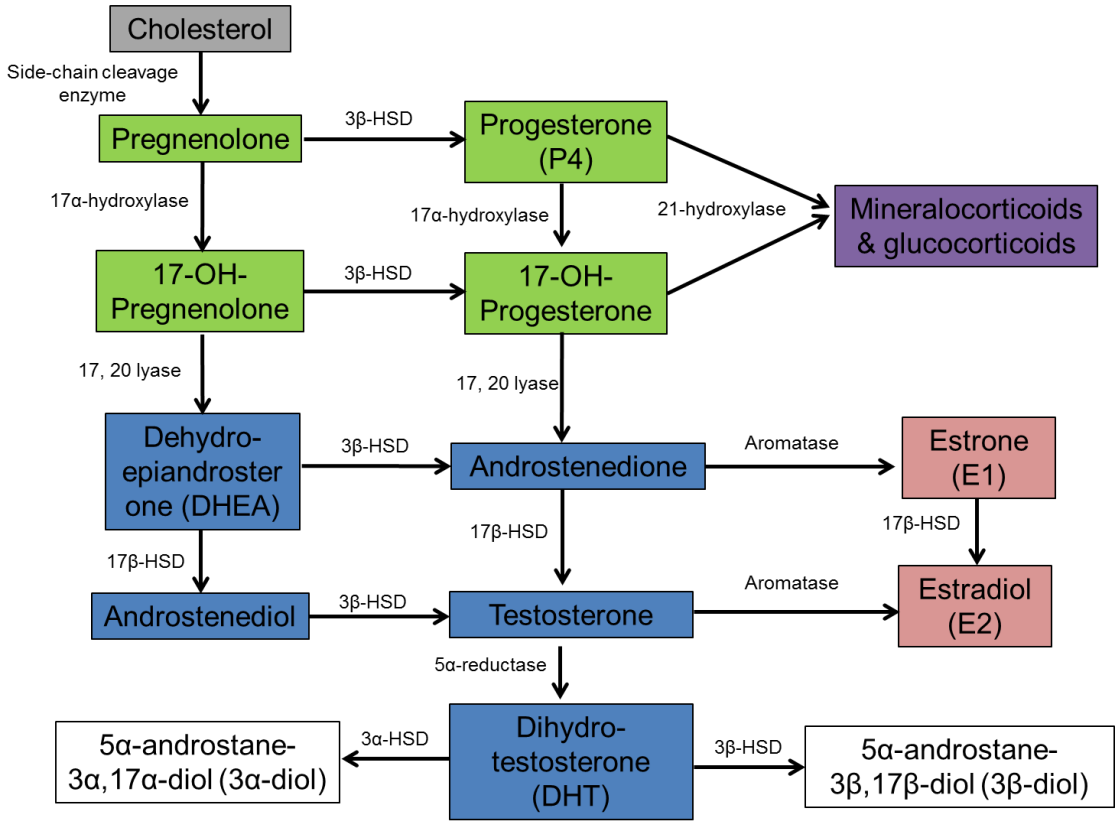



**Figure 1.3. Biosynthesis and metabolism of androgens.** Green boxes represent progestogens, blue boxes represent androgen and proandrogens and pink boxes represent estrogens.

### 1.4.3 Androgen receptor (AR)

AR is a member of the steroid/thyroid/retinoic acid receptor superfamily. Members of this superfamily, including AR, contain several functional domains encoded by 8 exons: exon 1 encodes the large N-terminal, exons 2 and 3 separately encodes the DNA-binding domain consisting of two zinc fingers, and exons 4-8 encodes the information for the ligand-binding domain (Figure 1.4) (Chang et al., 1988, Trapman et al., 1988). AR exists in a non-DNA-binding state as an inactive complex with heat shock proteins in the absence of androgens. The AR has higher affinity for DHT than for testosterone (Nicolas Diaz-Chico et al., 2007).

The *Ar* gene is only located on the X chromosome with no relation to the Y allele. Hence, in males it functions solely as a single-copy gene, as shown by the complete loss of androgen effect in XY (male) hemizygous individuals with an inactivating mutations of the *Ar* (Avila et al., 2001, Chang et al., 1995). This is evident in ARKO male mice where they display female external genitalia, typical for complete androgen insensitivity syndrome (cAIS) phenotype in men and male mice (Notini et al., 2005b, Quigley et al., 1995). Men and male mice with cAIS phenotype are infertile due to loss of spermatogenesis; hence, ARKO females cannot be produced by natural breeding.

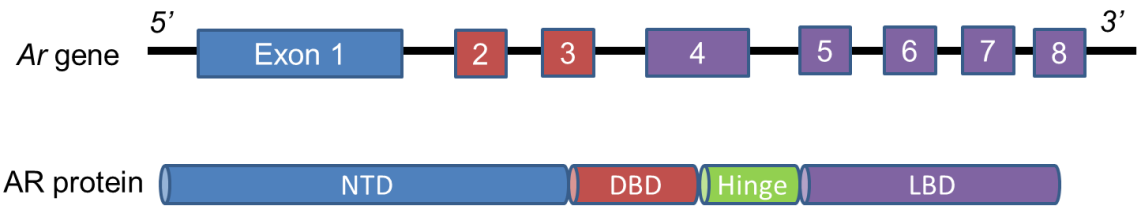



**Figure 1.4. Schematic structure of human *Ar* gene and protein.** Top, The *Ar* gene: contains eight coding exons. Bottom, AR protein: the N-terminal domain (NTD), the DNA-binding domain (DBD), which contains a pair of zinc finger motifs; the hinge region and the ligand-binding domain (LBD).

### 1.4.4 AR signaling pathway

In the absence of androgens, AR is located in cytoplasm and exists in a non-DNA-binding state bound to heat shock protein as chaperones (Figure 1.5). When androgens bind to AR, heat shock protein disassociates and AR dimerises in an anti-parallel configuration to reveal its DNA-binding domain and translocates to nucleus. In the nucleus, AR binds to the androgen responsive element (ARE) of the regulated gene with two zinc fingers and thereby activates specific gene transcription (Figure 1.5) (Carson-Jurica et al., 1990). In addition, it is suggested that AR transactivation could also be induced by growth factors such as epidermal growth factor, insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (Culig et al., 1994) and cytokines like interleukin-6 (IL-6) in a ligand independent manner (Hobisch et al., 1998).

Furthermore, non-genomic signalling of androgens has been suggested which occurs through cell-surface receptors. *In vitro* studies have shown that androgens can rapidly induce second messengers such as kinase signalling

cascades and these effects have been observed in the presence of transcription and translation inhibitors (Kousteni et al., 2001, Heinlein and Chang, 2002b). These rapid changes are regarded as non-genomic actions but still require binding of androgens to the cell surface receptor. However, full molecular characterization of this mechanism has yet to be reported.

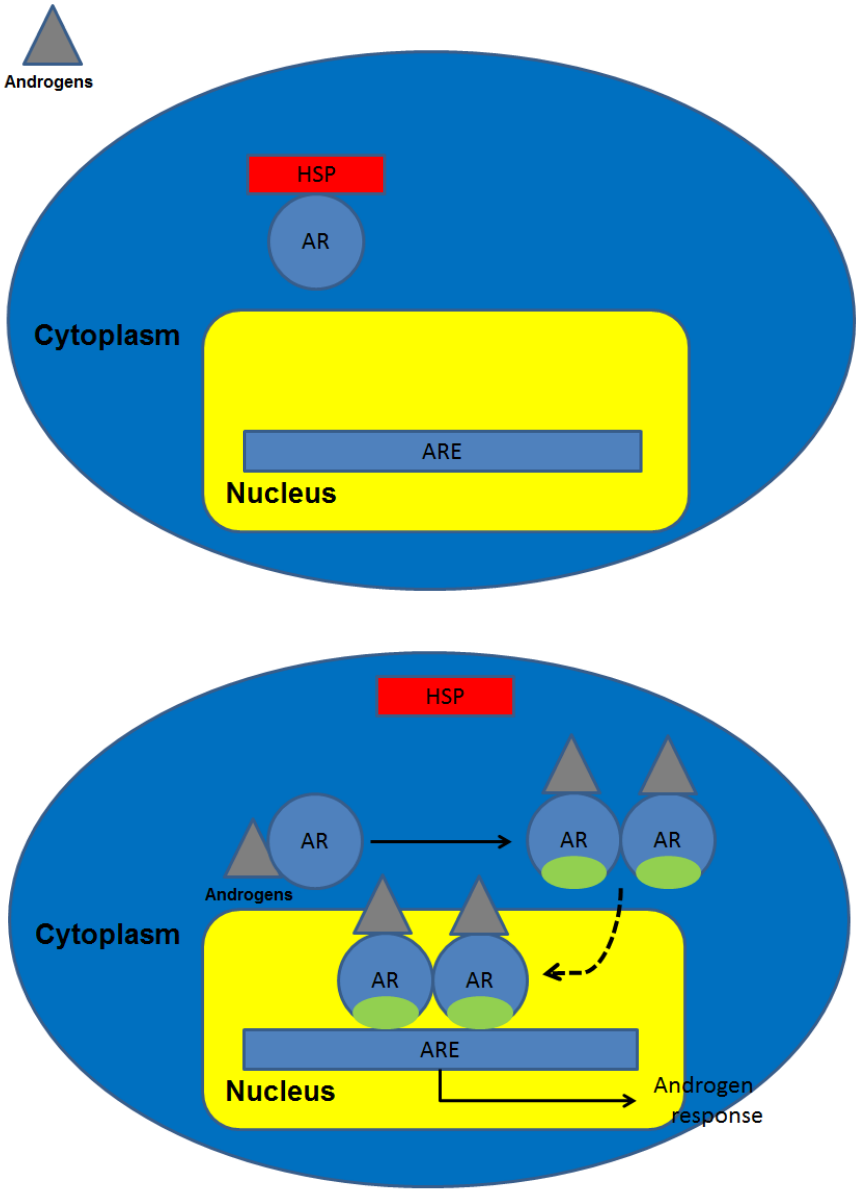



**Figure 1.5. AR signaling pathway.** *Top:* inactivate AR signaling pathway. *Bottom:* active AR signaling pathway. AR=androgen receptor, HSP=heat shock protein, ARE=androgen responsive element, green ovals=DNA-binding domain.

### 1.4.5 AR Coregulators

The functional properties of AR (via transcriptional activity of AR) can be regulated by AR coregulators (Heinlein and Chang, 2002a, Heemers and Tindall, 2007). By a definition, AR coregulators are proteins that are recruited by AR and either act as coactivators or corepressors of transcription activity. But AR coregulators do not alter the basal transcription rate and do not typically possess DNA binding ability. Instead, coregulators influence AR-mediated transcription by acting at the target gene promoter region to facilitate DNA occupancy, chromatin remodelling, and/ or recruitment of general transcription factors associated with RNA polymerase II, or by assuring the competency of the AR to enhance gene expression directly (Heemers and Tindall, 2007). There are more than 200 nuclear receptor coregulators identified. In-depth reviews on AR coregulators have been published by Heinlein and Chang (Heinlein and Chang, 2002a) and Heemers and Tindall (Heemers and Tindall, 2007). Androgen-responsive tissues like prostate and breast rely on AR-coregulators as suggested by studies in various mouse models and cell lines (reviewed in (Heemers and Tindall, 2007, Heinlein and Chang, 2002a)).

There are coregulators reported to either positively or negatively modulate or modify ligand-dependent transcription by nuclear receptors (reviewed in (Heemers and Tindall, 2007)). Some AR coregulators are specific to AR alone but most other coregulators have broader range of interaction and coregulation with other nuclear receptors (reviewed in (Heemers and Tindall, 2007)). For example, ARA70 was initially regarded as AR specific coregulators but has since been shown to modulate transcription by several other nuclear receptors

such as ER, PR and glucocorticoid receptor (GR) (Alen et al., 1999, Yeh and Chang, 1996).

## 1.5 Androgens, AR and uterus

There is increasing evidence that androgens and AR play a vital role in uterine physiology. This section discusses the expression, regulation and role of androgens in uterus.

### 1.5.1 AR expression in the uterus

AR is expressed in the uterus both in rodents (Pelletier et al., 2004, Hirai et al., 1994) and humans (Kimura et al., 1993, Ito et al., 2002) in a similar manner. AR protein was detected in the epithelial and stromal cells in endometrium and myometrial cells of the human uterus (Ito et al., 2002, Kimura et al., 1993). Similarly in the mouse uterus, AR mRNA was expressed in the luminal and glandular epithelial cells, stromal cells and smooth muscle cells (Pelletier et al., 2004) and the intensity of AR mRNA labelling is quite uniform throughout the different uterine compartments (Figure 1.6).

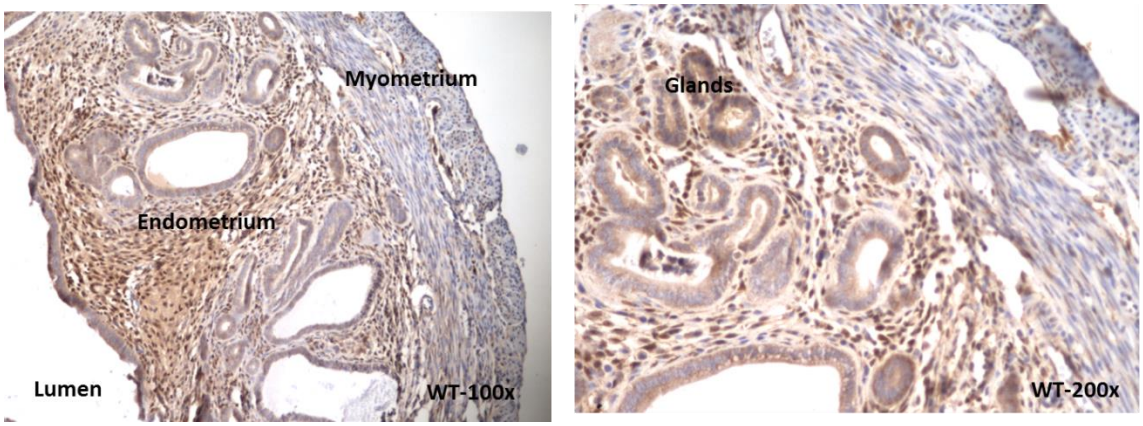



**Figure 1.6. AR immunopositivity in adult mouse uterus.** AR immunopositivity (brown staining) is abundant throughout different compartments of uterus including glandular and luminal epithelial cells, stroma and myometrium at diestrus stage (Choi, unpublished).

## 1.5.2 Regulation of AR expression in the uterus

Expression and localization of AR is highly regulated by E2 as ovariectomy significantly reduces uterine *Ar* mRNA expression (18% in the epithelial cells, 23% in the stromal cells and 50% in the myometrial cells compared to WT controls) and the expression was completely restored after E2 administration (Pelletier et al., 2004). In addition to E2, AR expression is also regulated by P4 with P4 treatment in women and rhesus macaques reducing uterine AR expression that was reversed by P4 antagonists (RU 486) (Slayden et al., 2001). Furthermore, RU 486 treatment caused striking up-regulation of glandular epithelial AR staining and enhanced stromal AR signaling (Slayden et al., 2001). Due to the hormonal regulation, AR expression also varies depending on the estrous cycle. In human uterus, AR was immunolocalized predominately in stromal cells during the proliferative phase and in epithelial cells during the secretory phase (Ito et

al., 2002). AR expression is not detectable during the late secretory phase (Mertens et al., 2001). In addition, AR expression may be regulated by androgens in an auto-regulation manner as shown in the rat prostate where castration reduced AR expression (Prins and Birch, 1993).

### 1.5.3 Androgen effects on uterine growth

Androgens may have growth promoting role in the rodent uterus as shown in previous studies (Armstrong and Papkoff, 1976, Schmidt and Katzenellenbogen, 1979, Nantermet et al., 2005, Walters et al., 2009). In 1976, Armstrong and Papkoff demonstrated that the administration of androgens (testosterone or DHT) for 3 days in immature hypophysectomised rats at three weeks old caused marked increase in uterine weights (Armstrong and Papkoff, 1976) suggesting the growth promoting role of androgens in uterus. This was further supported when DHT treated immature rats showed increased uterine growth which was reversed by antiandrogen treatment (flutamide) (Schmidt and Katzenellenbogen, 1979).

The growth promoting effect of androgens was suggested to involve interaction of androgens with both the AR and ER in the uterus. This was supported as high dose of DHT (5 or 10mg) causes nuclear translocation and cytoplasmic depletion of the ER (Schmidt and Katzenellenbogen, 1979). In addition, a more recent study suggested that in E2 induced epithelial cell proliferation, ERα induces stromal AR and AR in reverse amplifies the ERα signal by induction of IGF-1 (Weihua et al., 2002) an effect that is blocked by AR antagonist (bicalutamide) (Nantermet et al., 2005). This indicates an important proliferative

role of androgens acting via AR in uterus where estrogens and androgens may act via one pathway in sequential steps (Weihua et al., 2002). Furthermore, it has been suggested that the non-aromatizable androgen, DHT, requires ERα to promote uterine growth as DHT could not induce uterine growth in ERαKO mice (Nantermet et al., 2005). However, DHT does not appear to directly activate ERα as multiple ERα dependent genes were not induced by DHT. The growth promoting role of androgens in uterus was further supported by global AR knockout (ARKO) transgenic mouse model. The ARKO female mice have thinner uterine wall and reduced total uterine area compared to the controls (Walters et al., 2009), suggesting the growth promoting or morphogenesis role of androgens in uterus is via AR. Hence, these *in vivo* studies collectively build evidence to suggest a growth promoting AR dependent role of androgens in uterus.

However, an *in vitro* study suggested a local, uterine specific AR mediated inhibitory role of aromatizable pro-androgen, androstenedione on the proliferation of human endometrial epithelial cells. The effect was mediated via AR as it was reversed by antiandrogen (cyproterone acetate) (Tuckerman et al., 2000). Therefore, the role of androgens on uterine growth still remains contradictory from *in vivo* and *in vitro* studies; hence, part of this PhD project aimed to clarify the contradicting findings in the past by confirming the *in vivo* role of AR mediated androgen actions in uterine growth. In addition it was intended to determine the extrauterine and intrauterine androgen effects as well as cell specific role of AR in uterus as AR is expressed in all the uterine cells.

## 1.6 Uterine cancer

### 1.6.1 Uterine cancer statistics

Globally, uterine (endometrial) cancer is the seventh most common malignant disorder and most commonly diagnosed gynaecological cancer (Parkin et al., 1999). Uterine cancer incidence varies among regions with less developed countries (i.e., African countries) having low endometrial cancer incidence but higher specific mortality when compared to developed countries (i.e., European countries) (Hill et al., 1996, Madison et al., 2004). In 2010, uterine cancer was the second most common cause of gynaecological cancer death in Australia, accounting for 2% of all cancer deaths in women (Welfare, 2012a) and around 4% of all new cancers in Australian women (Welfare, 2012a). The time of uterine cancer diagnosis is at 64.1 years of age with the risk of developing uterine cancer before the age of 85 years in women was 1 in 48 (Welfare, 2012a). The incidence of uterine cancer has increased between 1982 and 2009 in Australia. The age-standardised incidence increased from 13.8 to 17.1 cases per 100,000 women (Welfare, 2012a). However, the age-standardised mortality rate of uterine cancer in women decreased from 3.3 to 2.7 deaths per 100,000 women between 1982 and 2010 (Welfare, 2012a), likely due to the improved diagnosis and treatment. The relative survival rates for uterine cancer have increased in recent years in Australia. Five-year relative survival rate was 82% between 2006 and 2010 which has been steady in previous reports (Welfare, 2012a). Therefore, uterine cancer remains a fatal and frequent cancer among women in developed countries and warrants further investigation into uterine cancer prevention and treatment.

Despite the improving mortality rate and high five-year survival rate, uterine cancer is estimated to be the leading cause of the burden of disease in Australia (Welfare, 2012a) as removal of the uterus as a treatment results in females becoming infertile. In 2012, uterine cancer was estimated to account for 5,300 disability-adjusted life years (DALYs) in Australia; of these, 3,700 were years lost due to premature death and 1,600 were years of health life lost due to disease, disability or injury (Welfare, 2012b).

### 1.6.2 Hormonal risk factors for uterine cancer

Although the mechanisms still remain unknown, endogenous hormones appear to play an important role in the development of uterine cancer. Increased uterine cancer risk has been associated with early menarche and late menopause suggesting a relationship with greater lifetime estrogen exposure at premenopausal levels (Kaaks et al., 2002). Other factors related to hormone exposure include nulliparity (exposure to P4 in pregnancy), long term use of tamoxifen as a breast cancer treatment (tamoxifen acts as estrogen agonist in uterus), hormone replacement therapy (progestagens) and years of menstruation (estrogen exposure) (Kaaks et al., 2002, Weiss and Hill, 1996, Emons et al., 2000, Rose, 1996). Furthermore, plasma concentrations of increased estrogens and androgens and decreased P4 are known to increase risk of uterine cancer (Potischman et al., 1996).

The most popular theory describing the relationship between endogenous steroid hormones and endometrial cancer risk is known as the *"unopposed estrogen hypothesis"* (Siiteri, 1978, Key and Pike, 1988). This hypothesis

proposes that endometrial cancer risk increased in women with high plasma estrogens and/or low plasma P4, so that overall mitogenic effects are increased causing proliferation. This theory was proposed based on two important observations:

i) *Increased endometrial proliferation rates during the follicular phase of the menstrual cycle, during which progesterone levels are low, whereas E2 levels are at normal premenopausal concentration* (Key and Pike, 1988, Ferenczy et al., 1979).

ii) *Increased endometrial cancer risk among women using exogenous estrogens without progestins* (1999).

In addition, to classical ovarian hormones, recent studies have suggested androgens and AR play a vital role in uterine cancer (Ito et al., 2002, Rodriguez et al., 2006). The role of androgens has been associated with 5-fold increased risk of uterine cancer in patients with polycystic ovarian syndrome (PCOS) (Kaaks et al., 2002). The PCOS is a metabolic and endocrine alteration that results in hyperandrogenism. However, the excessive androgens that are produced can be converted to estrogens to produce an estrogen enriched environment and therefore the specific role of androgens is not clear (further discussed in section 1.9).

On the other hand, factors that are suggested to reduce uterine cancer risk include multiparity (increase P4 exposure due to pregnancy) (Hinkula et al., 2002), oral-contraceptives (P4 based) (Deligeoroglou et al., 2003), smoking (decreases estrogen sensitivity) (Viswanathan et al., 2005), physical activity (Schouten et al., 2004) and phyto-estrogens reduced diet (Unfer et al., 2004).

The relevant factors that regulate the risk of uterine cancer have been summarised in table 1.2.

Table 1.2. Factors regulating the risk of uterine cancer (adapted from Amant *et al*, 2005).

| **Factors increasing risk** | |
|---|---|
| Increasing age | History of breast cancer |
| Long-term exposure to unopposed estrogen | High concentrations of estrogens postmenopausal |
| Residence in North America or Europe | Long-term use of tamoxifen |
| Nulliparity | Genetic disorders (i.e. PTEN mutation) |
| First-degree relative with endometrial cancer | Hormone-replacement therapy with less than 12-14 days of progestogens |
| Polycystic ovarian syndrome (PCOS) | Years of menstruation |
| **Factors decreasing risk** | |
| Multiparity | Physical activity |
| Oral-contraceptive use | Reduced diet of some phyto-estrogens (*i.e.* soy) |
| Smoking | |

These hormonal risk factors mentioned above were supported by experimental studies (Takasugi et al., 1962, Rustia and Schenken, 1976, Rustia, 1979, Troisi et al., 2007). The protective effect of P4 on uterine cancer has been shown using progestins (synthetic analogs of P4) (Mortel et al., 1990). The human endometrial carcinoma transplants treated with high doses (2 and 5mg) of progestin resulted in the better control of uterine cancer growth (Mortel et al., 1990). The protective role of P4 in uterine cancer was further supported using PR knockout mouse (PRKO) model (Kurita et al., 1998, Mulac-Jericevic et al., 2000). P4 treatment significantly decreased uterine size in mice with endometriosis, while PRKO females did not respond to P4 treatment,

demonstrating that the effect of P4 was PR mediated. In addition, the E2 treatment induced growth of uterus was suppressed by P4 treatment in WT but not in PRKO females (Fang et al., 2004). This finding suggests P4 actions through PR are responsible for opposing E2 dependent uterine growth. However, as the model has PR inactivation not only in the uterus but also in other organs such as ovary and pituitary (Lydon et al., 1995), the study cannot conclude whether the protective role of P4 is via intra-uterine or extra-uterine PR, however, this was confirmed in another study (Kurita et al., 1998).

### 1.6.3 Prevention and treatment of uterine cancer

As described above, uterine cancer is highly hormone dependent cancer and accordingly hormonal prevention and treatment methods have been widely implemented. As mentioned previously (Section 1.3), P4 is known to inhibit estrogen-induced hyperplasia of the luminal and glandular epithelial compartments (Martin et al., 1973a). Hence, progestins have been widely used to treat uterine cancer. However, not all uterine cancers respond well to progestin treatments, this is mainly due to the variable PR expressions in uterine cancers. Some studies have reported ineffectiveness of progestin treatments in uterine cancer (reviewed in (Banno et al., 2012)) and also PTEN (phosphatase and tensin homolog) inactivation induced uterine cancers in mice are nonresponsive to progestin treatments (Fyles et al., 2008). On a positive note, there are other treatments used for uterine cancers which include use of anti-estrogens (i.e. tamoxifen), aromatase inhibitors (i.e. letrozole) and

gonadotropin-releasing hormone agonists (i.e. leuprolide), and also surgery (total hysterectomy) and radiotherapy (Bender et al., 2011, Amant et al., 2005).

## 1.7 PTEN and uterine cancer

In addition to hormonal factors, genetic factors such as first-degree relative with endometrial cancer and PTEN mutations may increase the risk of uterine cancer (Risinger et al., 1997). Deletions and mutations in *Pten* gene are found in a high frequency in many sporadic human cancers, including glioblastomas, as well as endometrial, prostate, and breast cancers (Dahia, 2000).

### 1.7.1 PTEN pathway

The PTEN protein consists of a 179-residue N-terminal domain (residues 7-185) and a 166-residue C-terminal domain (residues 186-351). The N-terminal domain contains the protein tyrosine phosphatase (PTP) signature motif and the large phosphatase active site in PTEN allows accommodation of a phosphotidylinositol-3,4,5-triphosphate ($PIP_3$) substrate. (Lee et al., 1999).

PTEN functions as a phosphatase with both lipid and protein phosphatase activities (Maehama and Dixon, 1998, Myers et al., 1998, Li and Sun, 1997). As a lipid phosphatase, PTEN dephosphorylates $PIP_3$, a product of phosphotidylinosital-3-kinase (PI3K) (Maehama and Dixon, 1998, Stambolic et al., 1998, Sun et al., 1999). By dephosphorylating $PIP_3$, PTEN inhibits the growth factor signals transduced through PI3K by inhibiting the AKT phosphorylation (Figure 1.7). PTEN deficiency leads to accumulation of $PIP_3$ that in turn activates several signaling molecules including the phosphotidylinositol-dependent kinases (PDKs), the serine/threonine kinases AKT/PKB, S6 kinase, and mTOR, as well as small GTPases Rac1 and Cdc42 (Anderson et al., 1998, Liliental et al., 2000, Pene et al., 2002, Stiles et al., 2002, Sun et al., 1999, Wu et al., 1998).

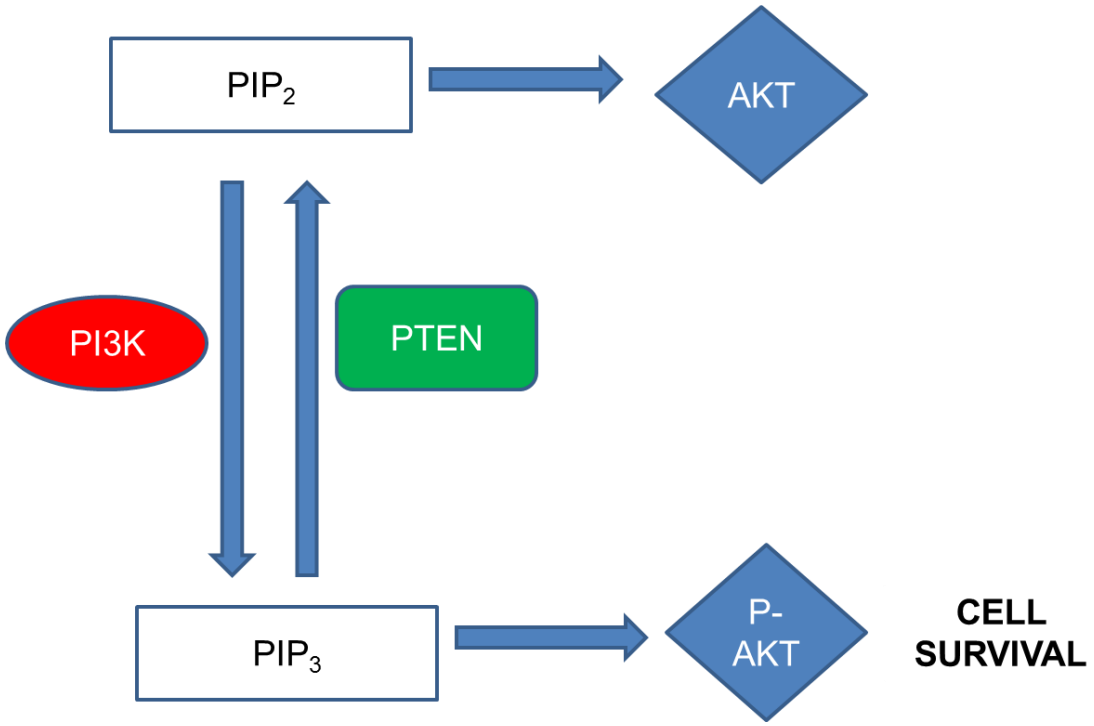



**Figure 1.7. Influence of PTEN and PI3K in AKT pathway.** PI3K phosphorylates $PIP_2$ to $PIP_3$ which promotes cell survival by phosphorylating AKT (P-AKT). Whereas, PTEN opposes PI3K pathway by dephosphorylating $PIP_3$ to $PIP_2$, hence AKT remains unphosphorylated.

## 1.7.2 Function and biological role of PTEN

PTEN has a broad impact on cell growth, cell migration, cell death, and cell differentiation, processes involved in normal development (reviewed in (Stiles et al., 2004)). An important role during development is demonstrated in PTEN knockout mouse models where the homozygous PTEN knockout is embryo lethal with fetal mice dying between gestation day 6.5-9.5 (Suzuki et al., 1998, Podsypanina et al., 1999). Mice with heterozygous PTEN inactivation do

survive but develop various cancers including uterine cancer during their lifetime (Stambolic et al., 2000). Therefore, PTEN is identified as a tumor suppressor gene which inhibits cell growth/survival (Liscovitch and Cantley, 1994, Vanhaesebroeck et al., 1997) and cell migration/adhesion (Tamura et al., 1998) through its lipid and protein phosphatase activities, respectively. PTEN induces growth suppression by at least two mechanisms. Firstly, it promotes cell-cycle arrest at the G1 phase and also increases apoptosis induced by multiple distinct stimuli. As an example, in the presence of ectopic expression of PTEN, glioblastoma and renal carcinoma cells arrest at G1 phase (Furnari et al., 1998, Ramaswamy et al., 1999). This function of PTEN is dependent on P-AKT, where P-AKT rescued the cell line from PTEN-induced G1 arrest (Li and Sun, 1998, Ramaswamy et al., 1999). PTEN also have ability to suppress growth and tumor progression by increasing cellular apoptotic rates (Li and Sun, 1998, Stambolic et al., 1998). PTEN inactivation in mice was shown to reduce the sensitivity to apoptosis induced by UV, heat shock and radiation, while the introduction of PTEN to these mice restored the apoptotic sensitivity (Stambolic et al., 1998).

### 1.7.3 PTEN and estrogen sensitivity

While the PTEN deletion and estrogen actions via ER are both associated with the development of uterine cancer, a relationship between PTEN and estrogen sensitivity has also been reported. A physiological and functional link between loss of PTEN and increase in ER activity was suggested by the development of estrogen-related neoplastic lesions in the non-hyperestrogenic mouse models

with PTEN inactivation (Vilgelm et al., 2006). Supporting this, several *in vitro* studies have further suggested the existence of co-operation between PTEN and ERα through P-AKT. It was suggested that activation of P-AKT pathway by loss of PTEN also activates ERα (Campbell et al., 2001, Sun et al., 2001). Similarly, in MCF-7 breast cancer cells the activation of PI3K/ AKT pathway increases ERα transcriptional activity (Stoica et al., 2003), these findings also supported by *in vivo* studies (Lian et al., 2006, Vilgelm et al., 2006). Increased activation of both P-AKT and ERα is required for uterine cancer development in heterozygous PTEN inactivated mouse models. Conversely, induction of PTEN resulted in reduction of P-AKT and parallel decrease in the ERα activation resulting in reduced uterine cancer in mice (Vilgelm et al., 2006). Furthermore, increased ERα activity following PTEN inactivation is supported by findings that heterozygous PTEN inactivation altered the known ERα regulated genes in the uterus, mimicking hyperestrogenic environment (Lian et al., 2006).

### 1.7.4 PTEN and AR interaction

The possible interaction of AR and PTEN has been established in hormone dependent cancers like breast and prostate cancers (Wang et al., 2011b). Recent *in vivo* and *in vitro* studies have shown that AR and PTEN can cross-regulate each other by reciprocal feedback (Carver et al., 2011, Mulholland et al., 2011).

In prostate cancer cells, depending on the growth stages of cell lines, AR transactivation was suppressed or not effected by PTEN via PI3K/AKT pathways (Lin et al., 2001, Mulholland et al., 2011, Li et al., 2001, Nan et al.,

2003, Lin et al., 2004). Suppression of AR transactivation was supported by finding that PTEN loss in the prostate epithelium suppressed androgen-responsive gene expression *in vivo* (Mulholland et al., 2011). Furthermore, PTEN was demonstrated to repress AR nuclear translocation and induce AR degradation in prostate cancer cells (Lin et al., 2004, Lin et al., 2002). PTEN can bind to the hinge region of AR resulting in the interruption of the nuclear localization signal (NLS) and thereby inhibiting nuclear translocation of AR (Lin et al., 2004). Alternatively, PTEN could also compete with other AR coregulators for the same binding region, inhibiting nuclear translocation (Lin et al., 2004). Moreover, it has been reported that the PI3K/AKT pathway promotes AR ubiquitination, leading to AR degradation (Lin et al., 2002). This suggests that both PTEN and the PI3K/AKT pathway can promote AR degradation. While the interaction has been demonstrated mainly in prostate cancers and to some extent in breast cancers, similar PTEN mediated inhibition of AR could occur in other PTEN-AR target tissues such as the uterus. As PTEN can regulate AR in prostate cancers, it strongly suggests the PTEN and AR interaction; however, in reverse whether AR can regulate PTEN has not been studied in depth. Although a recent study suggests the reverse interaction where AR regulates PTEN transcription and translation in breast cancer cells (Wang et al., 2011a). The study suggested that AR upregulates PTEN transcription in breast cancer cells (Wang et al., 2011a) and AR and PTEN may interact in certain apocrine type breast cancers (Banneau et al., 2010). However, there have been no previous reports suggesting the interaction in uterine cancer and our current study aims to investigate the role of AR in PTEN regulation in uterine cancers.

### 1.7.5 PTEN mutations

PTEN is frequently inactivated by somatic mutations in a wide array of human tumors including uterine cancer (Chow and Baker, 2006). In addition, germline mutation of one allele of PTEN results in hereditary cancer predisposition causing Cowden syndrome (CS) (Liaw et al., 1997). PTEN mutations in CS tend to cluster at, but are not limited to, exon 5, which encodes the phosphatase signature motif. CS is an autosomal dominant disorder which comprises multiple hematomas in tissues derived from the three major embryological layers, and is associated with a higher risk of endometrial cancer, breast cancer and thyroid cancer (25-50% of affected females), and in a subset of cases, a dysplatic gnaglyocytoma of the cerebellum, Lhermitte-Duclos disease, might also develop (Dahia, 2000).

Previous studies have shown that germline PTEN mutations are observed in 30-80% of type I endometrial carcinomas (EMCs) and in ~20% of complex atypical hyperplasia where complex atypical hyperplasia is a precursor stage to type I EMC (Risinger et al., 1998, Tashiro et al., 1997).

### 1.7.6 PTEN knockout mouse as uterine cancer model

Mice do not naturally develop uterine cancer; however, such cancers are only observed by using genetically modified mouse models (i.e. *Pten* inactivation) and/or hormone treatments (i.e. estrogens). In the context of genetically modified mouse models, PTEN knockout mouse models have been widely used

as these models develop atypical endometrial hyperplasia with ~20% of them progressing to develop well-differentiated uterine cancer by age of 40 weeks, although the timing and incidence varies between mice and studies (Daikoku et al., 2008, Vilgelm et al., 2006, Podsypanina et al., 1999, Stambolic et al., 2000). Therefore, the PTEN knockout females provide a good environment to investigate uterine cancer in short experimental time with low cost. PTEN knockout mouse models are generated using specific Cre/loxP transgenic system. Cre/loxP system allows generation of either global heterozygous PTEN knockout (global Cre deletor mouse line) or tissue specific, homozygous PTEN knockouts (tissue specific Cre deletor line).

**Heterozygous global *Pten* knockout:** Heterozygous global PTEN knockout mouse models have been created by homologous recombination strategy (Di Cristofano et al., 1998) and cre/loxP system using global-cre promoter (Stambolic et al., 2000). Both of these models generated by homologous recombination strategy and cre/loxP system induced wide range of tumors resembling CS patients with uterine cancers. A majority of the tumors exhibited loss of heterozygosity at the *Pten* locus and activated P-AKT and ERα pathway (Stambolic et al., 2000, Di Cristofano et al., 1998). All heterozygous PTEN knockout females generated using cre/loxP system (65 out of 65) developed endometrial hyperplasia and 14 of 65 mice developed uterine cancer (Stambolic et al., 2000).

The advantage of this global, heterozygous PTEN knockout mouse model is that uterine cancers in these mice resemble uterine cancers in CS patients; hence it is an excellent model to investigate PTEN-related hematoma syndromes as well as the role of PTEN in uterine cancer. However, the

disadvantage of this model in experimental studies is that there is still an intact copy of *Pten* as it is a heterozygous inactivation. This brings complexity of investigating the sole role of PTEN in experimental studies.

**Endometrial specific *Pten* knockout:** To generate homozygous endometrial specific *Pten* knockout mouse model (Soyal et al., 2005), the PR promoter was used to drive the Cre (PR-Cre) in the uterine endometrial cells. All these mice developed endometrial cancer as early as age of one month with myometrial invasion occurring by 3 months and had a lifespan of around five months compared to eight months in WT females (study only followed up to eight months of age so far) (Daikoku et al., 2008). All early stage lesions found in this mouse model exhibited elevated P-AKT levels. The disadvantage of this model is the non-uterine expression of Cre in the PR-Cre females. Cre expression and Cre mediated excision of DNA between the loxP sites was also detected in ovary, oviduct, mammary gland and pituitary (Soyal et al., 2005). Therefore, the knockout model generated using this model could also exhibit systemic effects making this mouse model not a truly endometrial specific knockout.

**Uterine endometrial epithelium and stroma specific PTEN inactivation:** Conditional gene recombination by adenovirus-driven cre has been used to generate homozygous epithelial and stromal specific PTEN knockout (advPTENKO) in the endometrium of mouse uterus (Joshi and Ellenson, 2011, Wang et al., 2006). advPTENKO had increased incidence of uterine carcinoma when compared to the global heterozygous *Pten* inactivated mouse model (Joshi and Ellenson, 2011). Furthermore, the uterine carcinomas

found in advPTENKO were more aggressive with invasion into myometrium and also surrounding adipose tissues.

The advantage of this model is that PTEN inactivation is specific to the endometrial layer of the uterus (epithelial and stromal cells). However, the disadvantage is the recombination strategy as it is difficult to control the amount and the localization of cre administered by adenovirus within the endometrial layer of the uterus. This may result in high variability of amount and localization of the PTEN knockout between individual experimental mice.

## 1.8 Androgens, AR and uterine cancer

As previously described in section 1.5, androgens and AR play an important role in regulating uterine growth and development, but androgens and AR are also suggested to play a significant role in uterine cancer. The possible role of androgens acting via AR is supported by previous studies showing AR expression in 65-80% of endometrial cancers (Ito et al., 2002, Prodi et al., 1980, Takeda et al., 1990, Mertens et al., 1996, Horie et al., 1992). In endometrial hyperplasia, AR was detected in the nuclei of both epithelial, stromal cells and in hyperplastic glands (Ito et al., 2002). In uterine cancer, weak AR immunoreactivity was detected in the stromal cells and strong AR was detected in the nuclei of carcinoma cells (Ito et al., 2002).

The indirect role of androgens in uterine cancer has been extensively suggested in patients with PCOS. PCOS is a complex metabolic syndrome, which is characterized by elevated plasma androgens and ovarian hyperandrogenism (Potischman et al., 1996, Austin et al., 1991, Gimes et al., 1986, Mollerstrom et al., 1993, Nagamani et al., 1986) and patients with PCOS have increased risk of developing uterine cancer (Coulam et al., 1983, Smyczek-Gargya and Geppert, 1992). Most widely accepted mechanism may explain the risk of uterine cancer in PCOS patients is known as "unopposed estrogen" as these patients have persistently elevated levels of circulating E2 from aromatization of excess androgens(further described in section 1.6.2).

The role of AR in uterine cancer is also supported by the findings that the increased AR activity in women may increase uterine cancer risk (McGrath et al., 2006) suggesting androgens via AR may increase uterine cancer. The

increased AR activity in these women is due to shorter CAG repeat in AR and it has been demonstrated that CAG repeat in exon 1 of the *Ar* gene is inversely correlated with androgen sensitivity (Simanainen et al., 2011a). In contrast, also a positive correlation between increased CAG repeat length in *Ar* gene (reduced androgen sensitivity) and uterine cancer risk has been reported (Sasaki et al., 2003, Yaron et al., 2001). The discrepancy among these studies could be due to the different ethnic differences in genotype distribution, sample size and control selection.

Despite the clinical findings suggesting androgen actions in uterine cancer, the experimental studies supporting these findings are sparse. In 1961, testosterone was shown to induce uterine cancer in mice, however, the histological characteristics of these specific uterine cancers differ from the uterine carcinomas and uterine sarcomas described previously (van et al., 1961). In addition, more recently, the 5α-dihydrotestosterone-benzonate (DHT-B) alone (implant) induced proliferation of uterine endometrial epithelium and myometrial smooth muscle cells (Zhang et al., 2004) and when co-administered with E2, the females developed severe endometrial and myometrial hyperplasia that was greater than induced by E2 alone. This study suggests carcinogenic effects of androgens in the uterus and also suggests the synergistic effects of androgen with already well known carcinogen, E2.

Contradicting the postulate that androgens increase uterine cancer risk, one study has shown that danazol (synthetic androgen with no estrogenic activity) has preventive effects on E2 induced-uterine cancer in mice (Niwa et al., 2000). The possible mechanisms suggested for the preventive effects of danazol include the suppression of endometrial cell growth by inhibiting estrogen-

induced c-fos/jun-expression, decreasing ovarian E2 synthesis (Niwa et al., 2000) and/or directly acting via AR in the uterus (Rigano et al., 1999). However, it should be noted that danazol has ability to shut down the hypothalamus-pituitary-gonad axis and activate PR; hence, these characteristics of danazol could have an indirect effect on uterine cancers. Furthermore, an *in vitro* study has shown proliferation of MFE-296 cells [AR-positive endometrial cancer cell line (unresponsive to estrogen)) was inhibited by DHT (Hackenberg et al., 1994). The effect of DHT was antagonized by anti-androgens, suggesting the involvement of the AR.

In summary, despite the contradictory studies on the role of androgens and AR in uterine cancer, it is certain that androgens and AR do play important roles in uterine cancer progression. Different results from previous studies could be due to the distinct types of androgens used in the experiments (i.e. aromatizable vs non-aromatizable). Also, the role of androgens acting via AR in uterine cancer could be dependent on presence of other steroid hormones such as E2. Hence further investigation on the role of androgens via AR in uterus and uterine cancer is warranted.

## 1.9 Objectives and aims

Previously, androgens have been known as “male hormones” where most extensive studies have been conducted investigating the role of androgens in male reproductive physiology and pathology such as prostate. However, although recent studies have shown important direct role of androgens via AR in female reproductive physiology and pathology, the role of androgens via AR in females remains not well characterized. Hence, the current project aims to provide novel evidence and specific mechanistic information on androgen actions in the highly hormone dependent organ, the uterus, both in developmental physiology and uterine carcinogenesis. This has been achieved by utilizing our laboratory’s novel female mouse model, where AR is inactivated. Furthermore, to induce uterine cancers, AR inactivated mouse model is combined with a unique mouse model with inactivation of PTEN and therefore susceptible for development of uterine cancers. Therefore our specific hypothesis and aims are:

**Hypothesis one:** Androgens acting via AR can support uterine growth and the growth promoting effect of androgens is mediated via AR in uterus.

**Aim one:** Generate and validate uterine glandular epithelial specific AR knockout (ugeARKO) mouse model using Cre/LoxP strategy. Thereby investigate the role of AR in uterine growth and functions (Chapter 3).

**Hypothesis two:** Androgen actions via AR may increase PTEN inactivation induced uterine pathology

**Aim two:** Investigate the role of global AR inactivation in PTEN deletion induced uterine cancer (Chapter 4).

**Hypothesis three:** Androgen actions via glandular epithelial AR may increase PTEN inactivation induced uterine pathology

**Aim three:**

Investigate the effect of glandular epithelial AR inactivation in PTEN inactivation induced uterine pathology (Chapter 5).

Thus, the combination of these unique mouse models provide exciting platform to determine the mechanisms of male hormone, androgen actions in initiation, progression and therapy of breast and uterine cancer.

# Chapter 2: General methods and materials

## 2.1 Mouse models

All experiments conducted in this PhD project were approved by the Animal Welfare Committee of the Sydney Local Health District within NHMRC guidelines for animal experiments. All experimental mice were bred and kept in the animal facility at the ANZAC Research Institute, Molecular Physiology Unit (MPU). This facility is maintained under the PC2 requirements and equipped with 12 hour light/dark cycle with lights on from 6am to 6pm daily. Mice were kept in a plastic cage with filter top lids under specific pathogen free (SPF) conditions and had unlimited access to food and water.

### 2.1.1 Mouse lines

**Tg(Sox2-cre)1Amc (denoted SOX2-Cre)**

SOX2-Cre mice with FVB/N background were kindly donated by Dr. L Rob (Walter and Eliza hall Institute, Australia) and Professor A McMahon (Harvard University, USA). The Cre expression is regulated by the SRY-box containing gene2 (Sox2) promoter to drive Cre expression in all cells already during early embryonic development (embryonic day 6.5) (Hayashi et al., 2002).

**Tg(Pbsn-cre)20Fwan (denoted PBSN-Cre)**

PBSN-Cre mice with FVB/N background were kindly donated By Dr. F Wang (Centre for Cancer Biology and Nutrition in Houston, USA). The Cre expression is regulated by probasin (PBSN) promoter to drive Cre expression in prostate

epithelial cells (Jin *et al.*, 2003) and in uterine glandular epithelial cells (Chapter 3).

**$Ar^{tm1Jdz}$ (denoted ARflox)**

ARflox mice were originally C57BL/6 background and were generated in collaboration with Professor J Zajac (University of Melbourne, Australia). For this PhD project, ARflox mice were backcrossed to the FVB/N background for at least 6 generations. In these mice, the exon 3 of the *Ar* gene is surrounded by the LoxP sites, where exon 3 encodes the $2^{nd}$ zinc finger of the AR DNA binding domain which is required for stabilizing DNA binding of the AR. Excision of exon 3 causes in-frame mutation leading to continuous transcription and protein translation (Figure 2.1) (Notini et al., 2005a). It is important to note that there are number of different *Ar* knockout models available which have been thoroughly compared in two recent review papers (Rana et al., 2014, Walters and Simanainen, 2010). The exon 3 deleted *Ar* knockout model was chosen due to its unique characteristic of in-frame mutation of *Ar* gene rather than frameshift mutation in other models. This creates a minimally truncated AR which is fully inactivated but is much closer to physiological than the major deletions of the AR protein created by introducing stop codons early into the translated protein which abrogates all co-regulator interactions.

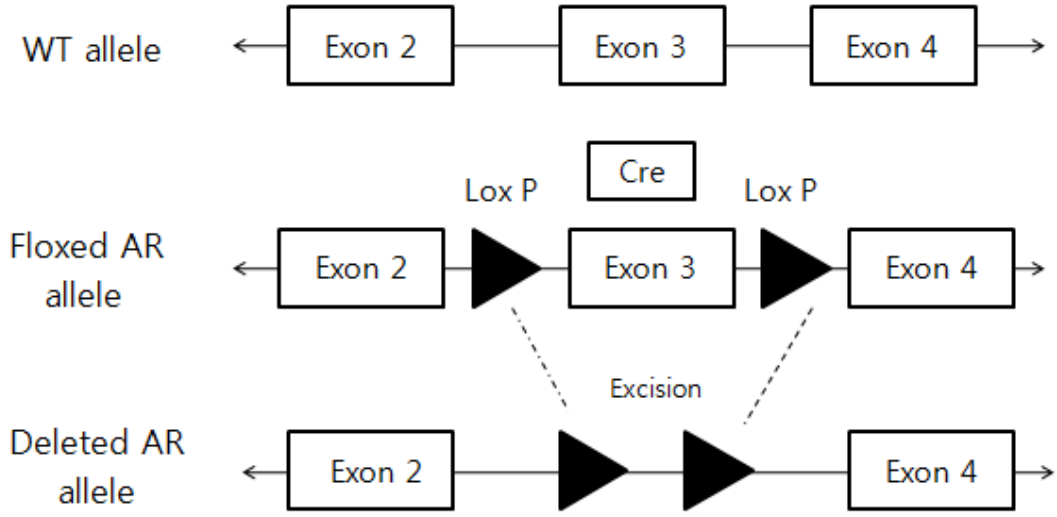



**Figure 2.1. Schematic presentation of Cre/LoxP mediated excision of exon 3 of *Ar*.**

**Pten$^{tm1Hwu}$ (denoted PTENflox)**

In PTENflox mice with FVB/N background, the exon 5 of the Pten gene is surrounded by the LoxP sites. Excision of exon 5 produces premature stop codon, leading to early termination of transcription and protein translation (Figure 2.2) (Lesche et al., 2002).

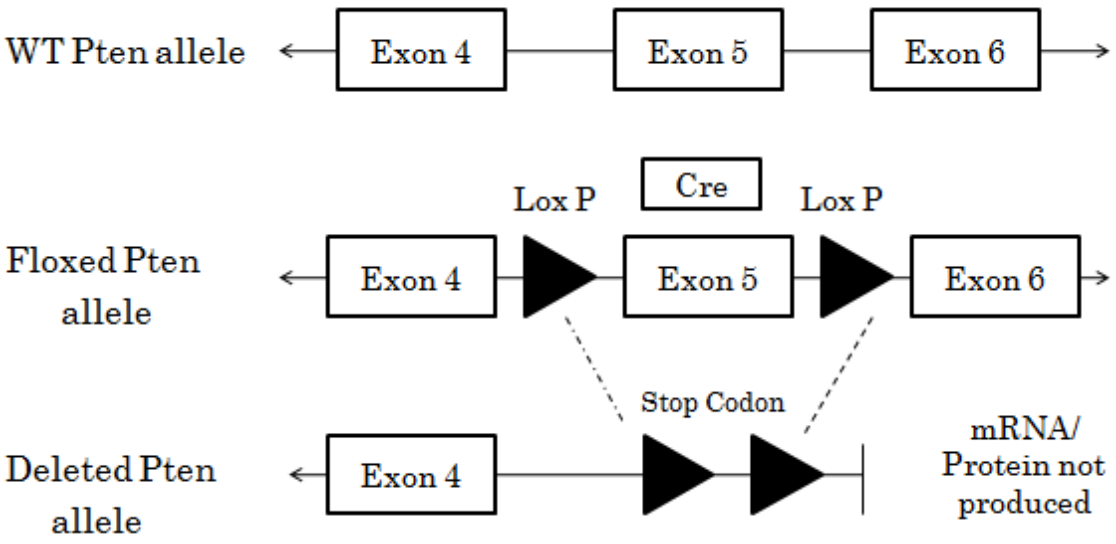



**Figure 2.2. Schematic presentation of Cre/LoxP mediated excision of exon 5 of *Pten*.**

**Gt(ROSA)26Sor (denoted R26R)**

R26R mice were kindly donated by Dr. W Alexander (Walter and Eliza hall Institute, Australia). R26R mice have a bacterial *lacZ* gene encoding the enzyme β-galactosidase (Soriano, 1999). In R26R mouse, a transcriptional stop codon (bacterial lacZ gene) is flanked by loxP sites. When intact, this stop codon prevents the expression of β-galactosidase (β-gal) from the downstream lacZ coding sequence. When Cre is present, stop codon is excised and permits expression of β-galactosidase (β-gal), which stains x-gal to blue cells (Figure 2.3).

All founder lines are viable, fertile, and normal in size and display no gross physical abnormalities.

### 2.1.2 Breeding protocol for experimental mice

The following breeding protocol outlines the generation of experimental female mice used in this PhD project: R26R (PBSN-Cre characterization), global knockout models and uterine glandular epithelial specific knockout models.

**R26R mouse model to localize PBSN-Cre activity in female mouse**

Characterization of PBSN-Cre model was achieved by crossing the PBSN-Cre mouse with R26R mouse to localize PBSN-Cre activity in female mouse. In R26R mouse, a transcriptional stop codon is surrounded by LoxP sites. When intact, this stop codon prevents the expression of β-galactosidase from the downstream LacZ coding sequence. When Cre is expressed (i.e. PBSN-Cre), excision of the stop codon permits expression of β-galactosidase which converts 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) to blue color in the cells, hence, Cre activity can be visually localized (Figure 2.3).

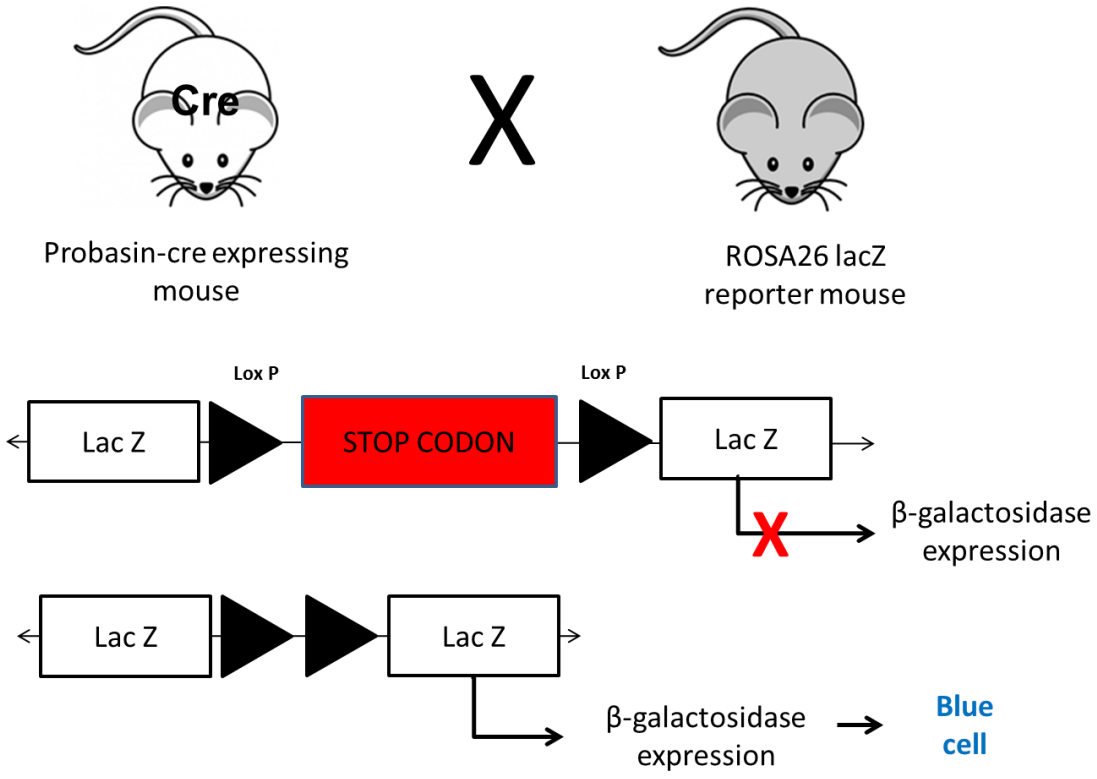



**Figure 2.3. R26R mouse model to characterize PBSN-Cre in female mouse.** PBSN-Cre will excise stop codon surrounded by LoxP sites permitting the expression of β-galactosidase to localize PBSN-Cre (blue staining).

### Generation of global knockout mouse model

For the generation of the global knockout mouse model, three founding lines have been used: a SOX2-Cre, PTENflox and ARflox mice. The following breeding steps were used (Figure 2.4) to generate the experimental mice.

Firstly, **PTENflox mouse** ($Pten^{flox/flox}$ male) is crossed with **ARflox mouse** ($AR^{flox/wt}$ female) to generate $Pten^{flox/wt}AR^{flox/wt}$ females (used in step 2 of breeding protocol ) as well as $Pten^{flox/wt}AR^{flox}$ and $Pten^{flox/wt}AR^{wt}$ males (step 3).

In step 2, $Pten^{flox/wt}AR^{flox/wt}$ female is crossed with **Sox2-Cre mouse** ($Sox^{cre/cre}$ male) to produce $Pten^{wt/wt}AR^{flox/wt}Cre^{+/-}$ female in which the exon 3 of floxed AR allele is deleted by Cre creating AR cut allele. This female is used to generate all the global knockout experimental mice used in this PhD project (step 3).

As a result, we generated: WT (wild-type AR and PTEN), ARKO (global homozygous AR inactivation), PTENKO (global heterozygous PTEN deletion) and PTENARKO (global homozygous AR inactivation with global heterozygous PTEN deletion) female mice (Figure 2.4).

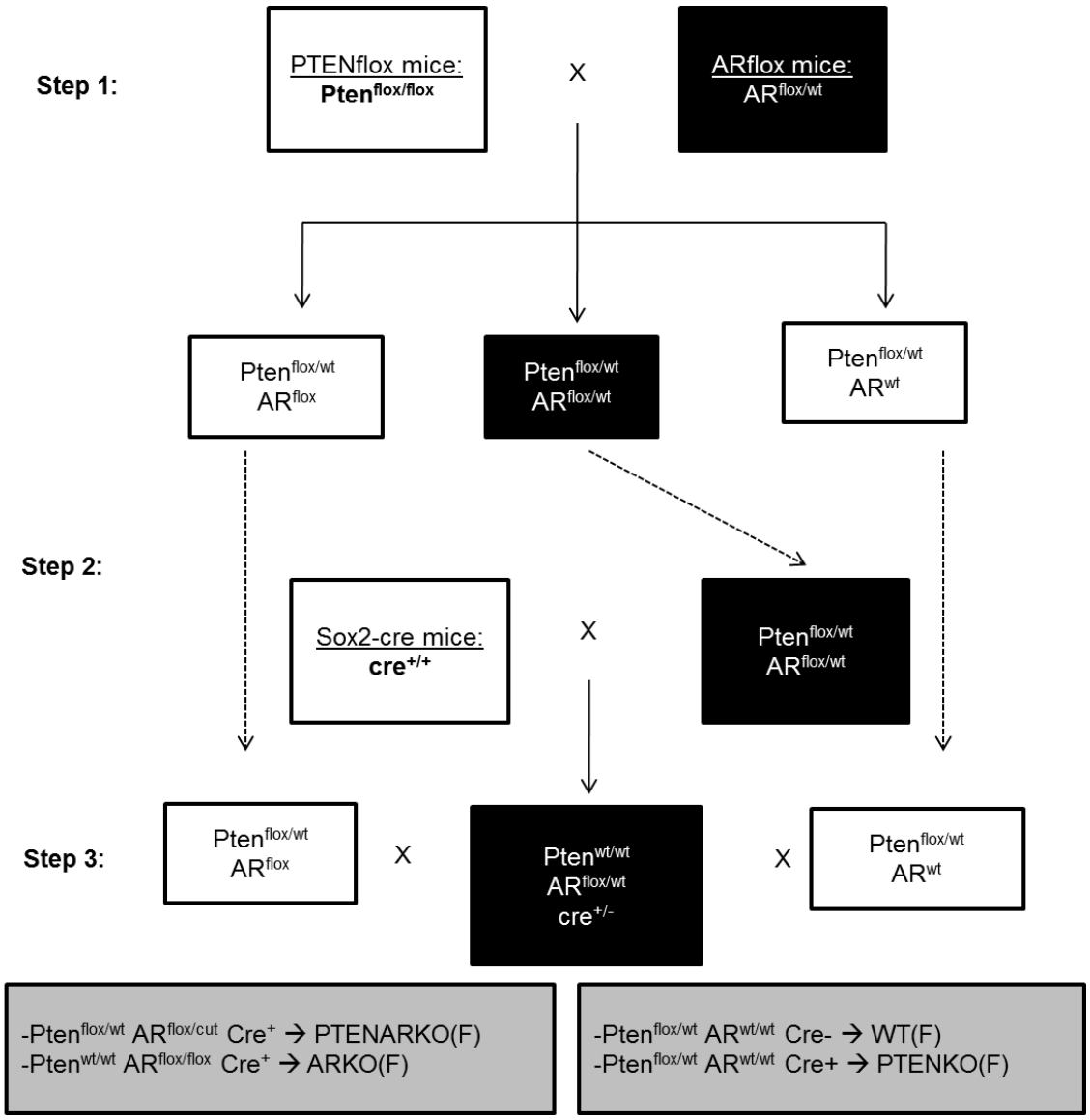



**Figure 2.4. Breeding protocol to generate global knockout mouse model.** White boxes represent male mice and black boxes represent female mice and grey boxes represent experimental groups. PTENflox mice have $AR^{wt/wt}$ and $cre^{-/-}$ alleles whereas ARflox mice have $PTEN^{wt/wt}$ and $cre^{-/-}$ alleles. Sox2-cre mice have $AR^{wt/wt}$ and $PTEN^{wt/wt}$ alleles.

**Generation of uterine glandular epithelial specific mouse model**

For the generation of the uterine glandular epithelial knockout mouse model, three founding lines have been used: a PBSN-Cre, PTENflox and ARflox mice. Similar breeding steps as global knockout mouse model was used (Figure 2.5).

Firstly, **PTENflox mouse** ($Pten^{flox/flox}$ male) is crossed with **ARflox mouse** ($AR^{flox/wt}$ female) to generate $Pten^{flox/wt}AR^{flox/wt}$ females (used in step 2 of breeding protocol) as well as $Pten^{flox/wt}AR^{flox}$ and $Pten^{flox/wt}AR^{wt}$ males (step 3).

In step 2, $Pten^{flox/wt}AR^{flox/wt}$ female is crossed with **PBSN-Cre mouse** ($PBNS^{cre/cre}$ male) to produce $Pten^{flox/wt}AR^{flox/wt}Cre^{+/-}$ female in which the exon 3 of floxed AR allele is deleted by Cre creating AR cut allele. This female is used to generate all the uterine glandular epithelial specific knockout experimental mice used in this PhD project (step 3).

As a result, we generated: WT (wild-type AR and PTEN), ugeARKO (homozygous AR inactivation in uterine glandular epithelial cells), ugePTENKO (homozygous PTEN deletion in uterine glandular epithelial cells) and ugePTENARKO (homozygous AR inactivation and PTEN deletion in uterine glandular epithelial cells) female mice (Figure 2.5).

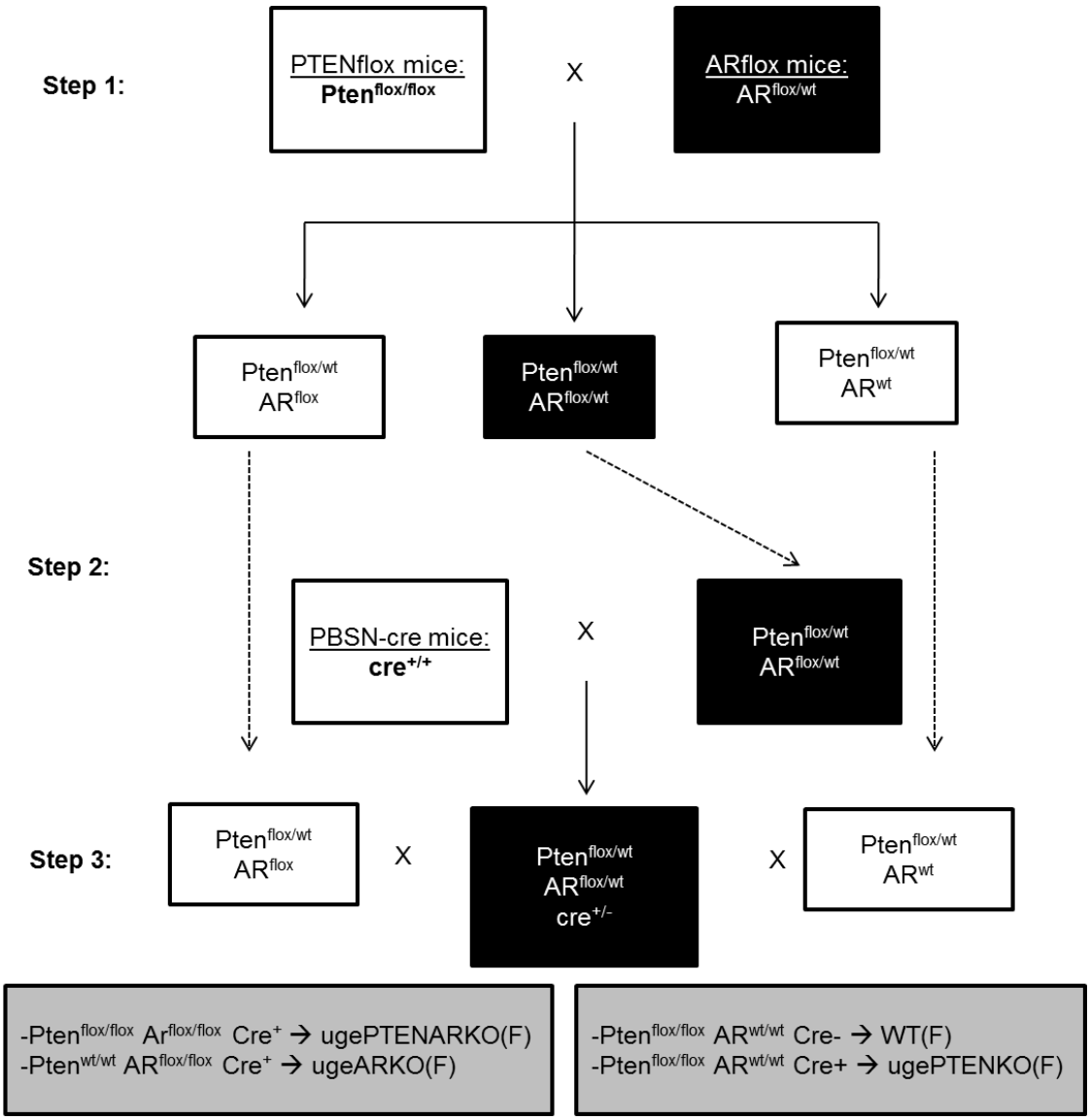



**Figure 2.5. Breeding protocol to generate uterine glandular epithelial specific knockout mouse model.** White box represent male mice and black box represent female mice and grey box represent experimental groups. PTENflox mice have $AR^{wt/wt}$ and $cre^{-/-}$ alleles whereas ARflox mice have $PTEN^{wt/wt}$ and $cre^{-/-}$ alleles. PBSN-cre mice have $AR^{wt/wt}$ and $PTEN^{wt/wt}$ alleles.

## 2.2 DNA extraction and PCR genotyping

### 2.2.1 DNA extraction

Experimental mice as well as their breeders were genotyped using genomic DNA (gDNA) extracted from tissue biopsies (Walters et al., 2007). Tissue biopsies were either from a toe from pups collected prior to weaning for identification or a small piece of tail following sample collection. Genotyping by PCR was performed using a Proteinase K lysed sample. Tissue samples were incubated with 250µl lysis mixture, containing 25µl PCR buffer (Fisher Biotech, Australia, TQRB-5), 25µl 20mM $MgCl_2$, and 1.7µl proteinase K (Roche Diagnostic, Mannheim, Germany, recombinant PCR grade 18±4mg/ml), for 2 hours at 55°C to break the tissue and release the DNA. This was followed by 15 minutes at 98°C to inactivate the proteinase K. Lysed samples were stored at 4°C.

In addition, for specific confirmation of Cre activity in tissues of interest, DNA was extracted using QIAGEN DNeasy Blood & Tissue kit (QIAGEN, Doncaster, Australia) from uterus to confirm excision of exon 5 of *Pten* and exon 3 of *Ar* at the DNA level. DNA extraction kit was used to obtain purified DNA. DNA extraction was performed according to the manufacturers' instructions (Appendix I). Briefly, small piece of tissue (<25mg) was placed in a 1.5ml microcentrifuge tube with 180µl of Buffer ATL with 20µl of proteinase K to lyse the tissue. Then, samples were vortexed and incubated at 56°C until tissue was completely lysed. Lysed sample was vortexed for 15 seconds and 200µl of buffer AL and 200µl of 100% ethanol was added to purify the DNA. Then, the solution was pipetted into a DNeasy Mini spin column in a 2 ml collection tube

and centrifuged. In the spin column, DNA binds to the column for extraction. DNA was extracted from spin column using 200µl of Buffer AE and stored at -20°C.

## 2.2.2 PCR

All primers (Table 2.1) for PCR genotyping were purchased from Sigma Aldrich Pty (Sydney, Australia). Water was used as a negative control instead of DNA and positive controls (gene of interest) was used in all PCRs for specific PCR reaction and determination. Details of all PCR reagents used are listed in Appendix 1.

**Table 2.1. Primers used for genotyping**

| | |
|---|---|
| Cre-F (forward primer) | 5'-CTGACCGTACACCAAAATTTGCCTG-3' |
| Cre-Rv (reverse primer) | 5'-GATAATCGCGAACATCTTCAGGTTC-3' |
| ARCut-F (forward primer) | 5'-CAGAAATCCACGTGCCTCTACC-3' |
| ARIN3-Rv (reverse primer) | 5'-GGGAGACACAGGATAGGAAATT-3' |
| AREx3-F (forward primer) | 5'-CTTCTCTCAGGGAAACAGAAGT-3' |
| AR-Neo-F (forward primer) | 5'-TAGATCTCTCGTGGGATCATTG-3' |
| PTEN-F (forward primer) | 5'-TCCCAGAGTTCATACCAGGA-3' |
| PTEN-$Rv_1$ (reverse primer) | 5'-GCAATGGCCAGTACTAGTGAAC-3' |
| PTEN $Rv_2$ (reverse primer) | 5'-AATCTGTGCATGAAGGGAAC-3' |

**Cre PCR:** The primer pair used for detecting the presence or absence of the Cre gene in gDNA was Cre-F and Cre-Rv (Table 2.1). The presence of Cre was confirmed by the PCR product at 213bps. PCR reaction steps were 94ºC for 3 minutes, then 30 cycles of 94ºC for 1 minutes, 68 ºC for 1 minutes, 72 ºC for 1 minutes, then 72 ºC for 5 minutes and hold at 10 ºC.

**ARCUT PCR:** The primer pair used for detecting the intact and exon3-deleted AR in gDNA was ARCut-F and ARIN3-Rv (Table 2.1). The deletion of AR exon 3 was confirmed by the PCR product at 510bps whereas the intact AR exon 3 was detected at 1650bps (sometimes this band is not produced which depends on the activity and efficacy of the enzyme). PCR reaction steps were 94ºC for 3 minutes, then 35 cycles of 94ºC for 1 minutes, 62 ºC for 1 minutes, 72 ºC for 1 minutes, then 72 ºC for 5 minutes and hold at 10 ºC.

**ARFLOX PCR:** The primer pair used for detecting the intact and floxed AR in gDNA was AREx3-F, AR-Neo-F and ARIN3-Rv (Table 2.1). The intact AR was confirmed by the PCR product at 613bps whereas the floxed AR was detected at 289bps. PCR reaction steps were 94ºC for 3 minutes, then 30 cycles of 94ºC for 1 minutes, 58 ºC for 1 minutes, 72 ºC for 1 minutes, then 72 ºC for 5 minutes and hold at 10 ºC.

**PTEN PCR:** The primer pair used for detecting the intact PTEN, floxed PTEN and exon5-deleted PTEN in gDNA was PTEN-F, PTEN-$Rv_1$ and PTEN $Rv_2$ (Table 2.1). The location of these primers is shown in Figure 2.6. The deletion of

PTEN exon 5 was confirmed by the PCR product at 300bps, floxed PTEN at 650bps and intact PTEN at 500bps. PCR reaction steps were 94ºC for 3 minutes, then 40 cycles of 94ºC for 45 seconds, 60 ºC for 30 seconds, 72 ºC for 1 minutes, then 72 ºC for 5 minutes and hold at 10 ºC.

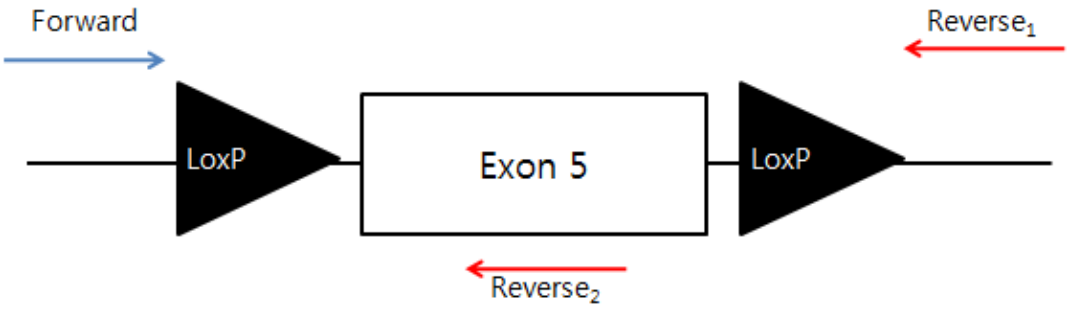



**Figure 2.6. Locations of PTEN primers in the *Pten* allele.**

## 2.2.6 Agarose gel

All the PCR samples in this PhD project were analysed on a 1.5% agarose (Amresco, Solon, OH, USA, Biotechnology grade) gel in 1X TBE buffer (Appendix II) containing 7.5 µl SYBR safe (Invitrogen, Oregon, USA) per 150 ml gel. SYBR safe binds the DNA and allows visualization of DNA band under the UV transilluminator (Bio-Rad Laboratories Pty Ltd, Gladesville, Australia). Each gel had a control lane containing 4 µl of control ladder (Bioline, Alexandrian, NSW, Australia, Hyperladder 1) for band size comparison.

## 2.3 Surgery

### 2.3.1 Anaesthetic for surgery

The anaesthetic mixture was prepared to keep the mice asleep for the duration of the surgery (approximately 10minutes). Anaesthetic for surgery contained 90mg/mL of Ketamine (Parnell Laboratories, Alexandria, NSW, Australia, 100mg/ml) and 10 mg/mL Xylazine (Troy laboratories, Smithfield, NSW, Australia, LLium Xylazil 100mg/ml) diluted with 0.9% saline solution (Appendix II). 100µl of anaesthetic mixture was injected subcutaneously per 10g of body weight using a 1ml insulin syringe (Thermo Medical Corporation, Elkton, USA). To make sure if the mice were anesthetized, paws were pinched and if there was no reflex, surgery was performed. After surgery, mice were wrapped in a towel and placed under a heat lamp until conscious and movement was observed. Health of these mice was monitored daily.

### 2.3.2 Ovariectomy and hormone implants

Ovariectomy and hormone implant surgeries were performed in MPU. Following the full anaesthesia, small incisions were made at both lateral abdominal skins. Then, another incision was made on the peritoneal wall to access ovaries. When ovaries were located they were removed under the microscope to make sure that the whole ovary was removed leaving the uterus intact. Peritoneal wall was sutured with 6-0 silk suture treads (Johnson and Johnson, North Ryde, NSW, Australia) and skin was sutured with 4-0 silk suture treads (Johnson and Johnson, North Ryde, NSW, Australia).

Following ovariectomy, silastic implants (inner diameter=1.47mm and outer diameter=1.95mm, Dow-Corning, Sydney, Australia) of 1cm length testosterone containing ~10mg crystalline testosterone (Merck, Darmstardt, Australia) or 2x0.5cm length DHT containing ~10mg crystalline DHT (Merck, Darmstardt, Australia) or empty (negative control) implants were inserted subdermally through a small incision at the back of the neck. Wounds were sutured with 4-0 silk suture tread (Johnson and Johnson, North Ryde, NSW, Australia).

Mr Mark Jimenez of the ANZAC Research Institute (ARI) kindly prepared the implants.

## 2.4 Fertility assessment

To evaluate effect of endometrial gland specific AR inactivation on fertility, the 8- to 10-wk-old WT and ugeARKO females were continuously mated with an individual WT male (at least 8 weeks old) for a 5-month period. Cages were monitored daily and the number of pups and litters were recorded.

## 2.5 Sample collection

### 2.5.1 Estrous cycle stage

In female mice, the cyclical physiologic changes induced by endogenous reproductive hormones are known as estrous cycle, with each cycle lasting 4-5 days. Mouse estrous cycle has four stages, proestrus, estrus, metestrus and diestrus (Figure 2.7). As uterus is a highly hormone dependent organ, all the mice used in this PhD project were collected at diestrus stage.

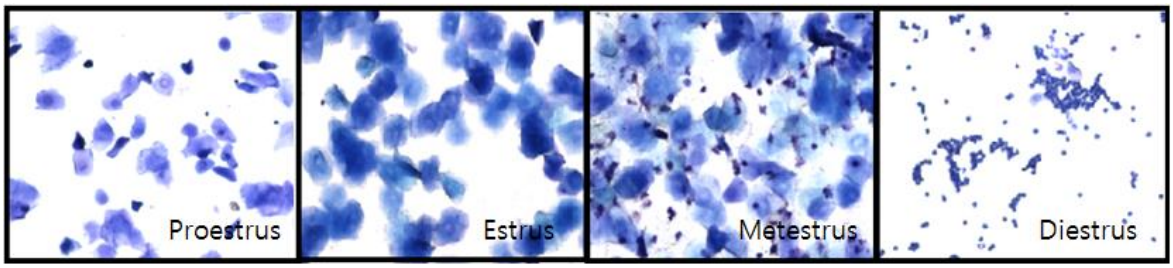



**Figure 2.7. Estrous cycle stage of mice: Proestrus, estrus, metestrus and diestrus.** Estrous stage was determined by vaginal smears stained by toluidine blue staining.

At proestrus, the smear consists of predominantly nucleated epithelial cells, with few keratinized epithelial cells or smaller leukocytes also present. Estrus is characterized by keratinization of all epithelial cells, which increase in size and the nuclei is degenerated. Metestrus is characterized by the presence of a mixture of both the small leukocytes as well as larger keratinized epithelial cells. Lastly, diestrus is predominantly leukocytes though some epithelial cells, nucleated or keratinized may be present. During diestrus the cells are often entangled within stringy mucus.

Estrous cycle of female mice was detected by vaginal smear. Using a pipette, 20µl of 0.9% saline was flushed in and out of vagina. Then, the solution in the pipette was smeared on a glass slide (Livingstone Premium Microscope Glass Slides). Vaginal smears were allowed to air dry and stained with 0.5% toludine blue (Appendix II) and examined under the light microscope.

### 2.5.2 Anaesthetic for sample collection

Anaesthetic for tissue collection contained 50 mg/mL Ketamine (Parnell Laboratories, Alexandria, NSW, Australia, 100 mg/mL) and 50 mg/mL Xylazine (Troy Laboratories, Smithfield, NSW, Australia, Ilium Xylazil 100 mg/mL) diluted with 0.9% saline solution. 100µl of anaesthetic was injected per 10g body weight using 1 mL insulin syringes (Terumo Medical Corporation, Elkton, USA). Mouse foot was lightly pinched to make sure that the mouse was under the influence of anaesthetics. If the mouse had reflex to pinched feet, more time was given or more anesthetic was injected before any procedures.

### 2.5.3 Blood and serum collection

Blood was collected from anaesthetized mice by cardiac puncture using 1cc/mL tuberculin syringe (Laguna, Philippines) and 25 G x 5/8 inch needle (Terumo, Tokyo, Japan). Blood was left at room temperature for at least 20 minutes to allow coagulation of the blood and centrifuged at 2300g for 5 minutes to separate the serum. Pipette was used to extract the serum (top clear layer) and it was transferred to a new Eppendorf tube and stored in -20°C.

### 2.5.4 Tissue collection

After blood collection, the mice were euthanized by neck dislocation. A small piece of tail was collected and stored at -20°C for genotype confirmation. Peritoneal wall was incised to collect uterus and ovaries. These organs were dissected clean of surrounding fat under a dissecting stereo-microscope (Olympus SZ40) using extra fine forceps (Dumoxel non-magnetic, World Precision Instruments Inc, Florida, USA) and fine spring scissors (Scissors, Vannas, 8cm, STR, World Precision Instruments Inc, Florida, USA) and weighed using a fine scale (Mettler AE 163). Uterus (without oviduct) and ovaries were either snap frozen or fixed in 4% paraformaldehyde (PFA) (Appendix II).

For the global knockout study, other organs such as mammary gland, spleen, liver and adrenal gland were also weighed and fixed in 4% PFA for histological analysis. All the abnormalities found were recorded and photographs were taken for future references.

### 2.4.5 Laser capture micro-dissection

Laser capture micro-dissection was used to capture glandular epithelial cells from formalin fixed paraffin embedded (FFPE) uterine section (chapter 3).

FFPE uterine sections were sectioned at 15µm thickness on to a membrane slides (MembraneSlide NF 1.0 PEN (D), Carl Zeiss, Sydney, Australia). Standard hematoxylin and eosin (H&E) staining was performed to visualise glandular epithelial cells. PALM Duoflex Combi System (Bosch Institute, The

University of Sydney) was used for laser capture micro-dissection. PALM system allows non-contact laser capitulation meaning the cutting and capitulation of the sample is performed out by the power of the laser beam alone. This system is advantageous in minimising sample degradation and contamination. Upon laser micro-dissection (Figure 2.8), glandular epithelial cells were captured in adhesive cap (AdhesiveCap 500 opaque (D), Carl Zeiss, Sydney, Australia).

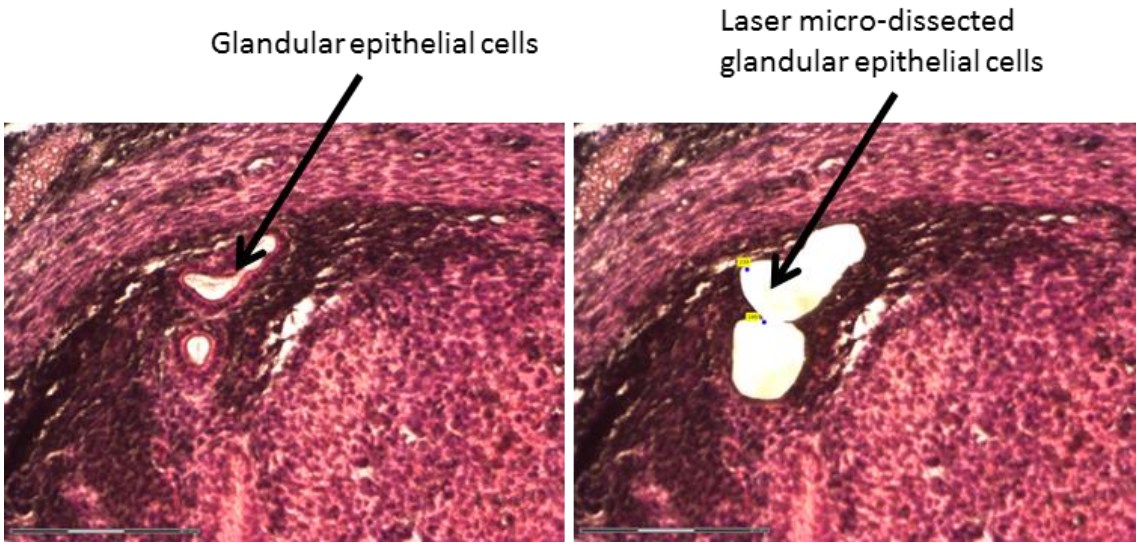



**Figure 2.8. Laser micro-dissected glandular epithelial cells.** *Left:* Histological section of uterus before the micro-dissection showing presence of glandular epithelial cells. *Right:* after the micro-dissection showing absence of glandular epithelial cells.

DNA was extracted from laser micro-dissected glandular epithelial cells and rest of the uterus using QIAGEN micro DNA kit (QIAGEN, Doncaster, Australia) as manufacturer's protocol.

## 2.6 Histology

### 2.6.1 Tissue fixation

Tissues were fixed in 4% PFA overnight at 4°C and transferred to 70% ethanol on the following day and kept in room temperature until further processing.

### 2.6.2 Tissue processing & embedding (paraffin)

Tissues in 70% ethanol were embedded at ANZAC research institute for paraffin embedding (Appendix III). For uterus, mid-section from a fixed uterine horn was used for all histology.

### 2.6.3 Tissue cutting (paraffin)

Rotary microtome (MicroTEC Cut 4060) was used to cut paraffin blocks. Paraffin blocked tissues were cut at 5µm thickness and floated on a water bath of 40°C to flatten the tissue. Then, the sections were put on a glass slide (Lomb Superfrost Plus, Menzel-Glaser) and left in the 37°C oven for overnight to bond the tissue to the slide.

## 2.7 Staining

### 2.7.1 Haematoxylin and Eosin (H&E)

H&E staining was used for basic histological analysis (Appendix III). Haematoxylin stains the nuclei of the cells of virtually all tissues in a bluish color and the cytoplasmic portions of the cells of most tissues stain pink by eosin. Nuclei of cells are stained by haematoxylin due to their high nucleic acid (DNA and RNA) content that makes them basophilic. On the other hand, cytoplasmic portions are generally not basophilic but acidophilic or eosinophilic and eosin readily combines with acidophilic or eosinophilic molecules.

Briefly, the tissue sections on slide were rehydrated and stained in Harris haematoxylin (Fronine, Thermo-Fisher Scientific, Waltham, USA) following bluing of the color in acid alcohol (1% HCl in 70% ethanol) and Scott's tap water (Fronine, Thermo-Fisher Scientific, Waltham, USA) and staining with Eosin (1% Alcoholic Eosin, Fronine, Thermo-Fisher Scientific, Waltham, USA). Following the staining the sections were dehydrated, left air dry and coverslipped next day using DPX mountant for histology (Sigma, St. Louis, USA) and 24mm by 50mm coverslip (Coverglass No.1, ProSciTech, Thuringowa, Australia).

### 2.7.2 Histomorphological analyses

Using light microscopy, different areas of uterine section were marked (total area, myometrium, endometrium and lumen) (Figure 2.9) and each area was calculated using CASTGRID software (Olympus, Aarhus, Denmark). Measurements were repeated on three different uterine sections for each

sample and measurements were averaged. All sections were counted two times in order to assess inter-observer variability.

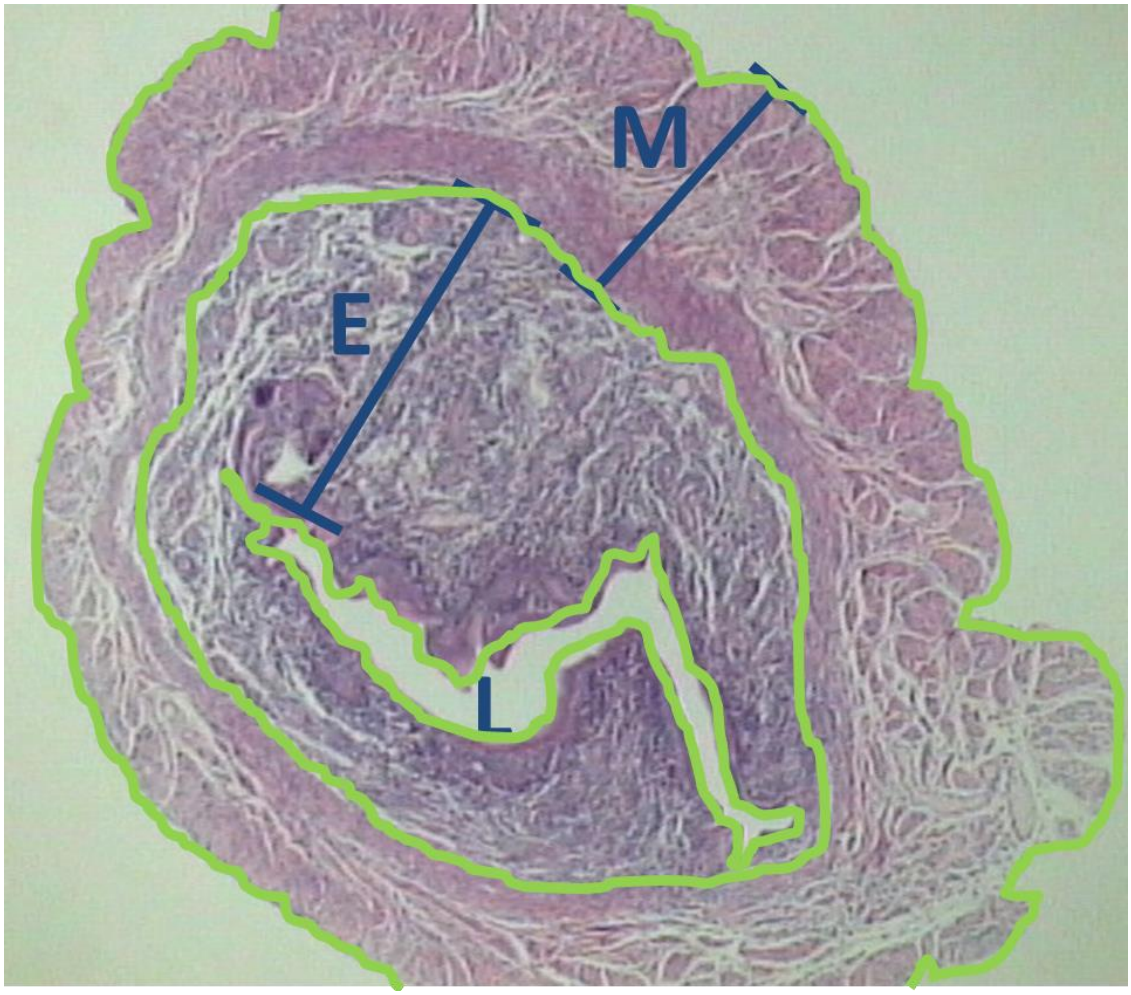



**Figure 2.9. Different areas of uterine sections.** E=endometrium, L=lumen, M=myometrium.

### 2.7.3 Immunohistochemistry

A detailed procedure for immunohistochemistry is provided in Appendix III. In brief, paraffin sectioned uterus was deparaffinised and rehydrated and underwent microwave-induced antigen retrieval with 10mM citric acid (pH 6) for

10 minutes to improve the demonstration of antigens by breaking the protein cross-links formed by PFA fixation. Sections were blocked using Pierce Superblock (containing 0.5% Bovine serum albumin) (Life Technologies Australia Pty Ltd. Mulgrave, Australia) and non-biotinylated rabbit primary antibodies against the proteins of interest (Table 2.2). Primary antibodies were visualized using anti-rabbit secondary Vectastain Elite ABC kit (Vector Laboratories, California, USA). Uterus sections from same pool of samples were used as negative controls by treating with PBS buffer instead of primary antibody (Appendix III).

**Table 2.2. Details of primary antibodies and dilutions used for immunohistochemistry**.

| Protein target | Name of Antibody | Manufacturer, catalogue #, and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used |
|---|---|---|---|---|
| **ERα** | MC-20 | Santa Cruz Biotech (sc-542) | Rabbit; polyclonal | 1:200 |
| **PR** | PR (C-19) | Santa Cruz Biotech (sc-538) | Rabbit; polyclonal | 1:100 |
| **PTEN** | PTEN (138G6) | Cell Signalling (9559) | Rabbit; monoclonal | 1:50 |
| **AKT** | 11E7 | Cell Signalling (4685) | Rabbit; polyclonal IgG | 1:50 |
| **P-AKT** | D9E | Cell Signalling (4060) | Rabbit; polyclonal IgG | 1:50 |
| **p27** | P27 (C-19) | Santa Cruz Biotech (sc-528) | Rabbit; polyclonal | 1:50 |

## 2.7.4 Immunohistochemistry quantification

ERα immunohistochemistry results in Chapter 4 were quantified to determine the % of ERα positivity and intensity of ERα staining. Immunopositivity and immunointensity of ERα was assessed by 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 accessing immunointensity (scale 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 two times in order to assess intra-observer variability.

## 2.7.5 Whole mount LacZ staining

To detect functional recombinase activity of a PBSN-Cre in females, PBSN-Cre mice were crossed with R26R (Section 2.1.2). In the R26R mice the Cre mediates activation of a floxed-*lacZ* gene and the following LacZ activity can be detected by whole mount β-galactosidase staining to localize the Cre activity. Whole mount β-galactosidase staining method has been used in previous studies on mouse uterus (Ismail et al., 2002, Mohamed et al., 2005).

Mice are sacrificed by cervical dislocation and uterus, ovary, mammary gland and pituitary were collected and immersed in fixing solution (Appendix II) for 1 hour at room temperature. Tissues were removed from fixing solution and washed 3 times in washing buffer (Appendix II) for 15 minutes each time. Then tissues were immersed in staining solution containing 1mg/ml of X-gal

(Appendix II) for overnight in the dark at 37ºC. Then tissues were postfixed in 4% PFA for overnight and paraffin embedded and sectioned in 10µm sections on glass slides as described in section 2.5. Slides were then deparaffinised and counterstained using Nuclear Fast Red (Sigma, St. Louis, USA). To confirm that the positive staining was not due to the endogenous cre activity, the PBSN-cre negative tissues were used as negative controls.

## 2.8 Reverse transcription (RT)-PCR

### 2.8.1 RNA extraction

RNA was extracted from snap frozen uteri and ovaries. Tissues were homogenized in 1ml (0.5ml for ovaries) of TRIzol reagent (Sigma-Aldrich, St Louis, USA) in 1.5ml RNase-free Eppendorf tubes, vortexed for 15 seconds and incubated in room temperature for 5 minutes. Chloroform (Sigma-Aldrich, St Louis, USA) was added (0.2ml per 1ml of TRIzol used) to the homogenate to denature the protein and RNAse. Tubes were vortexed for 15 seconds and centrifuged at 12000g for 15 minutes at 4ºC. Top aqueous phase (contains RNA) was carefully transferred into fresh RNA free 1.5ml Eppendorf tubes. Isopropanol (Ajax, Tarem Point, NSW, Australia) was added (0.5ml per 1ml of TRIzol used) to precipitate the RNA. Samples were vortex for 15 seconds and incubated in room temperature for 10minutes with gentle shake. Samples were centrifuged at 12000g for 10 minutes at 4ºC. The supernatant was removed and RNA pellets were washed with 80% ethanol (1ml per 1ml of TRIzol used) and centrifuged at 76g for 5 minutes at 4ºC. Supernatant was discarded and RNA pellets were air dried for 2 to 3 minutes (do not over dry) and pellets were redissolved in 20-60µl of RNAse free water. Samples were kept on ice and RNA concentrations and purity were measured on an Eppendorf Biophotometer. Purity of RNA was assessed with absorbance at 260nm and 280nm (ratio of 1.7-2.1). Extracted RNA was stored at -80ºC until DNase treatment and complementary DNA (cDNA) conversion.

### 2.8.2 DNase treatment and cDNA conversion

RNA samples were treated with DNase I, amplification grade (Invitrogen, CA, USA) to remove any DNA contamination. Then, DNase treated RNA samples were reverse-transcribed to produced cDNA using SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, CA, USA). Detailed protocol is listed in Appendix I.

After the cDNA conversion, PCR was performed to detect β-actin to confirm successful cDNA conversion. The primers used for this PCR was forward (5'-CCTAAGGCCAACCGTGAA-3') and reverse (5'-AACCGCTCGTTGCCAATA-3'). PCR reaction steps were 94ºC for 2 minutes, then 35 cycles of 94ºC for 5 seconds, 58 ºC for 15 seconds, 72 ºC for 30 seconds, then 72 ºC for 2 minutes and hold at 10 ºC.

### 2.8.3 RT-PCR

This PCR was designed and performed to detect the intact *Ar* and exon 3-deleted *Ar* at mRNA level. The primers used for this PCR were mARX2-F (5'-GGACAGTACCAGGGACCAT-3') located on exon 2 of *Ar* gene and mARX4-R (5'-CCAAGTTTCTTCAGCTTACGA-3') located on exon 4 of *Ar* gene (Figure 2.10). PCR reaction steps were 94ºC for 2 minutes, then 35 cycles of 94ºC for 5 seconds, 58 ºC for 15 seconds, 72 ºC for 30 seconds, then 72 ºC for 2 minutes and hold at 10 ºC. As a result, intact *Ar* was detected at 288bps whereas exon 3-deleted *Ar* was detected at 171 bps.

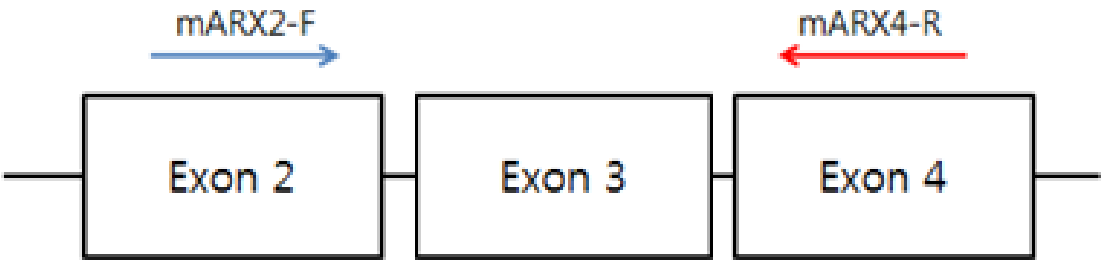



**Figure 2.10. Locations of ARCUT RT-PCR primers in the *Ar* allele to detect exon 3-deleted *Ar* gene.**

## 2.8.4 Real-time PCR

Quantitative real-time PCR was performed on cDNA samples using Corbett Rotor Gene 600 real time thermal cycler (Qiagen, Doncaster, Australia) in 0.1µl tubes. SensiMix SYBR Hi-ROX Kit (Bioline, Alexandria, NSW, Australia) was used as a master mix. The PCR reaction steps were 95ºC for 10 minutes, then 30-50 cycles of 95ºC for 20 seconds, 57-67 ºC for 20 seconds, 72 ºC for 20 seconds. Negative controls (RNase free water instead of cDNA) were included and a standard curve was used for each gene by serial dilutions (from PCR product generated by conventional RT-PCR made from the same primers used for the real-time PCR, Appendix I) between $10^{-2}$ and $10^{-14}$ (relative units). All quantifications were carried out in duplicates and the final volume of 10µl. Sample concentrations, relative to standards were established using mouse ribosomal protein L19 (*Rpl19*) or *Cyclophilin* as a cDNA loading control. Details of PCR reaction mixes used are listed in Appendix I. All primers used for real-time PCR were purchased from Sigma Aldrich Pty, Sydney, Australia and are listed in the Appendix IV.

## 2.9 Protein extraction and western blot

### 2.9.1 Protein extraction

Protein was extracted from snap frozen uterus for western blot analyses. Uterus (~30mg) was added to 500µl of RIPA buffer (Sigma, St. Louis, USA) containing PhosStop (1 tablet/ml of RIPA buffer) (Roche Diagnostics Australia, Castle Hill, Australia) and *cOmplete, Mini, EDTA-free* (1 tablet/ml of RIPA buffer) (Roche Diagnostics Australia, Castle Hill, Australia) in Lysing Matrix S tubes (MP Biomedicals Australia, Seven Hills, Australia). The tube containing tissue was homogenized at 375g for 15 sections up to 4 times and left on ice for 30 minutes. Tubes were vortexed and centrifuged at 13500g for 15 minutes in 4ºC. Top aqueous phase (contains protein) was carefully transferred into fresh 1.5ml Eppendorf tubes. Samples were kept on ice and protein concentration was measured on Enspire multimode plate readers (PerkinElmer, Waltham, USA) using Thermo Pierce 660nm protein assay (Life Technologies Australia Pty Ltd. Mulgrave, Australia) added with ionic detergent compatibility reagent (Life Technologies Australia Pty Ltd. Mulgrave, Australia). Extracted protein was stored at -80ºC for western blot.

### 2.9.2 Western blot

Western blots performed in chapter 4 were performed as previously described (Vignarajan et al., 2014). In brief, Bolt Western Blot and reagents from Life Technologies Australia Pty Ltd. Mulgrave, Australia was used. 10µg of protein was used for each sample. Proteins were denatured and loaded on to a gel

(Bolt 4-12% BT plus 10 well) and gel was ran on MES buffer at 165 volts for 1 hour. Protein was transferred to a membrane on NuPAGE transfer buffer at 30 volts for 2.5 hours at room temperature. Membrane was blocked with 5% Skim milk/TBST for 1 hour at room temperature followed by overnight primary antibody incubation at 4ºC. Primary antibody was washed three times in TBST for 10 minutes each and incubated with secondary antibody for 1 hour at room temperature and followed by TBST wash. Proteins on a membrane were visualized using Pierce ECL Western Blotting Substrate and detected under UV transilluminator (Bio-Rad Laboratories Pty Ltd, Gladesville, Australia). Image lab software (Bio-Rad Laboratories Pty Ltd, Gladesville, Australia) was used to quantify the intensity of proteins.

**Table 2.3. Details of primary antibodies and dilutions used for western blot.**

| Protein target | Name of Antibody | Manufacturer, catalogue #, and/or name of individual providing the antibody | Species raised in; monoclonal or polyclonal | Dilution used |
|---|---|---|---|---|
| **PTEN** | PTEN (138G6) | Cell Signalling (9559) | Rabbit; monoclonal | 1:500 |
| **AKT** | AKT 1/2/3 (H-136) | Santa Cruz Biotech (sc-8312) | Rabbit; polyclonal IgG | 1:500 |
| **P-AKT** | P-AKT 1/2/3 (Ser 473)-R | Santa Cruz Biotech (sc-7985) | Rabbit; polyclonal IgG | 1:300 |
| **p27** | P27 (C-19) | Santa Cruz Biotech (sc-528) | Rabbit; polyclonal | 1:500 |
| **B-actin** | Anti-beta Actin | Abcam (ab8229) | Goat; polyclonal | 1:3000 |

## 2.10 Liquid chromatography tandem mass spectrometry (LC-MS/MS)

LC-MS/MS was used to analyse steroid hormone levels in serum and ovaries by API-5000 triple-quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada).

### 2.10.1 Tissue preparation

Frozen ovary samples were transferred into 5 mL glass tubes. Ovary samples were then treated with 300 µL of homogenization buffer (0.5% BSA *(w/v)*, 5mM EDTA in PBS, pH 7.4) and homogenized on ice for 20 seconds using an IKA T10 basic disperser on the highest setting. The dispersion tool (probe) was cleaned between samples by rinsing in PBS. If any tissue sample remained, the probe was removed and the inside gently wiped. To pellet insoluble debris from the homogenate, samples were centrifuged at 840g for 20 minutes at 4°C before the supernatants were transferred to fresh 5 mL glass tubes. These were stored at -80°C until analysis. Blood serum was separated as described in above (section 2.4.3).

### 2.10.2 LC-MS/MS analysis

LC-MS/MS was operated by Ms Reena Desai at the Andrology laboratory, ANZAC research institute. The sample processing and analysis of serum and tissue steroids was performed as previously described in a study by Harwood and Handelsman (Harwood and Handelsman, 2009) and modified for mouse serum and tissue by McNamara and coworkers (McNamara et al., 2010a).

The lowest limits of quantification (defined as detectable with coefficient of variation <20%) were 10pg/mL for T, 20pg/mL for DHEA, 50pg/mL for DHT, 2.5pg/mL for $E_2$, 1pg/mL for $E_1$.

## 2.11 Corpus lutea (CLs) count

### 2.11.1 Ovary processing & embedding (resin)

PFA fixed ovaries were transferred into 70% ethanol and incubated for 1 hour. Then ovaries were transferred to 100% ethanol for 1 hour and this was repeated 3 times using fresh 100% ethanol each time. Then, in fume hood, ovaries were incubated in butanol for 1 hour twice.

Ovary was embedded in glycol methracrylate resin using TECKNOVIT KIT 7100 (Heraeus Kulzer, Chatswood, Australia). Infiltration solution was prepared by dissolving 1g of hardener I (1 sachet) in 100ml of methacrylate solution. Then embedding solution was prepared with 15ml of infiltration solution mixed with 1ml of hardner II. In fume hood, 1-2ml of embedding solution was placed in a mould to orientate ovary at the bottom centre of the mould. Then, pre-labelled block holder was place on top. The mould was left unmoved for 2 hours to maintain its orientation. After the embedding solution was hardened, mould with block holder was left in room temperature for 1-2 weeks until the block was completely solid.

Once, the blocks were completely solid, ovaries were serially sectioned at 20µm using Polycut S sliding microtome (Reichert Technologies, New York, USA). Sectioned were left to dry in 37ºC oven for overnight and Periodic acid Schiff staining was performed

### 2.11.2 Periodic acid Schiff staining and CLs count

Sectioned resin blocks were incubated in periodic acid (POCD Scientific, Artamon, Australia) for 30 minutes at room temperature followed by 10 minutes wash in running tap water. Then, in the fume hood, sections were incubated in Schiffs solution (Fronine, Thermo-Fischer Scientific, Waltham, USA) for 45 minutes at room temperature followed by 10 minutes wash in running tap water. Section were incubated in Mayers haematoxylin (Signma, St. Louis, USA) for 2 hours at 37ºC followed by 10 minutes wash in running tap water. Then, sections were incubated in Scott's bluing solution (Fronine, Thermo-Fischer Scientific, Waltham, USA) for 3 minutes and rinsed in tap water to remove Scott's bluing solution. Following the staining the sections were left air dry and coverslipped next day using DPX mountant for histology (Sigma, St. Louis, USA) and 24mm by 50mm coverslip (Coverglass No.1, ProSciTech, Thuringowa, Australia).

When the slides were completely dry, corpus lutea (CLs) was identified by morphological properties consistent with luteinized follicles and by being present in several serial sections. CLs were counted on each ovary using an Olympus microscope with Stereo investigator software (MicroBright Field, Williston, VT, USA) (Walters et al., 2009).

## 2.12 Data analysis

All the data were analysed using SPSS (SPSS, Chicago, IL, USA) software. Statistical significance was set at 95% confidence interval ($P<0.5$). Data were tested for normal distribution by homogeneity of variance test and evaluated using a one-way ANOVA (>2 groups), two-way ANOVA (2 variables) and independent samples t-test (2 groups) unless stated otherwise. *Post-hoc* tests were performed after a significant ANOVA ($P<0.05$) to determine the significance among groups. Non-parametric results were analysed using *Kruskal-Wallis* non-parametric test and combined with *Mann–Whitney U post-hoc* test.

# Chapter 3:

# The role of androgens acting via AR in uterine growth: Development and characterization of uterine glandular epithelium specific AR knockout mouse models

The content in this chapter has been published as:

**Jaesung (Peter) Choi**, Yu Zheng, Kate Skulte, David Handelsman, and Ulla Simanainen. Development and characterization of uterine glandular epithelium specific AR knockout mouse model. *Biology of Reproduction*. 93(5):120 (**2015**).

## 3.1 Abstract

While estrogens are the major driver of uterine development, androgens acting via the androgen receptor (AR) may also promote uterine growth as suggested by uterine phenotype in global AR knockout (ARKO) female mice. As AR is expressed in all the uterine cell types including glandular epithelial cells our aims of this experiment were to:

i) Generate and validate uterine glandular epithelial specific AR knockout (ugeARKO) mouse model using Cre/LoxP strategy.
ii) Investigate the role of glandular epithelial AR in uterine growth and function (fertility).
iii) Determine if AR mediated androgen actions are required for testosterone and DHT induced uterine regrowth upon ovariectomy.
iv) Determine the role of glandular epithelial AR in androgen induced uterine regrowth upon ovariectomy.
v) Determine if uterine glandular epithelial specific knockout mouse model could be used as a possible new uterine cancer model.

Our results show that endogenous androgen actions via AR in uterine gland epithelium may not be critical for normal uterine development and function as ugeARKO females had normal uterine development and fertility. To determine if exogenous androgens acting via AR can fully support uterine growth in absence of estrogens the ARKO and ugeARKO females were ovariectomised (OVX) and treated with supraphysiological doses of testosterone or DHT (non-aromatizable androgen). Both DHT and testosterone supported full uterine regrowth in wild-type (WT) females while ARKO females had no regrowth

(comparable to OVX only). These findings suggest that androgens acting via AR can promote full uterine regrowth in the absence of estrogens. The ugeARKO had 50% regrowth of the uterus when compared to intact indicating that androgens can act locally via AR within the uterus to support uterine growth. Histomorphologically, both the endometrial and myometrial areas were significantly ($p<0.05$) reduced, suggesting glandular epithelial AR located in endometrium may indirectly modify myometrial development. Additionally, to confirm the Cre function in endometrial glands, we generated uterine glandular epithelium specific PTEN knockout (ugePTENKO) mouse model. The ugePTENKO females developed severe endometrial hyperplasia and therefore present a novel model of uterine pathology for future research.

## 3.2 Introduction

Androgens are 19-carbon steroid hormones which mediate their effects via androgen receptor (AR). AR is encoded by an X chromosomal gene and belongs to the nuclear receptor superfamily (Lubahn et al., 1988). It is expressed in target organs including the uterus of both rodents (Pelletier et al., 2004, Hirai et al., 1994) and humans (Kimura et al., 1993, Ito et al., 2002) being detected in the luminal and glandular epithelial and stromal cells in endometrium as well as myometrial cells of the human uterus (Ito et al., 2002, Kimura et al., 1993). Similarly in the mouse uterus, *Ar* mRNA is expressed in the luminal and glandular epithelial cells, stromal cells and smooth muscle cells (Pelletier et al., 2004) and the intensity of *Ar* mRNA labelling is quite uniform throughout the different uterine compartments. The biological effects of androgens in male physiology are well known, but their physiological roles in the female beyond being the obligate precursors for conversion to estrogens by aromatase (Hillier et al., 1994) have only recently been recognized (Walters et al., 2007).

Androgens have growth and differentiation promoting actions in rodent uterus (Armstrong and Papkoff, 1976, Schmidt and Katzenellenbogen, 1979, Nantermet et al., 2005, Walters et al., 2009). Already in the 1970s, it was demonstrated that administration of androgens (testosterone or non-aromatizable dihydrotestosterone, DHT) to immature hypophysectomised rats significantly increased uterine weights (Armstrong and Papkoff, 1976, Schmidt and Katzenellenbogen, 1979). In addition, the global AR knockout (ARKO) female mice have thinner uterine wall and reduced total uterine area compared to the WT controls (Walters et al., 2009), suggesting the growth promoting role

of androgens in uterus is mediated via the AR. Hence, these *in vivo* studies collectively suggest a uterine growth promoting function of androgens, acting via AR. However, in contradiction, the aromatizable pro-androgen, androstenedione was shown to inhibit the proliferation of human endometrial epithelial cells, an effect which was reversed by an antiandrogen (cyproterone acetate) indicating local, uterine cell specific AR mediated actions may also inhibit uterine growth (Tuckerman et al., 2000). Furthermore, the role of androgens in clinical uterine cancers has been proposed (Gibson et al., 2014).

Androgens also have a role in optimal female fertility, as androgen insensitive ARKO female mice produce significantly fewer pups compared to the WT females in mating experiments (Walters et al., 2007). Glandular epithelial cells in the uterine endometrium synthesize, secrete and transport proteins and related substances that nourish the developing embryo (Bazer, 1975, Roberts and Szego, 1953, Simmen and Simmen, 1990) and these secretions are vital for the survival and development of embryos (Gray et al., 2001b). Therefore, successful morphogenesis of the endometrial glands is critical to the gestational capacity of the uterus. Furthermore, disrupted endometrial gland formation in adult mice can result in permanent infertility (Jeong et al., 2010, Franco et al., 2010). As the AR is also specifically expressed in the endometrial gland epithelia, we therefore aimed to develop (Cre/loxP) and characterize uterine endometrial gland specific AR knockout (ugeARKO) mouse model. Using the ugeARKO in comparison to ARKO females, we further characterized the role of androgens acting via AR in uterine development and function. In addition, as most endometrial carcinomas originate from glandular epithelia, we further characterized the endometrial glands specific Cre activity by generating uterine

glandular epithelium specific PTEN tumor suppressor (Dahia, 2000) knockout (ugePTENKO) as PTEN inactivation in uterus induces endometrial cancer in mice (Stambolic et al., 2000, Daikoku et al., 2008).

## 3.3 Experimental design

Experimental designs for this part of the PhD project were carried out as the following:

i) To validate the ugeARKO mouse model, WT, ugeARKO, R26R and ugePTENKO mice were generated as described in methods (see 2.1). WT and ugeARKO mice were collected at 20 weeks of age, R26R mice were collected at 10 and 20 weeks of age and ugePTENKO mice were collected at 30 weeks of age.

ii) The role of glandular epithelial AR in uterine growth and function (fertility) was investigated by comparing WT, ARKO and ugeARKO females at 20 weeks of age.

iii) To determine if AR mediated androgen actions are required for uterine regrowth and if glandular epithelial AR plays a role, WT, ARKO and ugeARKO females were ovariectomised and treated with androgens. Experimental details described below (Figure 3.1). These mice were collected at either 11 weeks or 20 weeks. Initially 20 weeks was chosen to be consistent with intact mice. However, 11 weeks were chosen for DHT treatment as we did not observe significant changes between 11 and 20 weeks.

iv) To determine if ugePTENKO mouse model could be used as a possible new uterine cancer model, uterine weight and histopathology as well as PCNA (proliferating cell nuclear antigen) was compared between WT and ugePTENKO at 30 weeks of age.

### *Genotype confirmation*

Excision of exon 3 of *Ar* in ARKO and ugeARKO uterus was confirmed on genomic DNA and mRNA level. Genomic DNA was released using proteinase K digestion of uterus or by DNA extraction kit (Qiagen DNeasy Blood and Tissue Kit, Hilden, Germany). PCR on gDNA was performed as previously described (Notini et al., 2005b). In addition, excision of exon 3 of *Ar* in the uterus was confirmed by RT-PCR on the RNA extracted from uteri as previously described (Simanainen et al., 2007). Two product sizes were obtained, 288 bps for intact *Ar* and 171 bp for Cre-mediated exon 3 excised *Ar*.

To confirm specific excision of exon 3 of *Ar* occurs solely in glandular epithelial cells of ugeARKO, genomic DNA was extracted from laser micro-dissected formalin fixed paraffin embedded (FFPE) uterus samples. In brief, FFPE uterine sections were sectioned at 15µm thickness on to a membrane slides (MembraneSlide NF 1.0 PEN (D), Carl Zeiss, Sydney, Australia). Standard H&E staining was performed to visualize glandular epithelial cells. PALM Duoflex Combi System was used for laser capture micro-dissection. Upon laser micro-dissection, glandular epithelial cells were captured in adhesive cap (AdhesiveCap 500 opaque (D), Carl Zeiss, Sydney, Australia). In addition, laser micro-dissection was performed to capture non-glandular uterus (i.e. uterus excluding glandular epithelium). Excision of DNA between floxP sites of *Ar* (exon 3) was confirmed by PCR using same primers as previously described (Gao et al., 2014) creating an exon 3 excised *Ar* at 510bps. Furthermore, to confirm that functional *Ar* was still present in non-glandular uterus tissue, PCR using same primers as for genotyping (Simanainen et al., 2012) detected

undeleted (floxed) *Ar* at 280 bps confirming tissue-specificity of Cre activity for glandular and not non-glandular uterus.

Excision of exon 5 in PTEN knockout was confirmed on genomic DNA. Exon 5 excision of *Pten* was confirmed using same primers as for genotyping (Byun et al., 2011). Two product sizes were obtained, 650 bps for intact *Pten* and 300 bp for Cre-mediated exon 5 excised *Pten.* β-Actin was used as a loading control. Exon 5 deletion in *Pten* was not confirmed by RT-PCR using RNA the Cre-mediated excision of DNA between loxP sites of *Pten* produces stop codon and no exons are produced after exon 4 (Vlietstra et al., 1998).

Real-time PCR was performed to determine *Pbsn* mRNA expression in the uterus. Prostate samples were used as a positive control. PBSN PCR was performed as previously described in (Simanainen et al., 2009, Doles et al., 2005).

## *Model characterization*

To characterize the uterine specificity of PBSN-Cre in females, the uterus, ovaries, mammary gland and pituitary were collected from $R26R^{Cre+}$ and $R26R^{Cre-}$ females at 10 and 20 weeks of age. In addition, to confirm that the PBSN-Cre mediated excision of tumor suppressor PTEN generates glandular hyperplasia, the ugePTENKO females were collected at 30 weeks of age to be subjected to PCR and immunocytochemistry for detection of Cre-mediated recombination. To determine the time of PBSN-Cre activation, ugePTENKO females were also collected at 3, 6 and 8 weeks.

### *Ovariectomy and implants*

To further characterize the androgen action in the uterus, a group of female mice were ovariectomised (OVX) under anaesthesia at 5 weeks and had 1 cm silastic implants (inner diameter=1.47mm and outer diameter=1.95mm, Dow-Corning, Sydney, Australia) either containing ~10 mg crystalline testosterone or no steroid placed subdermally for 15 weeks and subsequently collected at 20 weeks of age (Figure 3.1A). Initially, long term testosterone treatment was given to allow enough time for the testosterone to show effects on uterine regrowth. To investigate whether the non-aromatizable DHT behaved similarly to testosterone, a second group of female mice were ovariectomised at 8 weeks and received silastic implants subdermally containing either testosterone or DHT (2x0.5cm silastic implant filled with ~5mg crystalline DHT (Simanainen et al., 2009)) or empty implant for 3 weeks with mice sacrificed for tissue collection at 11 weeks old (Figure 3.1B). Short-term treatment was used based on the findings from our long-term treatment together with our laboratory's previous experiences with DHT treatment of male mice where significant changes occurred within three weeks of DHT treatment (Simanainen et al., 2011a). Intact females were also collected at the age of 11 weeks and 20 weeks.

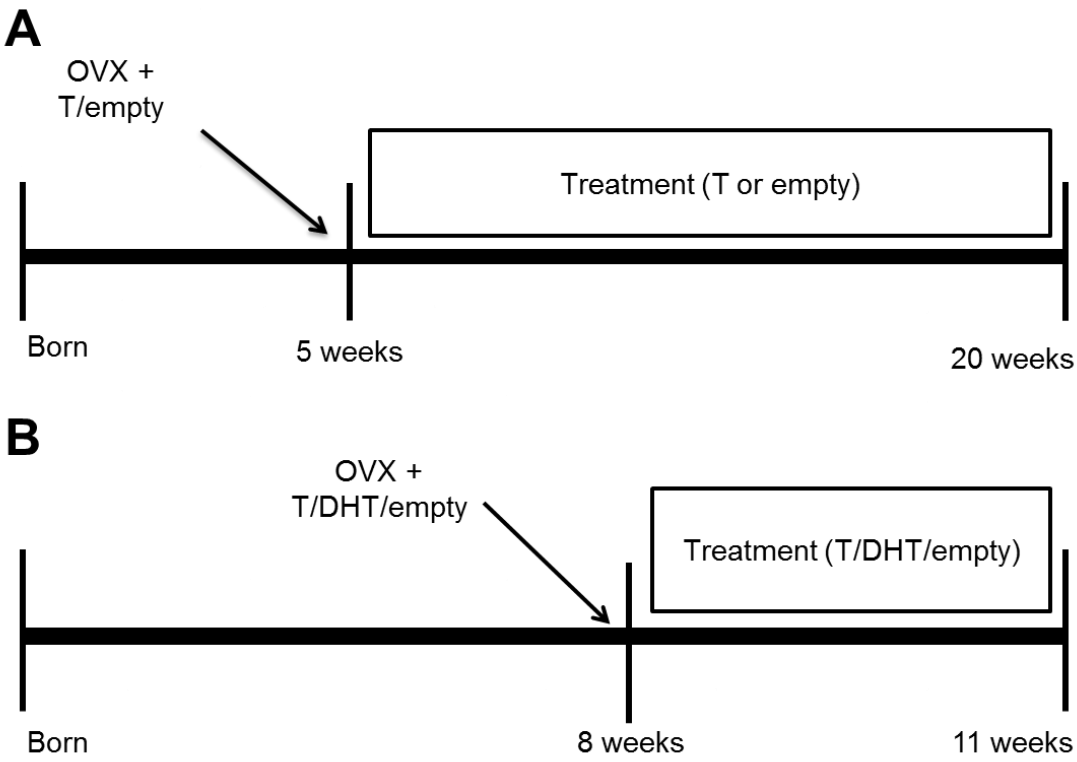



**Figure 3.1. Experimental design.** A) Experimental procedure for the group of females ovariectomised (OVX) at 5 weeks of age and treated with testosterone (T) or empty implant for 15 weeks. B) Experimental procedure for the group of females with OVX at 8 weeks of age and treated with T or dihydrotestosterone (DHT) or empty implant for 3 weeks.

## 3.4 Results

### 3.4.1 Successful generation and validation of endometrial gland specific AR knockout mouse model

PBSN-Cre mice are generally used to direct the Cre expression selectively into prostate epithelial cells (Simanainen et al., 2007). However, unexpectedly we observed possible uterine specific expression in female mice and therefore further validated the PBSN-Cre mouse model for uterine specific Cre expression. This was achieved by localizing the Cre activity in female mice. PBSN-Cre mice were crossed with R26R mouse. Tissues were collected at two different time points, at 10 and at 20 weeks of age. The Cre activity was analyzed in uterus, ovary, mammary gland and pituitary of both the 10 and 20 weeks of age. Blue lacZ staining was only detected in the glandular epithelial cells of the uterus (Figure 3.2A-B). No staining was detected in the Cre negative uterus (Figure 3.2C). Ovary, mammary gland, and pituitary (Figure 3.2D-F) were all negative for lacZ staining. Morphology of Cre positive and negative uterus is shown in Figure 3.2G-H.

PCR was used to confirm SOX2-Cre and PBSN-Cre mediated excision of exon 3 of *Ar* in ARKO and ugeARKO uterus. Exclusively native or exon 3 deleted *Ar* RT-PCR product was detected in WT or ARKO uterus, respectively. Due to uterine glandular epithelial cell specific Cre activity, ugeARKO uterus showed weak exon 3 deleted *Ar* product (glandular epithelial cells) and strong native *Ar* PCR product (non-glandular uterine cells) (Figure 3.2I). Similar to the WT ovaries, the ovaries of ugeARKO females only expressed native *Ar* (Figure 3.2I).

Furthermore, laser micro-dissection was performed to confirm excision of exon 3 of *Ar* in ugeARKO uterus is specific to glandular epithelial cells (Figure 3.2J-K). Exon 3 deleted *Ar* PCR product was only detected in glandular cells of ugeARKO uterus (Figure 3.2L) whereas undeleted (floxed) *Ar* PCR product was detected solely in the non-glandular tissue of the uterus in ugeARKO mice (Figure 3.2M).

Similarly, PCR was used to confirm PBSN-Cre mediated excision of exon 5 of *Pten* in ugePTENKO uterus. Due to uterine glandular epithelial cell specific Cre activity, ugePTENKO uterus shows weak exon 5 deleted *Pten* product (glandular epithelial cells) and strong naïve *Pten* PCR product (non-glandular epithelial cells) (Figure 3.2N). Similar to the WT ovaries, the ovaries of ugePTENKO females only expressed naive *Pten* (Figure 3.2N). To further confirm the gland specificity of PBSN-Cre activity, PTEN immunohistochemistry was performed in WT and ugePTENKO uterus. Uterine glands were strongly PTEN immunopositive in WT uterus (Figure 3.2O), but were negative for PTEN immunostaining in ugePTENKO uterus (Figure 3.2P). Negative control did not show any PTEN immunostaining in uterus (Figure 3.2Q).

Furthermore, to determine the age dependency of PBSN-Cre activation, the *Pten* PCR was performed on uteri at age of three, six and eight weeks. Exon 5 deleted *Pten* product was only detected at eight weeks of age but not earlier (Figure 3.3) showing that PBSN-Cre is activated after six weeks but before the age of eight weeks.

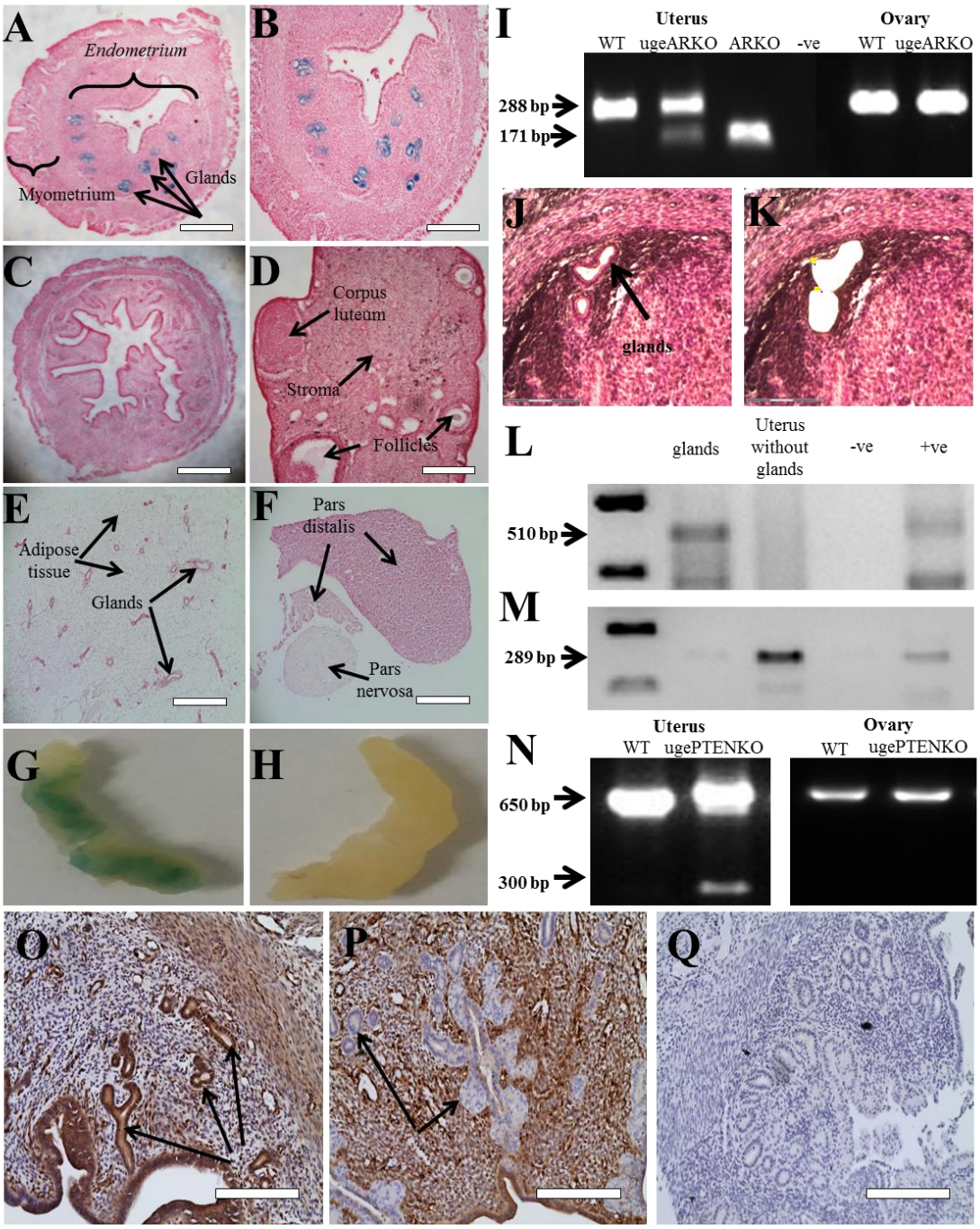



**Figure 3.2. Phenotypic (R26R mice) and genotypic characterization of endometrial glandular epithelial specific Cre expression.** A-B) β-Galactosidase staining of uterine section of PBSN-Cre positive 10 weeks old R26R mouse (n≥4; litter independent). C) β-Galactosidase staining of uterine section of PBSN-Cre negative 10 weeks old R26R mouse demonstrating lack of

endogenous β-galactosidase activity in uterus (n≥4). D-F) β-Galactosidase staining of ovary, mammary gland and pituitary sections of PBSN-Cre positive 10 weeks old R26R mouse demonstrating lack of β-galactosidase activity (n≥4). E-F) Morphology of 10 weeks old PBSN-Cre positive (G) and negative (H) uterus. I) RT-PCR showing WT *Ar* and exon 3 excised *Ar* products in the ugeARKO uterus and exon 3 excised *Ar* product only in ARKO as positive control. ugeARKO ovaries had only WT *Ar* product (20 weeks old mice). J-K) Representative photo of laser micro-dissection of uterine glands showing undissected FFPE section with glands (J) and the same section following dissection of glands (K). L) PCR products showing exon 3 excised *Ar* product in ugeARKO uterine glands only at 20 weeks old. Undissected FFPE ugeARKO was used as a positive control. M) PCR showing floxed *Ar* (functional *Ar*) product in ugeARKO non-glandular uterus at 20 weeks old. Undissected FFPE ugeARKO was used as a positive control. N) PTEN PCR showing WT *Pten* and exon 5 excised *Pten* present in ugePTENKO uterus while the WT uterus and ovaries of both WT and ugePTENKO females expressed only WT *Pten* at 20 weeks old. O-Q) Representative photo of PTEN immunohistochemistry demonstrating gland specific PTEN deletion (PBSN-Cre activity) in ugePTENKO uterus (P) (arrows pointing PTEN immunonegative glands) compared to WT (O) (arrows pointing to PTEN immunopositive glands) and negative control (Q) at 20 weeks old. Scale bars= 1000µm (A and C), 400µm (B, D, E, F, O and Q), 200µm (P).

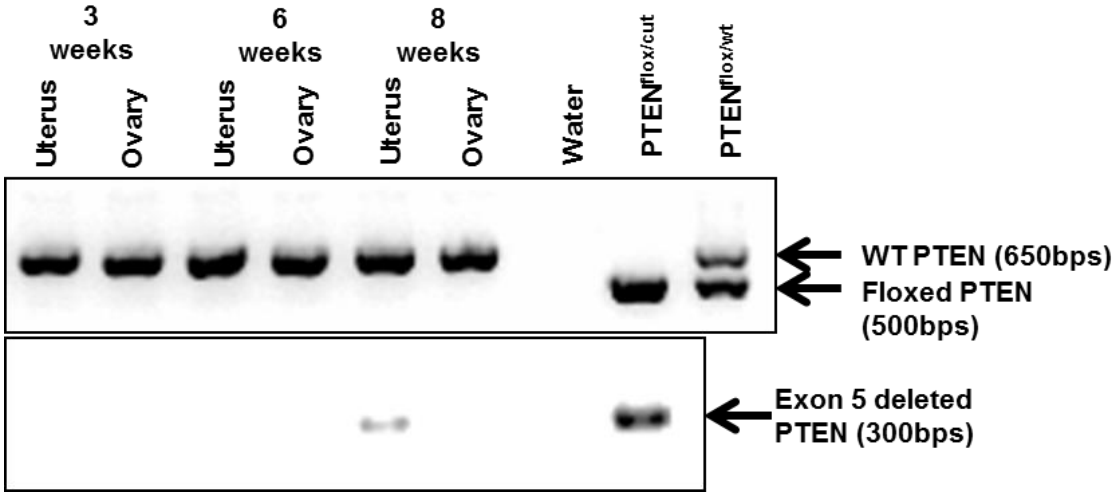



**Figure 3.2. Determining time of PBSN-Cre activity using PTEN PCR.** PTEN PCR has been conducted at 3, 6 and 8 weeks old uteri and ovaries to show time of PBSN-Cre activation in the uterus. Exon 5 deleted PTEN band (300bps) was only present at 8 weeks old uterus but not in other time points. Hence, the data indirectly shows that Pbsn-Cre is active at 8 weeks old uteri but not in ovaries.

To further validate the functional PBSN-Cre activity in ugePTENKO mice, the uterine weights and morphology were compared between WT and ugePTENKO females. ugePTENKO uterine weight was significantly increased compared to WT females (Figure 3.4A). Macroscopic analysis of ugePTENKO uterus demonstrated clearly abnormal uterus compared to WT (Figure 3.4B-C). Histological analysis of ugePTENKO uterus suggested that the increased weight and abnormal appearance was due to the presence of hyperplastic and

neoplastic uterine glands in ugePTENKO uterus not present in WT uteri (Figure 3.4D-F).

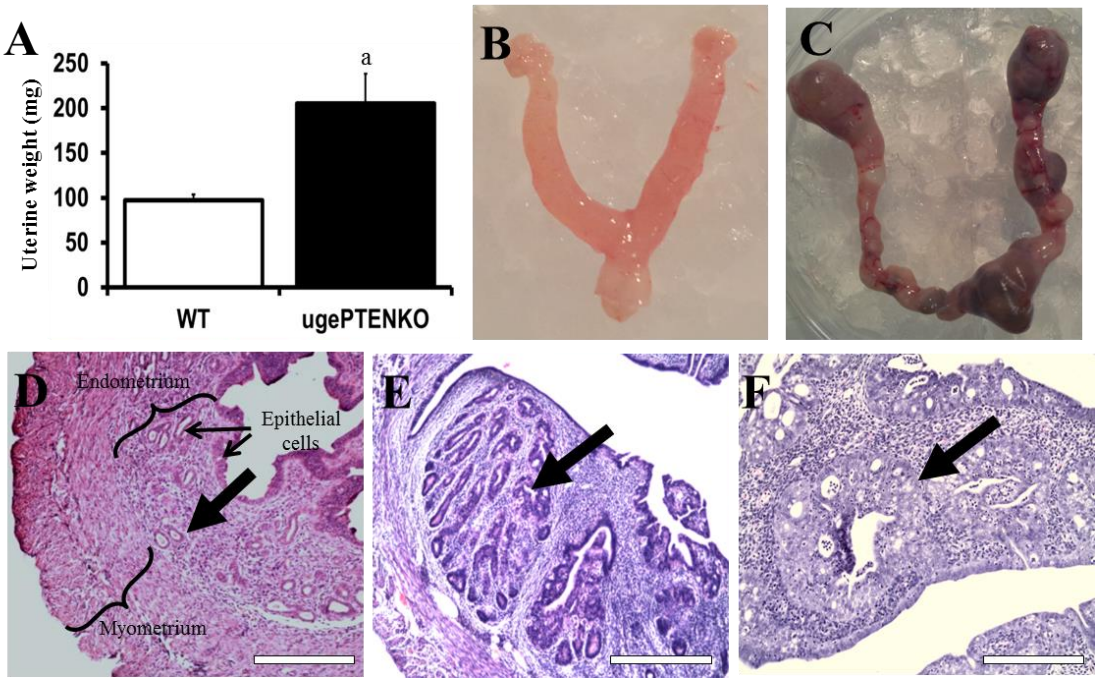



**Figure 3.4. Uterine weight and histology of endometrial gland PTEN knockout (ugePTENKO) uterus at 20 weeks of age.** A) Uterine weights were compared between WT and ugePTENKO at 20 weeks of age [n≥9; a=significantly different to WT ($p<0.05$); T-test]. B-C) Morphology of WT (normal) (B) and ugePTENKO (abnormal) uterus (C). D-F) Representative histology (H&E) of normal WT uterus (D) (endometrium layer mainly consists of stroma and epithelial cells whereas myometrium area mainly consists of smooth muscle cells) and hyperplastic glands (E) and intraepithelial neoplastic glands (F) in ugePTENKO uterus. (Arrows indicating endometrial glands which are lined by epithelial cells; scale bars=200µm (D) and 400µm (E and F)).

*Pbsn* mRNA was not detectable in our uterine samples at the age of 20 weeks by real-time PCR (Figure 3.5).

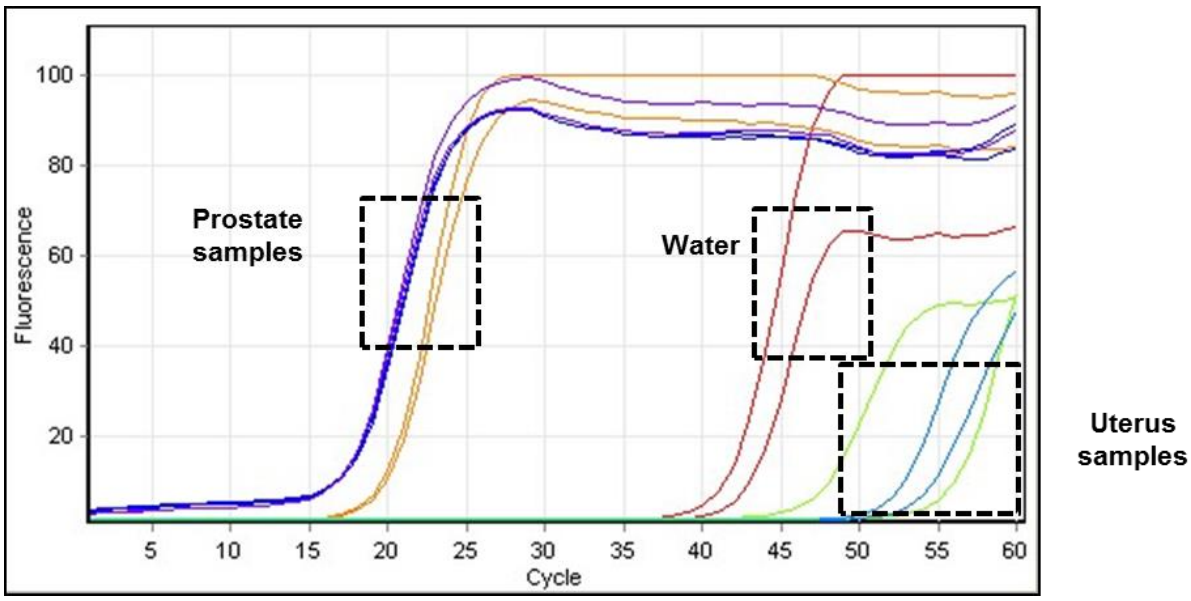



**Figure 3.3. Real-time PCR comparing *Probasin* gene expression in the uterus at 20 weeks.** *Probasin* gene expression was compared between prostate and uterus. Prostate samples (n=3) show fluorescence before water (negative control; ~40 cycles) at around 20 cycles. Whereas uterus samples (n=2) show fluorescence well after water at around 50 cycles.

### 3.4.2 Endometrial gland specific AR inactivation does not modify uterine growth or function (fertility)

No significant differences in uterine weight were detected between WT females from ARKO or ugeARKO colonies (Figure 3.6) and therefore the data from both WT female lines were combined. The uterine weights were similar between WT, ARKO and ugeARKO females at 20 weeks of age (Figure 3.7 Table). Also, there were no changes in histomorphometric measurements such as total uterine area or endometrial and myometrial area (Figure 3.7 Table).

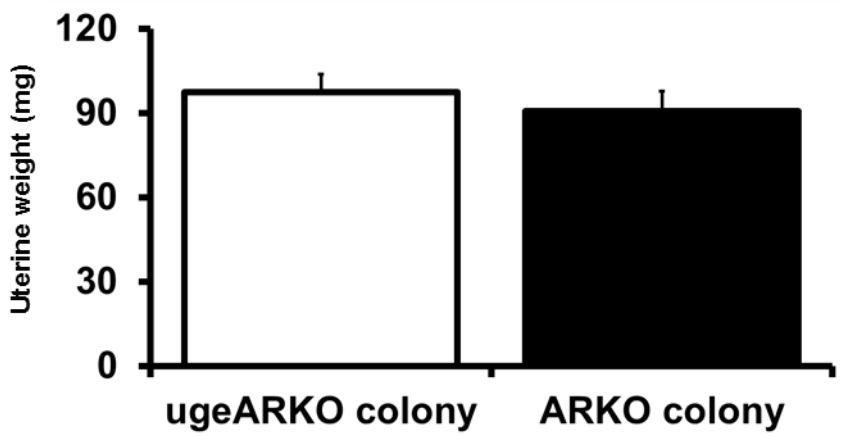



**Figure 3.4. Uterine weight of WTs from ugeARKO colony and ARKO colony.** There was no difference between WTs from ugeARKO colony and ARKO colony. Hence, in our experimental data both WTs were combined. Data shown as mean±SE; n≥8.

Fertility of WT and ugeARKO females was compared by determining the pups per litter and total number of pups produced by the female over 5 months period. No difference in pups per litter was observed between WT and ugeARKO females where both produced approximately 11 pups per litter (Figure 3.7A). Also, total number of pups produced over 5 months period for each female did not differ between WT and ugeARKO, both groups producing approximately 50 pups over 5 months period (Figure 3.7B). This suggests that inactivation of endometrial glandular epithelial AR did not affect fertility of female mice aged between 8 weeks to 5 months.

| | Uterine Weight (mg) | Total area ($mm^2$) | Endometrial area ($mm^2$) | Myometrial area ($mm^2$) |
|---|---|---|---|---|
| WT | 94.2±4.7 | 2.1±0.2 | 0.9±0.1 | 1.2±0.1 |
| ARKO | 100.2±9.2 | 1.9±0.1 | 0.7±0.1 | 1.2±0.1 |
| ugeARKO | 93.8±11.8 | 2.4±0.4 | 1.0±0.2 | 1.4±0.2 |

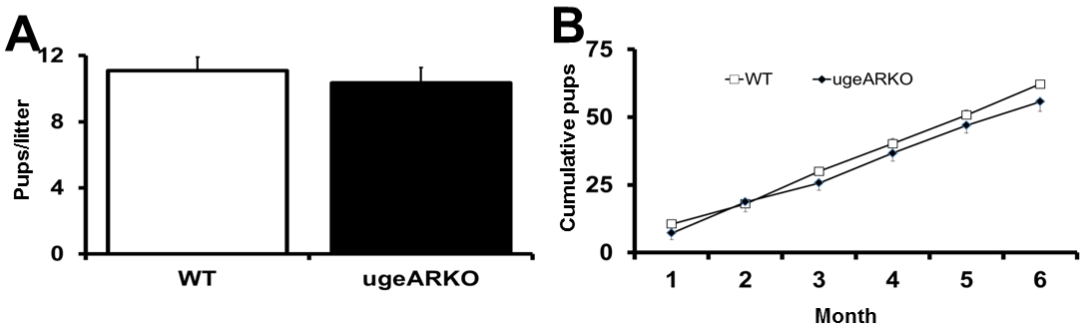



**Figure 3.5. Uterine weight, morphological measurements and fertility.** Uterine weights and morphology (Table) was compared between WT, ARKO and ugeARKO females at 20 weeks of age. In addition, the fertility (pups per litter, cumulative pups) was compared between ugeARKO and WT females (A-B). Data shown as mean±SE; n≥4.

### 3.4.3 AR mediated androgen actions are required for testosterone induced uterine growth

As expected, OVX at five weeks of age prevented normal uterine growth in all females with the uterine weights in OVX groups being about 15% of the intact female uterine weights at 20 weeks of age (Figure 3.8A). In WT females, T treatment following OVX (OVX+T) fully restored the uterine weight to the intact level at 20 weeks of age (Figure 3.8A). However, no uterine growth was detected following OVX and T treatment in ARKO females and the uterus weight remained similar to OVX only females (Figure 3.8A), suggesting that AR mediated T effects are required for uterine growth. Interestingly, in ugeARKO females, testosterone treatment in OVX+T group partial restoration (approximately 50%) of uterine weights were observed upon T treatment in OVX+T group when compared to intact ugeARKO uterus weights (Figure 3.8A).

### 3.4.4 Endometrial glandular AR modifies testosterone induced uterine growth (both the endometrium and myometrium)

Morphological measurements notably total area, endometrial and myometrial area were measured in the uterus. However, these measurements could not be made in OVX group and OVX+T treated ARKO as these uteri were too underdeveloped to make these measurements accurately (Figure 3.8B-D). OVX group (Figure 3.8K-L) had similar histological appearances to OVX+T treated ARKO uteri (Figure 3.8G-H).

OVX+T treated WT uterus had full regrowth of all the areas (endometrial and myometrial area) and they were similar to WT intact (Figure 3.8B-D). Uterine areas (total, endometrial and myometrial) of OVX+T treated ugeARKO were approximately 50% of OVX+T treated WT uterus (Figure 3.8B-D). This suggests that endometrial glandular epithelial AR plays a role in the uterine development modifying not only the endometrium but myometrium as well. This results in the maintenance of a stable endometrium to myometrium ratio such that both the WT and ugeARKO in OVX+T group had similar endometrium to myometrium ratio (Figure 3.8E). Representative figures of each OVX+T treated uterus are shown below (Figure 3.8F-M).

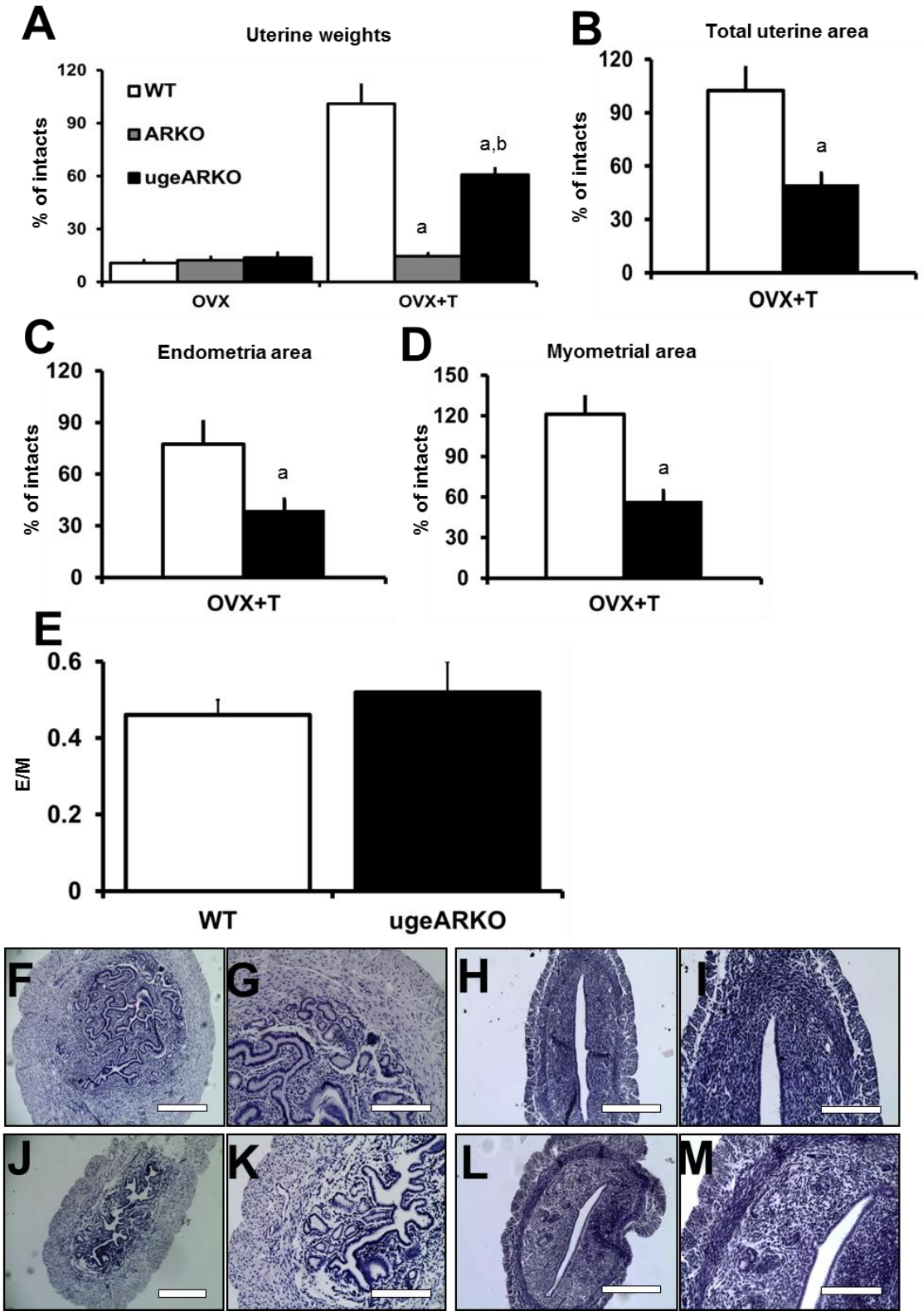



**Figure 3.6. Uterine weights and morphological measurements of ovariectomised and T treated mice at 20 weeks of age relative to respective intacts.** Uterine weights (A) and uterine morphology (B-D) following

ovariectomy with or with testosterone (T) treatment [a=significantly different to WT ($p<0.05$; one-way ANOVA), b= significantly different to ARKO ($p<0.05$; one-way ANOVA); mean±SE; n≥5]. E) Endometrium to myometrium ratio between OVX+T treated WT and ugeARKO. Endometrium to myometrium ratio was similar between WT and ugeARKO uterus. E/M=endometrium/myometrium. Data shown as mean±SE; n≥5. F-M) Representative photos of uterine histology (H&E staining) of OVX+T treated WT (F and G), ARKO (H and I), ugeARKO (J and K) and OVX alone (L and M). (Scale bars=400μm in F and J; scale bars=200μm in G, H, K and L; scale bars=100μm in I and M).

### 3.4.5 Androgens alone can promote full uterine regrowth and do not require E2 in mice

As T can be aromatized into $E_2$ via aromatase activity, we used non-aromatizable androgen, DHT to determine if AR mediated androgen effect on uterine growth requires $E_2$. Intact WT and ARKO uterine weights were similar, while the OVX group at 11 weeks of age (ovariectomised at eight weeks old) significantly reduced ($p<0.05$; non-parametric test) uterine weights in both WT and ARKO (Figure 3.9A). Both T and DHT treatment at the time of OVX for three weeks, restored the uterine weights to intact level in WT but had no effect on uterine weights in ARKO females. This finding suggests that androgens (testosterone and DHT) alone acting via AR can promote full uterine regrowth in the absence of $E_2$ (Figure 3.9A).

### 3.4.6 Treatment protocols produced supraphysiological serum levels of testosterone and DHT

To determine if the comparability of the intact female mouse serum T and DHT levels to those produced by the steroid hormone implants used in the present study, serum T, DHT, DHEA, $E_1$ and $E_2$ levels were measured using LC-MS/MS. Serum testosterone was 20-fold higher ($p \leq 0.05$; non-parametric test) in OVX+T treated compared to intact WT and ARKO females, suggesting that T implant produced supraphysiological testosterone levels (Figure 3.9B). In addition, OVX reduced serum T levels when compared to intact females.

Serum DHT levels were increased 8 and 36-fold in OVX+DHT treated compared to intact WT and ARKO females, respectively, demonstrating that also DHT implants produced supraphysiological DHT levels (Figure 3.9C). Unexpectedly, serum DHT was significantly higher in OVX+DHT treated ARKO females compared to OVX+DHT treated WT females despite the similar implants used. Serum DHT was also increased in OVX+T treated WT females (compared to intact and OVX) but not in ARKO suggesting significant conversion of T to DHT in WT but not in ARKO females. Serum DHEA, $E_1$ and $E_2$ were below the assay detection limits.

### 3.4.7 Testosterone and DHT via AR induced uterine regrowth in WT uterus and AR and ER may share the same signaling pathway

To determine if androgen induced uterine regrowth was via AR and if AR and ER share a signaling pathway, we investigated ERα dependent gene, *Lactoferrin* mRNA expression (Figure 3.9D). As expected, ovariectomy in WT and ARKO significantly reduced ($p<0.05$) *lactoferrin* expression compared to intacts. *Lactoferrin* expression was significantly increased in OVX+T and OVX+DHT groups compared to OVX alone in WT (which was comparable to WT intact) but there were no changes in ARKO suggesting androgen induced *lactoferrin* expression was via AR. Similar results were shown in other ERα dependent genes, Complement component 3 (*C3)* and Glucose-6-phosphate dehydrogenase *(G6PDH)* (Figure 3.9 E-F). Furthermore, AR and ER may share same signaling pathway. Surprisingly, in intact females, *lactoferrin, C3* and *G6PDH* expression was significantly increased ($p<0.05$; non-parametric test) in ARKO uterus compared to WT uterus.

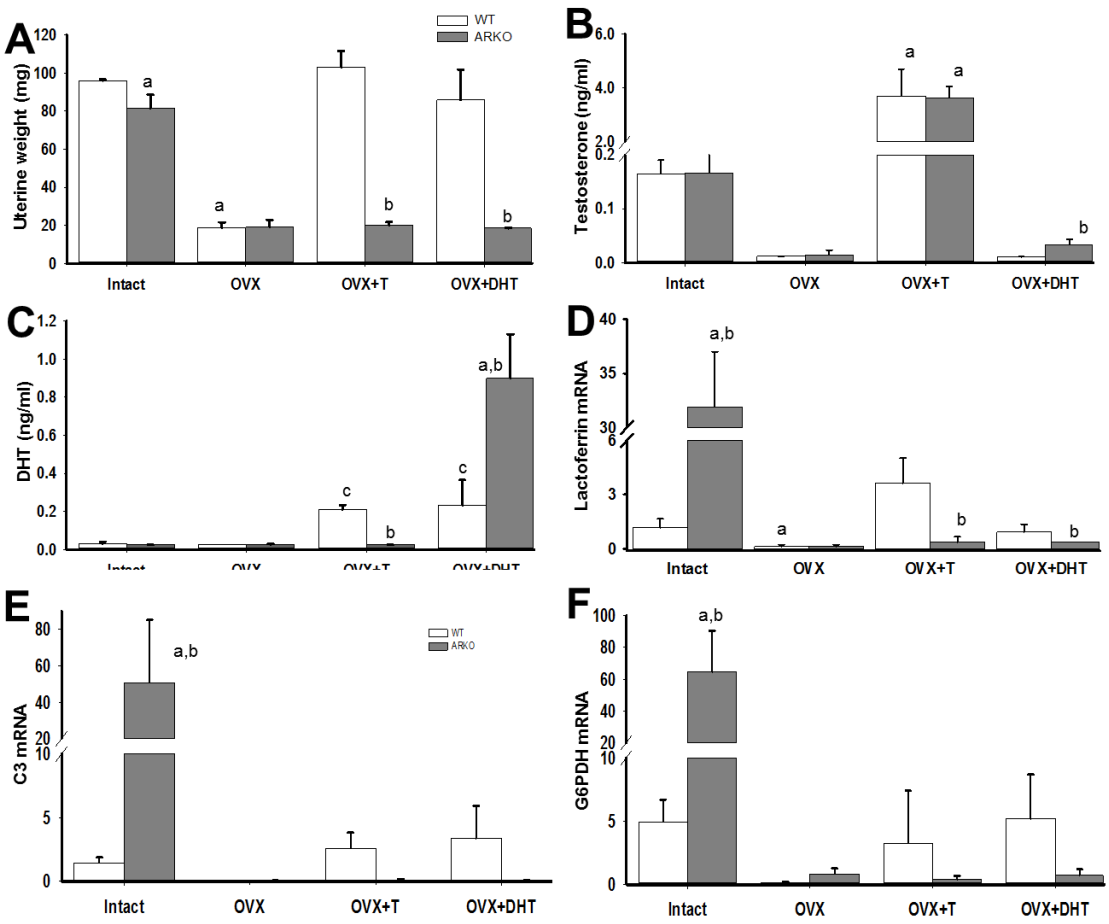



**Figure 3.7. Uterine weights, steroid hormone levels and ERα dependent gene mRNA expressions following ovariectomy at eight weeks of age and androgen (testosterone (T) or DHT) treatment for three weeks.** Uterine weight (A), serum T (B) and DHT (C) levels, *lactoferrin* (D), C3 (E) and G6PDH (F) mRNA expression was compared between WT and ARKO female upon ovariectomy and treatment with T or DHT [a= significantly different to other treated groups in respective genotype, b= significantly different to WT in the respective treatment group, c=significant to intact and OVX WT ($p \leq 0.05$; non-parametric test); mean±SE; $n \geq 3$].

## 3.5 Discussion

AR is expressed in the mouse (Pelletier et al., 2004, Hirai et al., 1994) and human (Kimura et al., 1993) uterus and direct androgen actions via AR has been suggested to regulate uterine physiology and growth (Walters et al., 2010). In mouse, AR is abundantly expressed throughout the uterus including endometrial glands (Pelletier et al., 2004). We therefore explored the role of androgens via the AR in experimental uterine development and, for the first time, generated and characterized endometrial gland specific knockout mouse model to investigate the role of endometrial gland specific AR in uterine development and fertility. Our discovery that the PBSN-Cre directs selective endometrial gland expression of Cre may be of value to further experimental research into uterine physiology and pathology.

To determine the role of AR mediated, endogenous androgen actions in murine uterus development, we compared uterus weights and uterine structure between sexually mature WT, ugeARKO and ARKO females. AR inactivation in either endometrial glands or globally, did not significantly modify uterine weights or uterine structure in our study. Similarly, in our previous study uterine weights were similar between WT and ARKO females whereas the total, endometrial and myometrial uterine areas were significantly reduced in ARKO when compared to WT at 8 weeks old females (Walters et al., 2009). While the model of AR inactivation between the studies is similar, the discrepancy in the affected uterine areas between the previous and present study could be due to the different age and genetic background of the mice. Uterine function (as indicated by fertility) appeared to be unaffected by endometrial gland specific AR inactivation, as pups per litter and total number of pups over five-month mating

study were similar between ugeARKO and WT females. Although there are no studies suggesting the role of endometrial gland AR inactivation in fertility, our previous study has shown that global AR inactivation significantly reduced fertility by 60% compared to the control group (Walters et al., 2009). Despite endometrial glands synthesizing essential substances for survival and development of the embryo (Gray et al., 2001a), our study suggested that lack of endogenous androgen actions via endometrial gland AR does not have significant effect on uterine development and fertility in mice, presumably due to redundancies in molecular pathways to provide this essential support for embryo development.

However, as androgens are suggested to support uterine growth (Armstrong and Papkoff, 1976, Schmidt and Katzenellenbogen, 1979), we further investigated the role of global AR and endometrial gland specific AR inactivation by removing ovarian steroids (estrogens, progesterone) by ovariectomy and administering a testosterone implant. As expected, ovariectomy reduced uterine weights to about 15% of intact weights without any significant difference among genotypes. On the other hand, while WT uterus displayed full regrowth to intact weights upon testosterone treatment after ovariectomy, the ARKO uterus displayed no regrowth and uterine weights remained at ovariectomy level; demonstrating that AR mediated androgen action alone can fully support uterine regrowth following ovariectomy.

In addition, we show that the growth promoting effects of androgens are via AR present in uterus as the ugeARKO females showed only 50% regrowth of uterus following ovariectomy and testosterone treatment. This suggests a significant role of endometrial gland specific AR in androgen effects on uterine

regrowth following ovariectomy. The 50% regrowth is striking as uterine glands constitute only <5% of the uterine structure. Hence, AR activity in the endometrial uterine glands affects the myometrium suggesting AR mediated paracrine signaling between the endometrial and myometrial cells. However, the Cre expression in Tg(Pbsn-cre)20Fwan mice is driven by ARR2PB composite promoter, which contains two AR response elements in order to increase the efficacy for AR-driven transcription of Cre (Jin et al., 2003). Hence, it is possible the Cre-expression pattern in PBSN-Cre mice can change upon testosterone treatment, in other words, Cre could be expressed outside glandular epithelial cells as we have shown in this study. In future, this issue can be addressed by investigating Cre expression in PBSN-Cre females upon ovariectomy and testosterone treatment.

As estrogens may modify androgen actions (Pelletier et al., 2004) and uterine function is estrogen dependent, we further explored if the testosterone conversion to $E_2$ is required in AR mediated androgen effects on uterine regrowth. Using the non-aromatizable androgen DHT we observed full restoration of uterine growth in WT females whereas ARKO uterus had no regrowth remaining similar to the estrogen deficient uterus. This further supports our finding that androgens acting via AR alone can promote full uterine regrowth without requiring $E_2$. This could be due to AR and ERα sharing similar molecular pathways as we demonstrated that androgens (T and DHT) acting via AR (lack of induction in ARKO) induced expression of ERα dependent genes, *lactoferrin*, *C3* and *G6PDH*. However, further studies are required to understand the possible sharing of pathways by ER and AR as there could also be other pathways involved. Furthermore, the ERα knockout

(ERαKO) and aromatase knockout (ArKO) female mice with supraphysiological levels of circulating T lacked uterine growth suggesting the essential role of $E_2$ in normal peripubertal uterine growth and development in mice (Hewitt et al., 2010, Lubahn et al., 1993, Fisher et al., 1998). In addition, although we expect $E_2$ to be absent in the ovariectomised mice and $E_2$ was under the detection limit (2.5pg/mL) of LC/MS-MS, we must consider the phytoestrogens that may be present through diet and may play a role uterine regrowth as high phytoestrogen diet elevated estrogen dependent genes in mice (Wang et al., 2005, Kaitu'u-Lino et al., 2007).

In our ugeARKO model, the floxed *Ar* gene creates a highly selective deletion of only the exon 3 (the second zinc finger of the DNA binding domain) of the AR. This creates a minimally truncated AR which is fully inactivated but is much closer to physiological than the major deletions of the AR protein created by introducing stop codons early into the translated protein. Unfortunately, the flip side of such highly selective, minimal deletion from a large protein is that it has proved impossible to obtain commercially or to custom generate specific antibodies specific to the truncated exon 3 deleted AR. Hence, AR protein is detectable to all commercial N and C terminal antibodies as well as any custom antibodies we tried to generate. However although the AR protein remains present immunohistochemically in its mutated form, it is functionally inactive (Notini et al., 2005b). Hence, as an alternative to prove the cellular-specificity of the Cre-mediated excision of exon 3, we micro-dissected uterine glandular epithelium from ugeARKO uterus and confirmed that the exon 3 deleted, inactive AR is only present in glands of ugeARKO uterus with no functional

(undeleted) *Ar* was detected in glands of ugeARKO uterus. Conversely, non-glandular uterine tissue expressed only the undeleted *Ar*.

We further validated and characterized the functional Cre activity in endometrial glands using ugePTENKO mouse model. *Pten* is a tumor suppressor gene (Dahia, 2000) and PTEN knockout causes endometrial cancer in mice (Stambolic et al., 2000, Daikoku et al., 2008). Firstly, we demonstrated PBSN-Cre activity is only present in endometrial glands by PTEN immunolocalization, where only the endometrial glands of ugePTENKO uterus were PTEN immunonegative compared to PTEN immunopositive glands in WT. Furthermore, we demonstrated significantly increased uterine weight in ugePTENKO females with increased cell proliferation (PCNA staining) when compared to WT. As PTEN deletion mouse model has been widely used in uterine cancer research (Stambolic et al., 2000, Daikoku et al., 2008), our model would be a novel model to investigate uterine cancer as it has been suggested that adenocarcinoma in uterus arise from endometrial glands (Newbold et al., 1990). Hence, the model would provide new platform to future uterine cancer research.

To create the endometrial gland specific knockout, we used PBSN-Cre mouse line for Cre/loxP system. PBSN has been known as a prostate specific protein which has been used to generate prostate specific genetically modified mouse models (Simanainen et al., 2007). Surprisingly, we have shown that the PBSN-Cre mouse line (Jin et al., 2003) can also be used to generate uterine gland specific knockouts in females. Although, *Pbsn* mRNA was not detectable in our uterine samples at 20 weeks diestrus, there are possibilities that *Pbsn* may be expressed at earlier during uterine development or it may be dependent on the

estrous cycle or the specific expression generated using PBSN promoter is specific for this PBSN-Cre model with the mechanism remaining unknown (Jin et al., 2003). Also, we have shown that *Pbsn* is selectively active in glandular epithelial cells in the uterus. Hence, the PBSN-Cre mouse line (Jin et al., 2003) used can effectively generate uterine gland specific knockout mice.

While other mouse models are also available to generate knockouts in the uterus, including endometrial knockouts using progesterone receptor (PR)-cre mouse line (Soyal et al., 2005) or conditional gene recombination by adenovirus-driven cre (Wang et al., 2006), these mice have some disadvantages. The disadvantage of PR-cre mouse line is that PR is also expressed in other tissues such as pituitary, ovary and mammary gland (Soyal et al., 2005) which depending on the knockout target could have systemic effects including changes in hormone profile hence effecting uterine physiology. The disadvantage of adenovirus-driven cre model is the recombination strategy is difficult to control the amount and the localization of cre delivered by the adenovirus vector. This may result in high variability of amount and localization of the knockout between individual experimental mice.

In conclusion, our study has suggested that androgens acting solely via AR can support full uterine growth, however, it is not known if potential estrogenic activity from the standard chow may play a role. Furthermore, we have generated and characterized the first endometrial gland specific genetically modified mouse model and using the model we have shown endometrial gland AR mediates uterine (including myometrial) growth via AR mediated androgen actions. These findings warrant further investigations of the role of AR and cross-talk involving AR expression in different type of uterine cells such as

luminal, stromal and myometrial cells. Also, the role of endometrial gland AR should be investigated in uterine disorders such as endometrial cancer which could lead to better biomarkers and more effective novel treatment of uterine disorders.

# Chapter 4:

# Androgen actions via androgen receptor promote PTEN inactivation induced uterine cancer

The content in this chapter has been published as:

**Jaesung (Peter) Choi**, Reena Desai, Yu Zheng, Mu Yao, Qihan Dong, Geoff Watson, David Handelsman, and Ulla Simanainen. Androgen actions via androgen receptor promote PTEN inactivation induced uterine cancer. *Endocrine-Related Cancers*. 22(5):687-710 (**2015**).

## 4.1 Abstract

Haploinsufficient inactivating *Pten* mutations cause Cowden syndrome, an autosomal dominant risk genotype for hormone dependent reproductive cancers including uterine cancer. As androgen actions mediated via 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. Our aims of this experiment were to determine:

i) The role of global AR inactivation in PTEN deletion induced uterine cancer.

ii) If global AR inactivation modifies PTEN expression and its AKT pathway in uterus and also if uterine ERα expression is affected.

iii) If global AR inactivation modifies serum and ovarian steroid hormone levels which may modify uterine cancer incidence.

To test the hypothesis, we compared the PTEN knockout induced uterine pathology in heterozygous PTEN knockout (PTENKO) and combined heterozygous PTEN and complete AR knockout (PTENARKO) female mice. PTEN knockout induced uterine pathology was significantly reduced by AR inactivation with severe macroscopic uterine pathology present in 21% of PTENARKO vs 46% of PTENKO at a median age of 45 weeks. This could be due to reduced stroma ERα expression in PTENARKO compared to PTENKO uterus, while AR inactivation did not modify PTEN or P-AKT levels. Unexpectedly, while progesterone is assumed protective in uterine cancers, serum progesterone was significantly higher in PTENKO females compared to wild type (WT), AR knockout (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 PTEN knockout induced uterine pathology and suggests a potential role for anti-androgens in uterine cancer prevention and treatment.

## 4.2 Introduction

Endometrial cancer (EC) is a frequent gynaecological cancer. It is highly hormone dependent being promoted by estradiol ($E_2$) and inhibited by progesterone ($P_4$) (Bender et al., 2011), while the role of androgens remains controversial. Androgens are 19-carbon steroid hormones produced in females mainly by ovarian and adrenal gland secretion together with extraglandular conversion. Androgens mediate their effects via androgen receptor (AR) expressed in target tissues such as the uterus of women (Somboonporn and Davis, 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 and Katzenellenbogen, 1979) whereas AR inactivation in mice resulted in thinner uterus with reduced total uterine area compared to wild type (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 development and/or progression

of uterine carcinogenesis as increased androgen activity due to shorter CAG repeat in AR was related to increased endometrial cancer risk in women (McGrath et al., 2006). In addition, AR is strongly expressed in human endometrial disorders including different types of endometrial cancers (Ito et al., 2002), further supporting a role of androgens acting via AR in uterine cancer.

*Pten*, a phosphatase and tensin homolog, is a tumor suppressor gene located on chromosome 10 (Dahia, 2000). *Pten* is mutated or deleted in a wide range of human cancers including endometrial cancers (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 endometrial carcinomas (EMC) and in 20 to 70% of complex atypical hyperplasia, a premalignant stage of EMC (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 day 6.5 to 9.5 (Suzuki et al., 1998). Global heterozygous *Pten* knockout (PTENKO) in mice resembles Cowden syndrome in patients and it causes endometrial cancer and therefore PTENKO females are used as endometrial cancer models (Stambolic et al., 2000). AR and PTEN interact in breast and prostate cancers (Wang et al., 2011b) and they cross regulate by reciprocal feedback (Carver et al., 2011, Mulholland et al., 2011). Furthermore, PTEN knockout increases ERα expression in endometrial cancer cells (Lian et al., 2006) and activates ERα dependent pathways in mouse endometrial cancer (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 Cre/LoxP system to determine the effects of AR inactivation on PTEN inactivation induced uterine cancer susceptibility and possible mechanisms involved.

## 4.3 Experimental design

Experimental design was carried out as it follows:

i) The role of global AR inactivation in PTEN deletion induced uterine cancer was determine by comparing uterine weight and uterine cancer incidences in PTENKO (heterozygous PTEN deletion) and PTENARKO (combined heterozygous PTEN deletion and homozygous AR inactivation) females at 45 weeks old. Furthermore, to determine if global AR inactivation modifies serum and ovarian steroid hormone levels which may modify uterine cancer incidences, these steroid hormone levels were compared between WT, ARKO, PTENKO and PTENARKO females at 45 weeks of age using LC-MS/MS. WT, ARKO, PTENKO and PTENARKO females were generated as described in methods (see 2.1).

ii) To determine if AR mediated androgen actions are responsible for androgen induced uterine growth, PTENKO and PTENARKO females were ovariectomised at 5 weeks old and treated with testosterone or empty implants for 15 weeks and collected at 20 weeks of age. Upon collection, uterine weights were compared.

iii) To determine if global AR inactivation modifies expression of PTEN, AKT, P-AKT or ERα expression in uterus, the immunopositivity of these proteins in uterus was compared between WT, ARKO, PTENKO and PTENARKO females at 20 weeks of age. The immunopositivity was compared at an earlier age to reduce variability due to different types of histopathologies developed in older (45

weeks) group. This also allowed comparison of the early molecular changes in neoplastic pathways.

## *Genotype confirmation*

Excision of exon 5 in PTEN knockout and exon 3 in AR knockout were confirmed at genomic DNA and mRNA levels. The genomic DNA was released using proteinase K digestion of uterine tissues. Excision of exon 5 of *Pten* was confirmed using same primers as for genotyping (Byun et al., 2011). Excision of AR exon 3 was confirmed as described (Notini et al., 2005b). In addition, 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 bps 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 bps 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 ovariectomised (OVX) under anaesthesia at 5 weeks of age and implanted with 1 cm silastic implants (inner diameter=1.47mm and outer diameter=1.95mm, Dow-Corning,

Sydney, Australia) either containing ~10 mg crystalline testosterone or no steroid placed subdermally for 15 weeks and subsequently collected at 20 weeks of age.

### *Uterine cancer classification*

All uteri from experimental mice were classified into two categories based on macroscopic characteristics: normal or abnormal. Normal uteri (Figure 4.1a) had uterine weight less than 300mg and did not exhibit any macroscopic abnormalities such as extensive vascularization (Figure 4.1b) or presence of unusual growths (Figure 4.1c). Abnormal uteri had uterine weight greater than or equal to 300mg (upper range of WT uterine weight mean±SD) resulting in enlarged uterine horns with any of the macroscopic abnormal features.

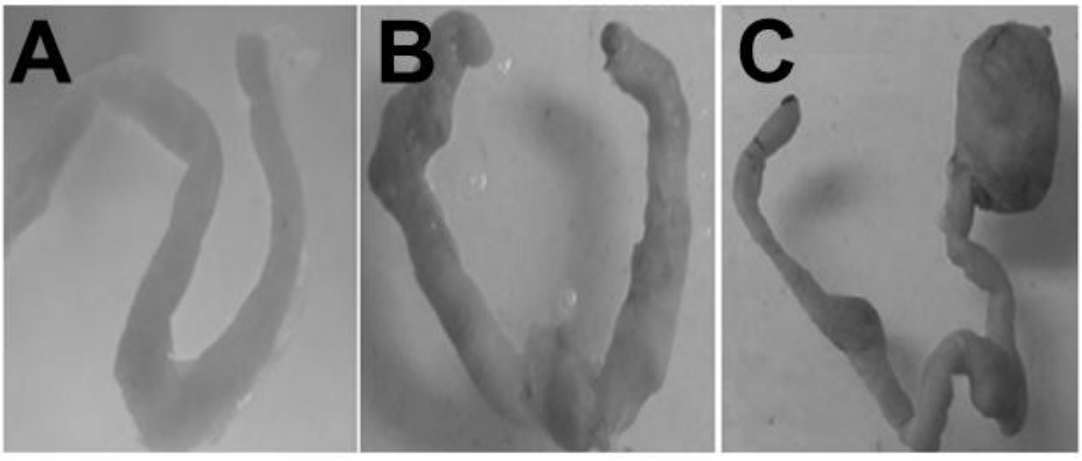



**Figure 4.1. Uterus morphological classification at the median age of 45 weeks.** A-C) Representative images of macroscopic uterine category: normal uterus (A) and abnormal uterus (B and C).

## 4.4 Results

### 4.4.1 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 (Figure 4.2A), while only native *Pten* product was detected in WT and ARKO uterus (Figure 4.2B). However, native *Pten* product was also detected in PTENKO and PTENARKO uterus as these mice were heterozygous for *Pten* deletion. In addition, presence of native *Ar* (larger product containing exon 3) was confirmed in 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 (Figure 4.2C). Exon 5 excision in *Pten* gene was not confirmed at the mRNA level because exon 5 excision produces stop codon and early termination of transcription of mRNA.

Western blot was used to compare PTEN protein levels between WT, ARKO, PTENKO and PTENARKO uterus (Figure 4.2D). While the PTEN levels were variable between individual uteri, the levels appeared to be decreased in PTENKO and PTENARKO uterus (note PTEN knockout is heterozygous thereby retaining a normal *Pten* allele) compared to WT and ARKO uterus.

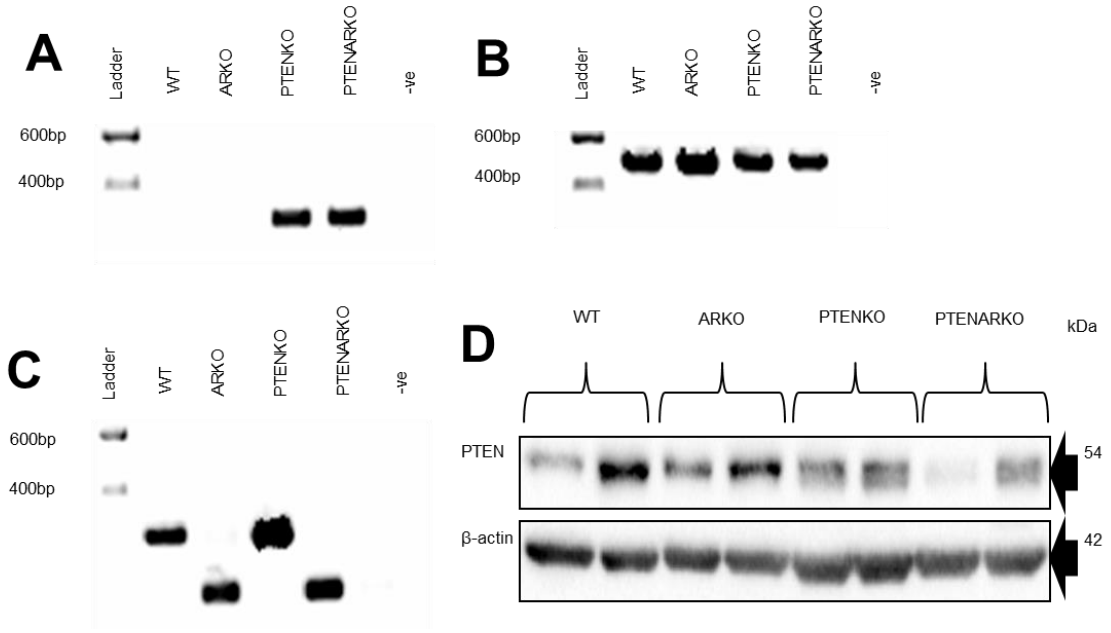



**Figure 4.2. Genotyping PCR on uterus cDNA.** A) PTEN PCR showing PTEN deletion. B) PTEN PCR showing WT PTEN. C) ARCUT RT-PCR showing WT AR and exon 3 excised AR. D) PTEN western blot showing PTEN reduction in PTENKO and PTENARKO.

### 4.4.2 AR inactivation modifies PTEN deletion induced uterine pathology

All 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 macroscopic abnormal uterus while only 21% (5/24) in PTENARKO females, mean age of 43 weeks (Table 4.1). PTEN deletion significantly increased ($p<0.001$; log-linear model) abnormal uterus incidence whereas AR inactivation significantly decreased ($p=0.0012$) the PTEN induced uterine cancer incidence; interaction between PTEN deletion and AR inactivation was not statistically significant.

**Table 4.1. Macroscopic categorization of uterine abnormalities.** a=significantly different to PTENARKO, #=% over total number of mice, N/A= no incidences of abnormal uterus found.

| | Number of mice | Age (mean±SD) (weeks) | Number (%[#]) of abnormal uterus |
|---|---|---|---|
| **WT** | 32 | 43±10 | N/A |
| **ARKO** | 14 | 46±9 | N/A |
| **PTENKO** | **52** | **46±8** | **24 (46)[a]** |
| **PTENARKO** | **24** | **43±7** | **5 (21)** |

Comparable to pathology, uterine weights at 45 weeks was increased in PTENKO and PTENARKO compared to WT and ARKO (Figure 4.3A). The uterine weights were significantly ($p=0.004$; two way ANOVA) increased by PTEN deletion but was 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 (Figure 4.10). In addition, PTENKO uterus was significantly heavier than PTENARKO uterus. Uterine weights were similar between ARKO and WT females (Figure 4.3A). Uteri that were categorized as normal in each genotype did not exhibit any abnormal histology and appeared similar (Figure 4.3B-E).

Histopathological analysis of the macroscopic abnormal uteri showed different types of histopathologies. In PTENKO, 58% (14/24) were high-grade (poorly-differentiated) carcinoma, (Figure 4.3F), 29% (7/24) were low-grade adenocarcinoma (Figure 4.3J), and 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.

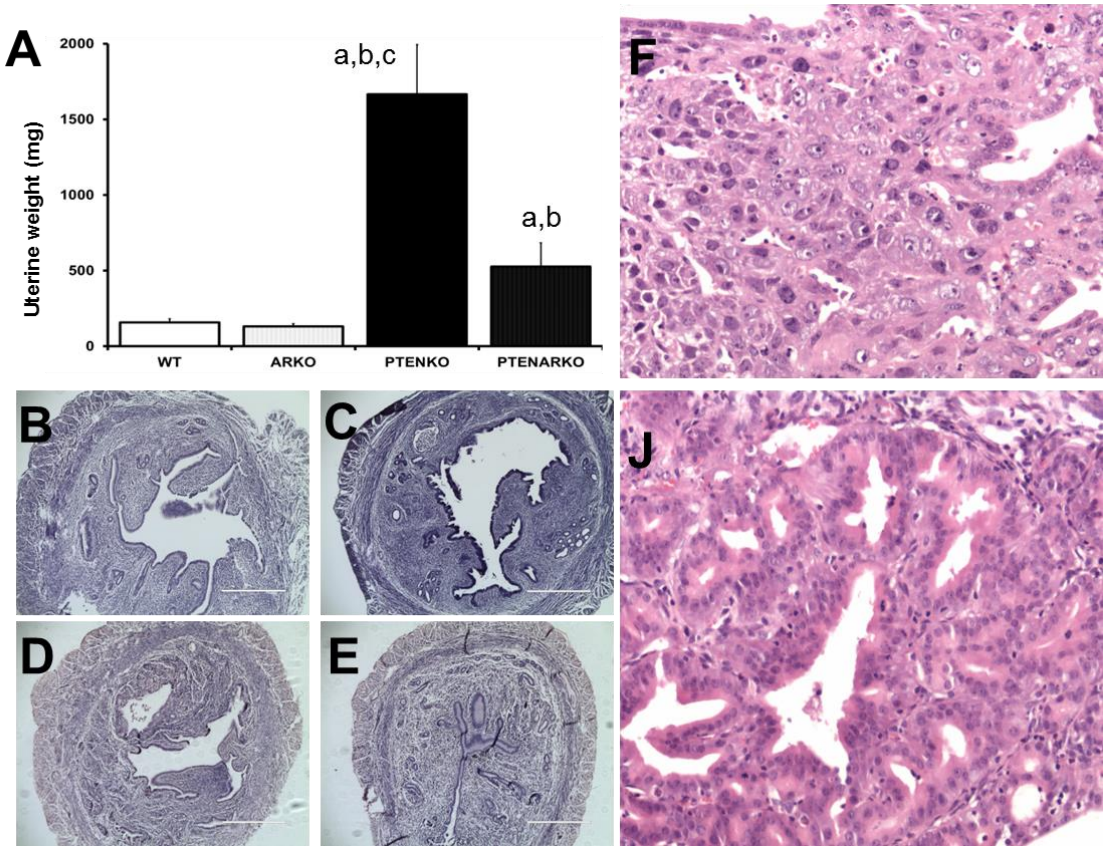



**Figure 4.3. Uterus weights and histopathology at the median age of 45 weeks.** A) Absolute uterine weight. [a= significantly different to WT, b= significantly different to ARKO, c= significantly different to PTENARKO ($p<0.05$; one way ANOVA) (mean±SE)]. B-E) Representative images of normal uterus of WT (B), ARKO (C), PTENKO (D) and PTENARKO (E) at 45 weeks (Scale bar=400μm). F-J) Representative images of major uterine histopathologies in PTENKO and PTENARKO: high-grade carcinoma with dedifferentiated morphology (F) and low-grade (focal) adenocarcinoma (J) (400x magnification).

Similarly to uterine weights at 45 weeks, uterine weight at 20 weeks were significantly (p=0.008; two way ANOVA) increased by PTEN deletion but was 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 (Figure 4.4A). Histologically, no uterine cancers were detected at 20weeks (Figure 4.4B-E) although PTENKO uteri were histologically abnormal with enlarged and disorganized endometrial glands not present in uteri from other genotypes (Figure 4.4D).

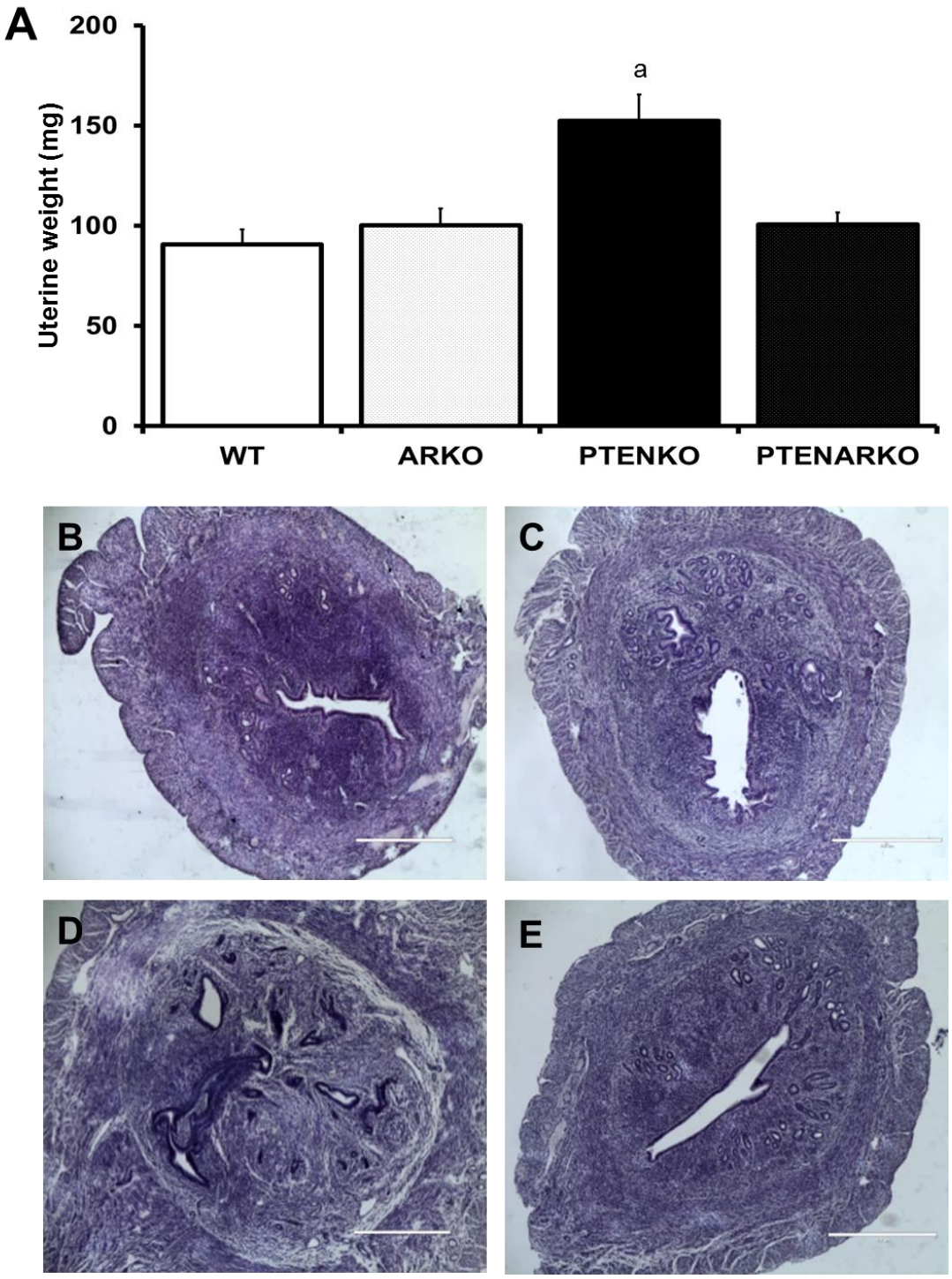



**Figure 4.4. Uterine weight and histology (H&E) at 20 weeks of age.** A) Uterine weight (mg). [a= significantly different to WT, ARKO and PTENARKO ($p<0.05$; one way ANOVA); mean±SE; n≥8]. B-E) Representative photos of H&E stained of WT (B), ARKO (C), PTENKO (D) and PTENARKO (E) at 20 weeks. (Scale bar=400um).

### 4.4.3 AR mediated androgen actions are required for testosterone induced uterine regrowth

OVX and testosterone treatment was performed on PTENKO and PTENARKO females to investigate the direct role of testosterone in uterus (Figure 4.5A). 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+T) fully restored the uterine weight to the intact level at 20 weeks of age. However, no uterine growth was detected following OVX and T treatment in PTENARKO females and the uterus weight remained similar to OVX only females. Histologically, uteri of PTENKO (OVX), PTENARKO (OVX) and PTENARKO (OVX+T) appeared similar (Figure 4.5B, D, and E).

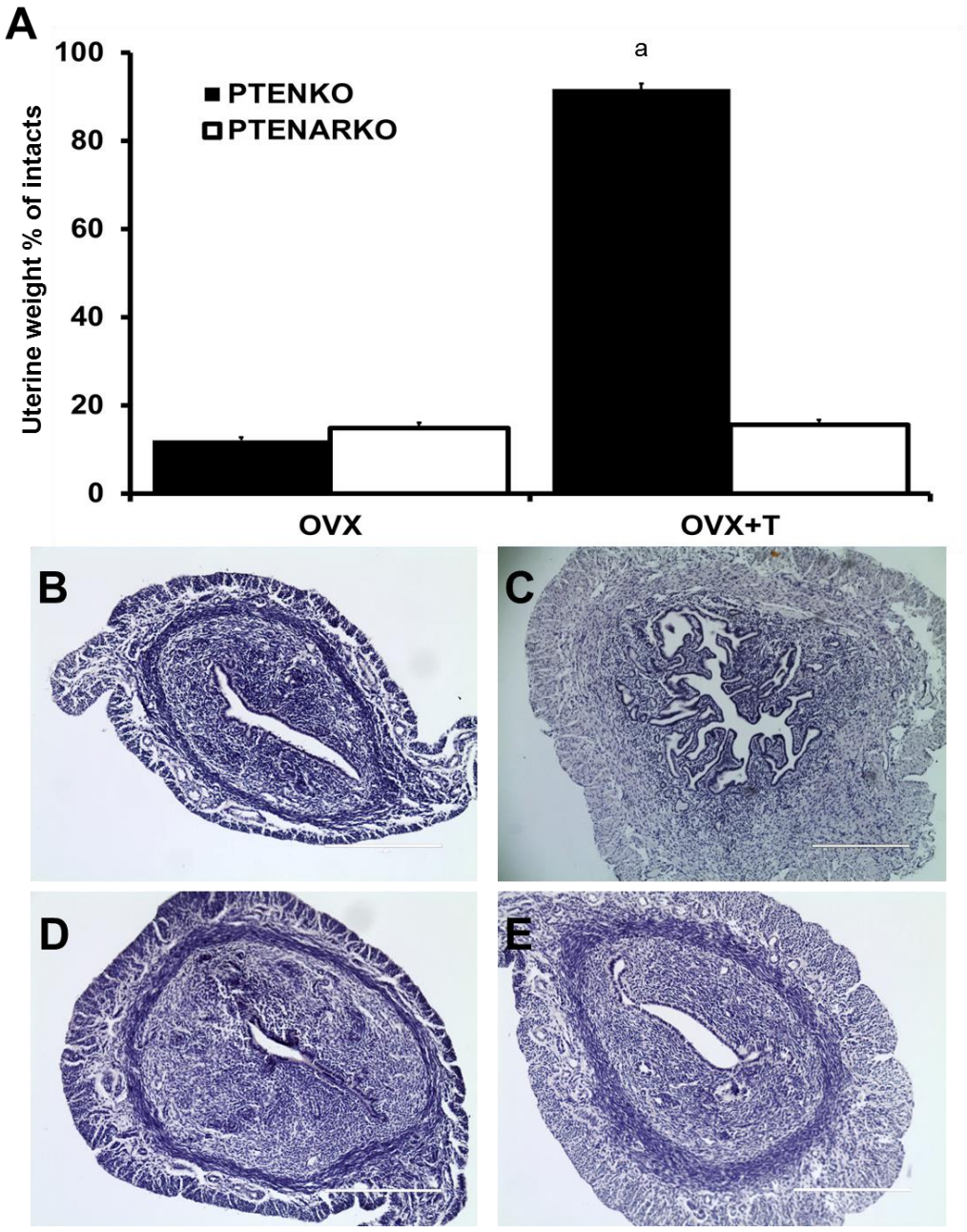



**Figure 4.5. Uterine weight and histology (H&E) at 20 weeks of age upon ovariectomy and testosterone treatment.** A) Uterine weight (relative to intacts). [a=significantly different to OVX group and PTENARKO (OVX+T) ($p<0.05$; one way ANOVA) (mean±SE)]. B-E) Representative photos of H&E stained of PTENKO (OVX) (B), PTENKO (OVX+T) (C), PTENARKO (OVX) (D) and PTENARKO (OVX+T) (E) (Note different size of scale bars. Scale bar=200um for image B, D, E and 400µm for image C).

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

As it has been suggested that AR can regulate PTEN expression and hence modify AKT pathway (Wang et al., 2011b), 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 (Figure 4.6A) between the PTENKO (native *Ar*) and PTENARKO (inactive *Ar*) uterus, the AKT levels (Figure 4.6B) were significantly ($p=0.02$; one way ANOVA) increased in PTENARKO uterus compared to PTENKO. However, the P-AKT (active form of AKT) levels (Figure 4.6C) were not affected by the AR inactivation in PTENKO uterus. As p27 is a key target of the growth regulatory activity exerted by AKT pathway (Liang et al., 2002) and is deregulated in multiple cancers including endometrioid uterus, however, p27 protein while reduced in PTENARKO (Figure 4.6D) was not significantly different ($p=0.082$) between PTENKO and PTENARKO uterus. Immunohistochemically, no differences were observed in PTEN (Figure 4.6E-F) and P-AKT (Figure 4.6H-I) expression in PTENKO and PTENARKO uterus in supporting the western blot results. Whereas, all cells of PTENKO were immunopositive for p27 while in PTENARKO uterus glandular epithelial cells were immunonegative supporting the lower protein levels observed by western blots (Figure 4.6K-L).

To further examine the effect of androgens on PTEN, P-AKT and p27 expression, we compared the levels between PTENKO and PTENKO (OVX+T) uterus. OVX + T treated PTENARKO uterus was not included as the uteri were underdeveloped (Figure 4.5). PTEN expression was decreased in all cells upon

OVX and testosterone treatment compared to intact PTENKO (Figure 4.6G) whereas P-AKT expression was increased in glandular and luminal epithelial cells (Figure 4.6J). p27 expression was increased in all cells upon OVX and testosterone treatment in PTENKO uterus compared to intact PTENKO (Figure 4.6M).

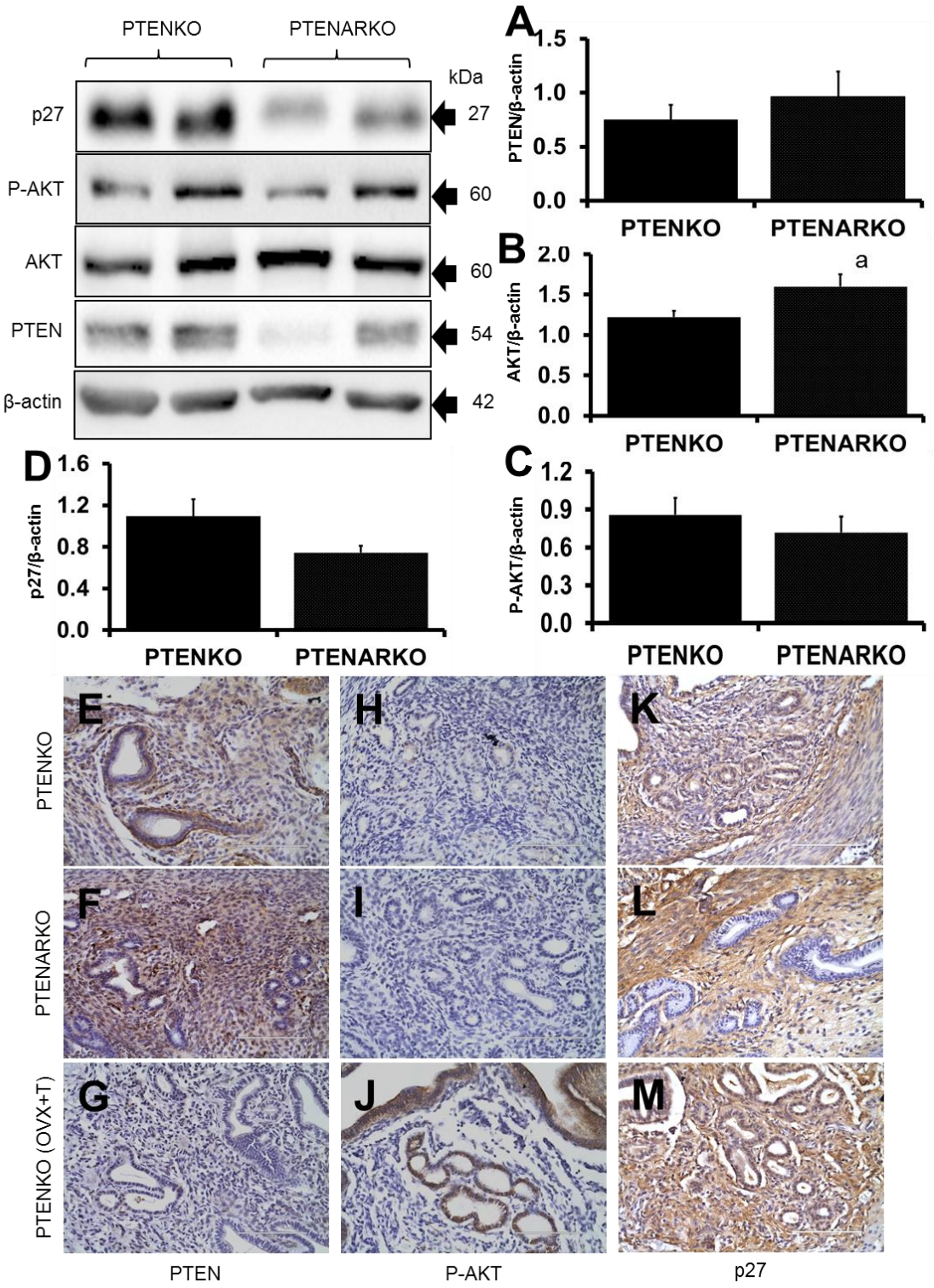



**Figure 4.6. p27, AKT, P-AKT and PTEN western blot and immunohistochemistry on PTEN deleted uterus at 20 weeks.** Representative western blots for each proteins are shown in the figure with name of the protein labelled on the left and protein band (kDa) shown on the

right. A-D) Quantification of western blot bands by measuring the intensity of each band standardized using B-actin as a housekeeper (a= significantly different to PTENKO ($p=0.043$; one way ANOVA); mean±SE; $n\geq5$). E-M) Representative images of immunohistochemistry of PTEN, P-AKT and p27 in PTENKO, PTENARKO and PTENKO (OVX+T) uterus at 20 weeks (Scale bar=100μm). Additional blots for PTEN have been included in Appendix V.

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

As ERα expression is significantly increased in PTENKO uterus (Vilgelm et al., 2006, Lian et al., 2006), we examined if AR inactivation could influence this PTEN knockout induced increase in ERα. ERα positivity (Figure 4.7A) and intensity (Figure 4.7B) were quantified according to different cell types: glandular epithelial cells, luminal epithelial cells, endometrial stroma and myometrium. ERα positivity in each cell types was not significantly affected by genotype. However, ERa 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. ERα intensity in endometrial stromal cells was significantly increased in 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 Figure 4.7C-F.

Real time RT-PCR was performed to determine ERα gene expression. ERα mRNA expression was increased (p=0.064; one way ANOVA) in the PTENKO uterus compared to WT but this effect was prevented by simultaneous AR inactivation in PTENARKO uterus (Figure 4.7G). Congruent findings were observed in the expression of ERa target genes, G6PDH expression was significantly increased (p=0.019; t-test) in PTENKO uterus compared to WT and again this effect was prevented by simultaneous AR inactivation in PTENARKO uterus (Figure 4.7H). Although not statistically significant, other ERa dependent genes, lactoferrin (p=0.064; t-test) (Figure 4.7I) and complement C3 (p=0.097;

t-test) (Figure 4.7J) were also increased in PTENKO uterus compared to WT and similarly this effect was prevented by simultaneous AR inactivation in PTENARKO uterus.

Furthermore, when compared to intact PTENKO uterus, OVX+T treated PTENKO uterus had marked increase in ERα expression immunopositivity (Figure 4.7K), which was observed in all cell types.

| A | Glandular epithelial | Luminal epithelial | Stroma | Myometrium |
|---|---|---|---|---|
| WT | 57.7±7.7 | 59.5±18.4 | 43.1±11.6 | 33.9±10.7 |
| ARKO | 24.0±13.0 | 20.8±11.4 | 24.5±10.0 | 16.7±9.5 |
| PTENKO | 64.3±17.4 | 87.0±4.9 | 58.5±6.8 | 27.5±4.6 |
| PTENARKO | 32.9±11.6 | 30.8±12.9 | 22.9±8.1 | 10.9±4.6 |

| B | Glandular epithelial | Luminal epithelial | Stroma | Myometrium |
|---|---|---|---|---|
| WT | 0.39±0.06 | 0.39±0.18 | 0.20±0.05 | 0.16±0.05 |
| ARKO | 0.11±0.06[a] | 0.08±0.04 | 0.11±0.05 | 0.09±0.06 |
| PTENKO | 0.34±0.13 | 0.54±0.08 | 0.37±0.05[b] | 0.15±0.02 |
| PTENARKO | 0.14±0.05[a] | 0.13±0.07 | 0.10±0.03 | 0.04±0.02 |

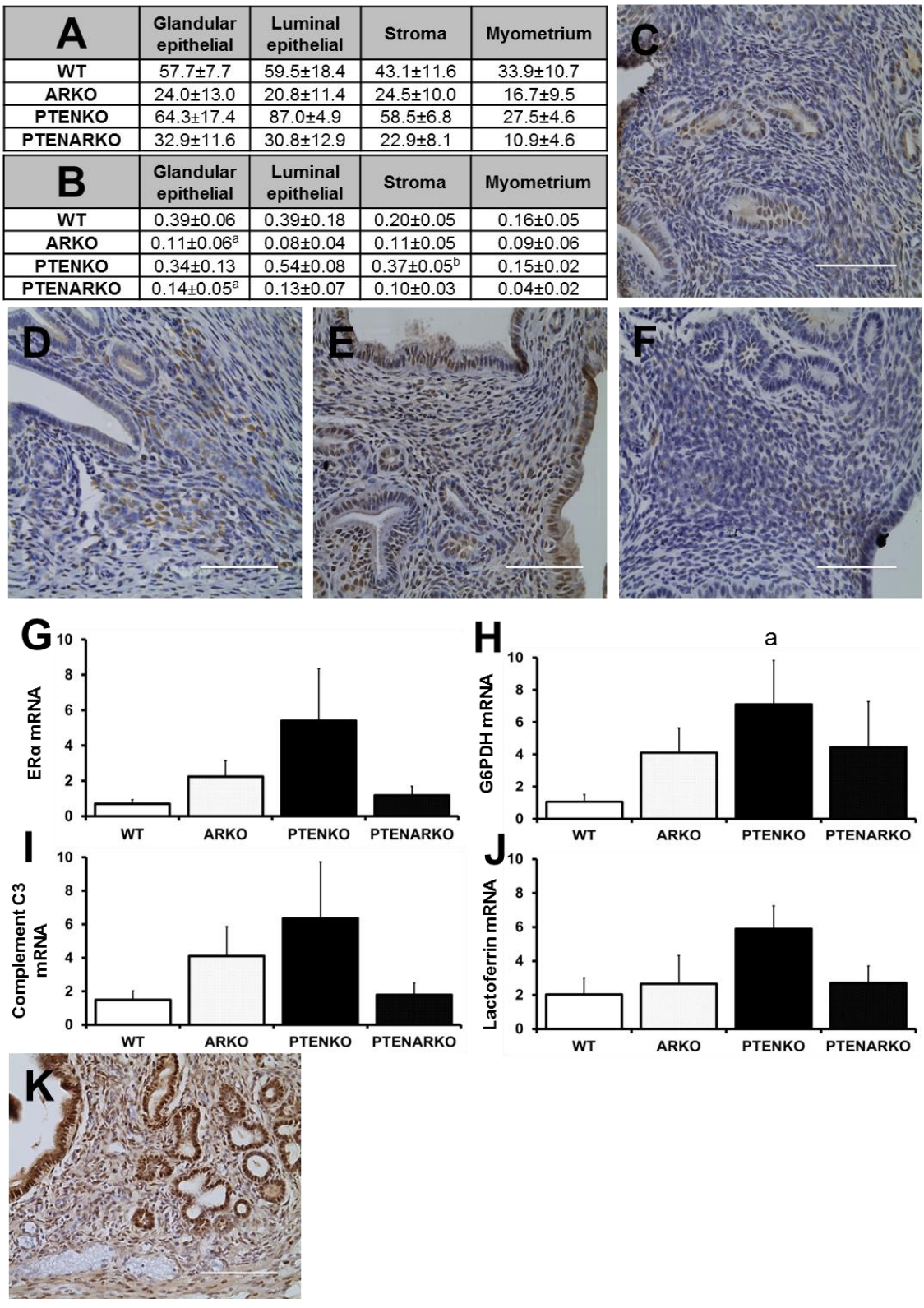



**Figure 4.7. Expression of ERα and ERα dependent genes in the uterus of 20 weeks old females.** A-B) Percentage of ERa positive cells (A) and intensity (B) in glandular epithelial cells, luminal epithelial cells, stroma and myometrium

in experimental groups [a= significantly different to WT, b=significantly different to WT, ARKO and PTENARKO (one-way ANOVA; mean±SE; n=4)]. C-F) Representative images of ERa immunohistochemistry on WT (C), ARKO (D), PTENKO (E) and PTENARKO (F) (Scale bar=100μm) G-J) Relative expression (relative to 18s rRNA housekeeping gene analyzed by real-time RT-PCR) of ERa mRNA (G) and ERa dependent genes (H-J) in the uterus at 20 weeks of age [a= significantly different to WT; (p=0.019; t-test) (mean±SE; n≥6)]. K) Representative image of ERa immunohistochemistry on ovariectomised and testosterone treated PTENKO uterus (Scale bar=100μm).

### 4.4.6 PTEN knockout in females increased serum P4

As uterine cancer is highly hormone dependent (Martin et al., 1973b, Martin et al., 1973a, 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 (Figure 4.8A) whereas serum P4 (Figure 4.8B) 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 $E_2$ content was analyzed as serum $E_2$ in intact female mice is usually below the detection limit of LC-MS/MS (2.5pg $E_2$/ml; (McNamara et al., 2010b)). Ovarian $E_2$ content was similar between the genotypes (Figure 4.8C). Ovarian P4 (Figure 4.8D) 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. CL number was significantly ($p=0.006$; two way ANOVA) reduced by AR inactivation and increased by PTEN deletion ($p=0.08$); significance of AR inactivation and PTEN deletion interaction was 0.053 (Figure 4.8E-F), suggesting that increased P4 levels could be due to increased CL number and that the increase in CL due to PTEN deletion was reversed by AR inactivation.

PR immunohistochemistry was performed on PTENKO (Figure 4.8G) and PTENARKO (Figure 4.8H) uterus at 20 weeks and both the PTENKO and PTENARKO uterus expressed PR without obvious differences. However, compared to intact PTENKO uterus, OVX+T treated PTENKO uterus had marked increase in PR immunopositivity (Figure 4.8I). This increase was observed in all cell types.

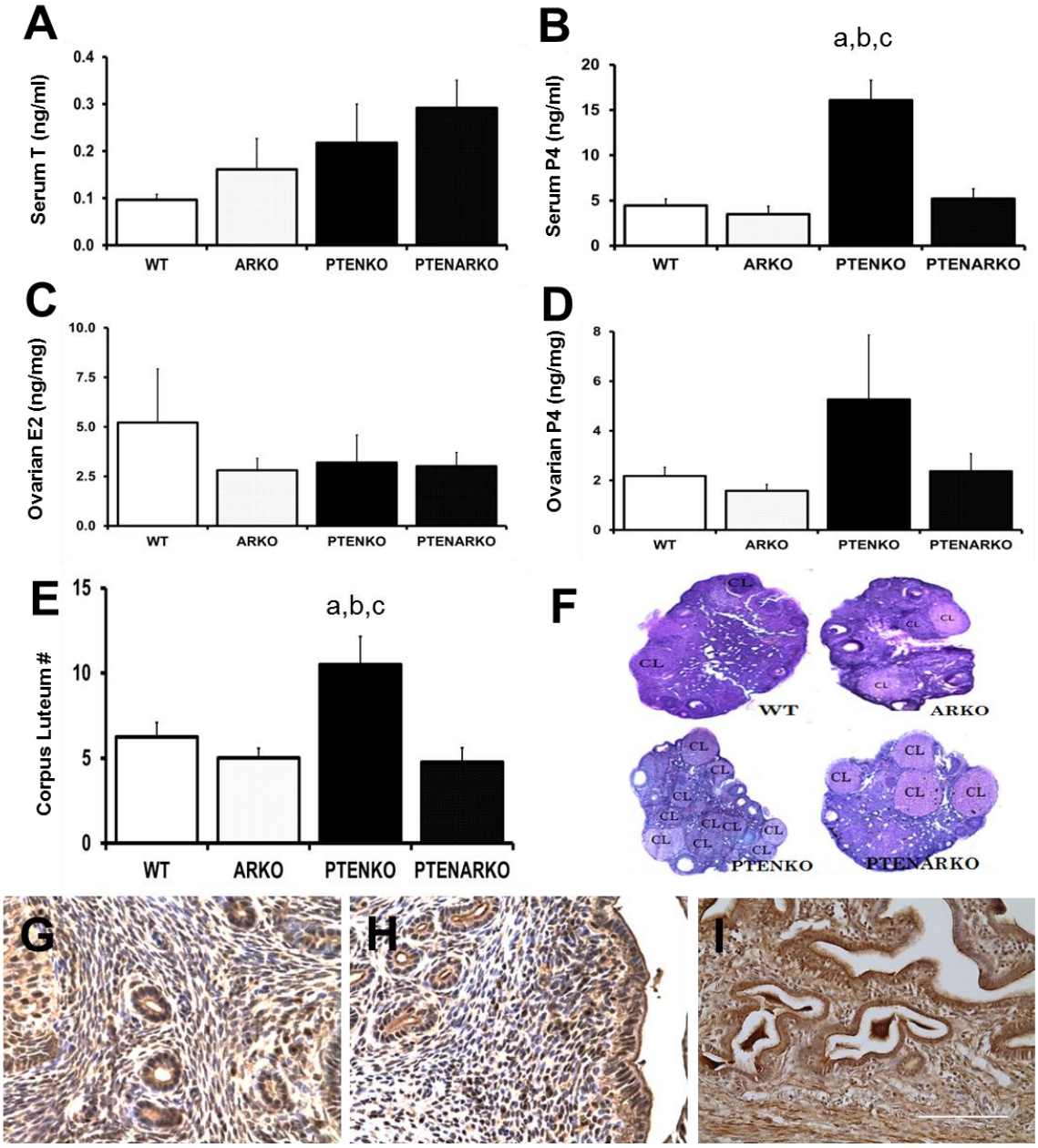



**Figure 4.8. Steroid hormone levels and CL count at 45 weeks and PR expression in PTENKO and PTENARKO uterus at 20 weeks.** Serum T (A) and P4 (B) levels [a= significantly different to WT, b= significantly different to ARKO, c= significantly different to PTENARKO ($p<0.05$; one way ANOVA); mean±SE; n: WT=32, ARKO=7, PTENKO=43, PTENARKO=23]. Ovarian P4 (C) and E2 (D) levels (mean±SE; n=4). E-F) Total number of Corpus Luteum (CL) in ovaries of WT, ARKO, PTENKO and PTENARKO females with

representative image (F) [a= significantly different to WT, b= significantly different to ARKO, c= significantly different to PTENARKO ($p<0.05$; nonparametric test); mean±SE; n=4]. PR immunohistochemistry on PTENKO (G), PTENARKO (H) and PTENKO (OVX+T) (I) at 20 weeks old (Scale bar=100μm).

### 4.4.7 Multiple organs were affected by PTEN deletion and AR inactivation in females

Body weight was significantly ($p<0.001$; two way ANOVA) increased by PTEN deletion but AR inactivation did not affect body weight; interaction between PTEN deletion and AR inactivation was significant ($p=0.001$) (Figure 4.9). As a result, other organ weights were evaluated with and without adjustment for body weight.

Ovary and adrenal gland weights were significantly ($p<0.02$) increased by PTEN deletion but was not affected by AR inactivation; there was no significant interaction between PTEN deletion and AR inactivation (Figure 4.9). However, when adjusted for body weight, ovary weights were not affected by PTEN deletion whereas adrenal gland remained similar (Figure 4.10). 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 (Figure 4.9). When adjusted for body weight only the interaction between AR inactivation and PTEN deletion was significant (0.043) in spleen but not in liver. (Figure 4.10).

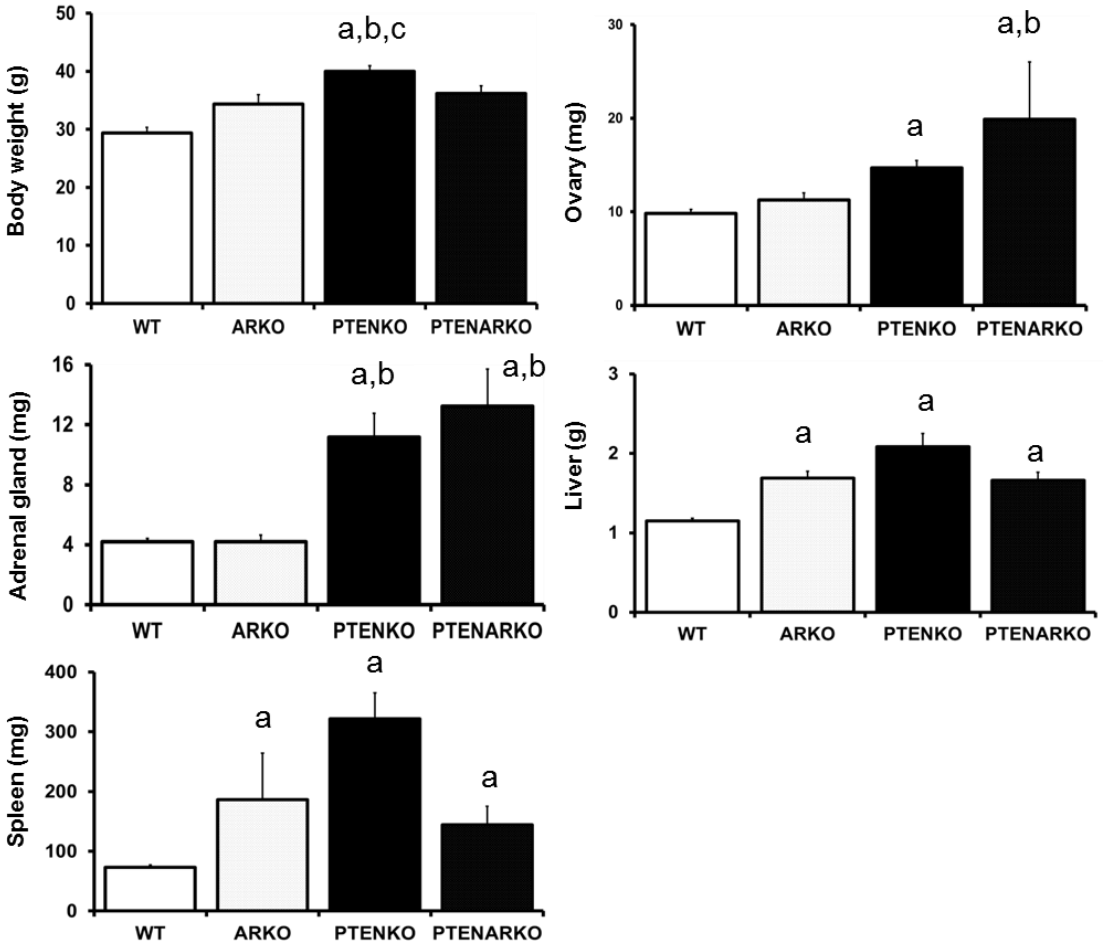



**Figure 4.9. Body weight and absolute organ weight of female mice at the median age of 45 weeks.** a= significantly different to WT, b= significantly different to ARKO, c= significantly different to PTENARKO [($p<0.05$; oneway ANOVA) (mean±SE); n: WT=32, ARKO>7, PTENKO>43, PTENARKO>23].

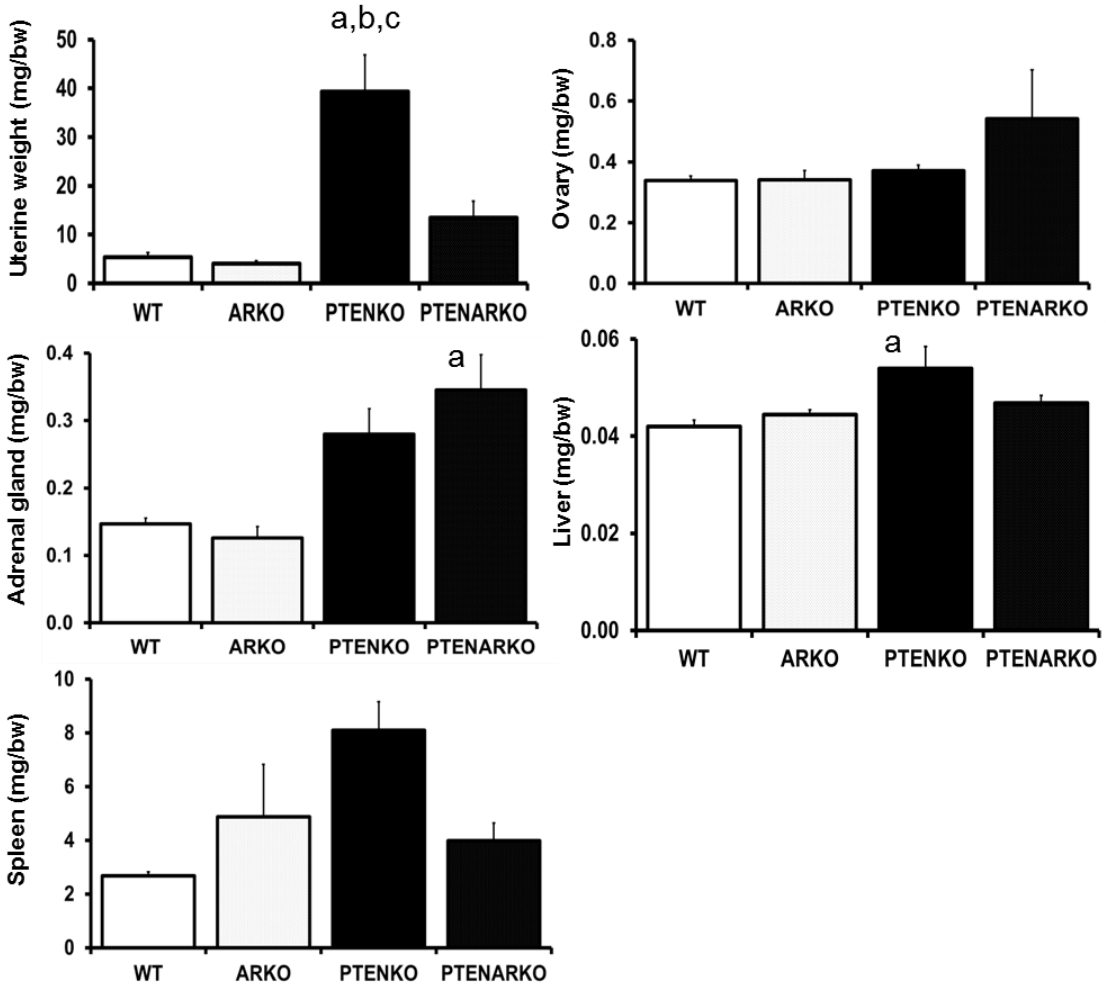



**Figure 4.10. Standardized organ weight of female mice at the median age of 45 weeks.** a= significantly different to WT, b= significantly different to ARKO, c= significantly different to PTENARKO, ($p<0.05$; mean±SE; n: WT=32, ARKO>7, PTENKO>43, PTENARKO>23).

### 4.4.8 Other incidental abnormalities

Global heterozygous PTEN knockout also induced abnormalities other than uterine pathology in female mice (Table 4.2). Mammary gland abnormalities were the most frequent with 54% of PTENKO and PTENARKO females bearing abnormal mammary glands (palpable tumors or alveolarized mammary gland with milk production (Li et al., 2002)) compared with none detected in WT and ARKO. Histologically, the palpable tumors within 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 4.2).

Very low frequencies of macroscopic abnormalities were also observed in adrenal glands, liver, lung, ovaries, pancreas and spleen (Table 4.2). Among these, the most frequent abnormality was the appearance of unusual black spots on the organ surface.

**Table 4.2. Abnormalities found in 45 week old experimental female mice.**

% shown are calculated over total number of mice.

| Genotype | Number of mice | Mammary gland | | | Liver | Ovary | Other |
|---|---|---|---|---|---|---|---|
| | | Palpable | | Alveolarization/ milk production | | | |
| | | Lym-phoma | Fibro-adenoma | | | | |
| WT | 32 | N/A | N/A | N/A | N/A | N/A | N/A |
| ARKO | 11 | N/A | N/A | N/A | N/A | N/A | N/A |
| PTENKO | 52 | 9 (17%) | 12 (23%) | 7 (13%) | 6 (12%) | 2 (4%) | 3 (6%) |
| PTENARKO | 24 | 5 (21%) | 7 (30%) | 1 (4%) | N/A | N/A | 1 (4%) |

## 4.5 Discussion

Direct AR mediated androgen actions on female reproductive physiology have been established (Walters et al., 2010) leading to suggestions that they may have impact on the physiology and pathology of the uterus and breast (Walters et al., 2010, Somboonporn and Davis, 2004). As AR is expressed in uterus of mice (Walters et al., 2010) and humans (Somboonporn and Davis, 2004), we therefore explored the role of androgens acting via the AR in experimental, PTEN knockout 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 elimination of heterozygous PTEN knockout induced increase in uterine ERa expression. Furthermore, it is important to note that our uterine cancer model resembles Cowden syndrome, the germline mutation of one allele of PTEN resulting in hereditary cancer predisposition (Liaw et al., 1997). Hence, the prevention of uterine cancers by interrupting androgen action in our model may represent not only an effective simulation of the endometrial cancers in Cowden syndrome but also point to the potential role of anti-androgens in its treatment.

This study demonstrates that androgens acting via AR decrease uterine cancer incidence in PTENKO experimental uterine cancer model. Heterozygous PTEN knockout induced uterine cancers as previously reported (Stambolic et al., 2000, Risinger et al., 1997). However, these changes in 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 endometrial cancer. While not experimentally tested before, an influence of androgens on uterine cancer development is consistent with a previous clinical study showing an excess of shorter CAG repeat in AR gene, which increases tissue androgen sensitivity (Simanainen et al., 2011a), being associated with increased endometrial cancer risk (McGrath et al., 2006). Furthermore, there is also evidence that PCOS patients with elevated plasma androgens (Nagamani et al., 1986) have increased risk of uterine cancer (Smyczek-Gargya and 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., 2011b) and therefore we determined if the PTEN expression was modified by AR inactivation in 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 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 modification of 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 PTEN (OVX+T) 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, where DHT via AR inhibited PTEN transcription and translation (Wang et al., 2011b). Diminished PTEN expression in PTENKO (OVX+T) uterus resulted in increased P-AKT expression in glandular and luminal epithelial cells supporting previous reports of negative correlation between PTEN and P-AKT in uterus (Stambolic et al., 2000, Stambolic et al., 1998). Hence, further investigations are warranted to investigate AR and PTEN interaction in the origins of uterine cancers.

Interestingly, we found that the expression of tumor suppressor p27 appeared to be reduced ($p=0.082$) in PTENARKO uterus when compared to PTENKO. This could be due to reduced p27 expression in glandular epithelial cells of PTENARKO uterus. This was unexpected as the p27 levels are usually negatively correlated with malignancy with the level of p27 being reduced in approximately 50% of human cancers (Slingerland and Pagano, 2000, Sgambato et al., 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 endometrial carcinoma (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 suggests the positive correlation between p27 and uterine cancer which would require further investigations. The increase in 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 increased p27 expression throughout PTENKO (OVX+T) uterus compared to 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 $E_2$ levels but, as they are frequently anovulatory, they lack the usual $P_4$ opposition against excessive estrogen action (Lobo et al., 1981). While we were unable to measure serum $E_2$ concentrations as they were below sensitivity of our LC-MS/MS methodology (limit of quantitation 2.5pg /ml), ovarian $E_2$ content was not significantly different between the genotypes suggesting the circulating $E_2$ levels were probably similar for each genotype. Furthermore, serum T levels did not differ according to genotype. However, as testosterone could can be aromatized within the uterus (Huhtinen et al., 2012), uterine content of $E_2$ should be analyzed in the future. In addition, while an exclusively direct role of androgens has not been confirmed, AR expression is increased in 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 endometrial cancers (Ito et al., 2002), further supporting a role of androgens acting via AR. Hence, these findings raise the hypothesis that antiandrogens may have an unrecognized role in prevention and the early stages of uterine carcinogenesis or in treatment.

Interestingly, the increased uterine estrogen sensitivity due to PTEN inactivation in PTENKO females corresponded to increasing expression of not only ERα but also a variety of estrogen sensitive target genes (*lactoferrin, G6PDH* and *complement C3*) in the PTENKO uterus. However, further analysis is warranted to determine if the increase in estrogen-regulated genes in PTENKO uterus is due to increased estrogen sensitivity or due to increased epithelial cell number as well as increased E2 levels and prolonged proestrus in PTENKO mice. This finding extends to an *in vivo* model and previous *in vitro* study where PTEN inactivation increased ERα expression (Lian et al., 2006). Similarly, the loss of *Pten* and subsequent AKT activation in uterus was shown to activate ERα dependent pathways in mouse endometrial cancer (Vilgelm et al., 2006). Furthermore, our findings suggest that the reduced uterine pathology following AR inactivation in PTENARKO females could be due to reduced ERα expression, consistent with the reduced ERα immunointensity in endometrial stroma and ERα mRNA expression. There could be number of possible mechanisms for how global AR inactivation reversed increased ERα induced by PTEN deletion. Firstly, AR directly modifies ERα expression in the uterus. Secondly, AR could interact with PTEN to indirectly modify ERα expression. Also, the loss of AR could indirectly affect uterine ERα level through systemic alterations. Supporting the first mechanism, testosterone was shown to suppress ERα expression in mammary tissue (Zhou et al., 2000). However, our study has shown that testosterone treatment in ovariectomised PTENKO female caused marked increase in ERα expression in PTENKO uterus in all cells. The second mechanism is supported by our findings whereby AR mediated actions modify PTEN (noting our model still has one functional PTEN

allele) and results in alterations in ERα expression (Vilgelm et al., 2006). Either or both of these mechanisms could represent the molecular pathway involved in the impact of AR mediated androgen action on PTEN inactivation induced uterine cancer. Clearly further mechanistic studies are warranted to further elucidate the role of AR mediated androgen action at various stages of uterine carcinogenesis.

Our study is also informative about the role of $P_4$ action in uterine carcinogenesis; however, further analysis is required. Whereas progestins are known to protect against uterine cancer (Martin et al., 1973a, Persson, 1996), we observed unexpectedly (given that $P_4$ protects against uterine cancer) that serum $P_4$ was significantly increased in PTENKO females compared to other genotypes. However, in our study PTENKO uterus did not seem to benefit from the increased serum $P_4$ levels although PR was expressed in all 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 $P_4$ is consistent with a previous study showing increased CL numbers in ovaries of the granulosa cell specific PTEN inactivation in mice (Fan et al., 2008), a finding which might explain increased serum $P_4$ as CLs characteristically secrete $P_4$. 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 PTENKO and

PTENARKO uterus, we showed that testosterone treatment in ovariectomised PTENKO females increased PR immunopositivity throughout 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 which contained mainly lymphoma and fibroadenomas (Stambolic et al., 2000) but also 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 have suggested 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 $P_4$ level in PTENKO but not in PTENARKO females. It has been shown $P_4$ may promote breast tissue proliferation and could thereby increase the risk of breast cancer 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 PTEN knockout induced fibroadenomas. Mammary glands with

alveolarization could be due to the increase $P_4$ levels, as $P_4$ 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 $P_4$ 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 uterus such as 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, enlarged liver, epithelial hyperplasia in lung, altered ovarian physiology, and 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 suggested that the global AR inactivation reduced PTEN inactivation induced uterine carcinogenesis by possibly decreasing stroma ERα expression and thereby estrogen sensitivity. However, further investigation is required to understand how PTEN and AR may modify ERα expression in the uterus. Most importantly, our study provides first *in vivo* evidence that androgen actions via AR play a significant role in the uterine cancer development and raises the hypothesis that antiandrogen therapy may have a role in prevention and early stages of uterine carcinogenesis and or its treatment.

# Chapter 5:

# Glandular epithelial AR inactivation enhances PTEN inactivation-induced uterine pathology by cell specific modification of PR expression

The content in this chapter is under revision in response to reviewers' comments in Endocrine-Related Cancer journal as of February 2016.

## 5.1 Abstract

Experimentally PTEN (phosphatase and tensin homolog) deletion induces uterine pathology while androgen actions via androgen receptor (AR) support uterine growth so thereby may modify uterine cancer risk. We hypothesized that the androgen actions mediated via uterine glandular epithelial AR could modify PTEN inactivation-induced uterine pathology. Hence, our aims of this experiment are to determine:

i) The effect of glandular epithelial AR inactivation in PTEN inactivation induced uterine pathology as well as uterine morphology.

ii) If glandular epithelial AR inactivation modified uterine P-AKT, ERα, PR and *Cox-2* expression.

To test our hypothesis, we developed uterine glandular epithelial specific PTEN, AR and the double knockout mouse models with uterine pathology compared between 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 knockout-induced uterine pathology with development of intraepithelial neoplasia in ugePTENARKO by 20 weeks of age. In ugePTENARKO females, 6/10 (60%) developed intraepithelial neoplasia while 30% developed glandular hyperplasia whereas only glandular hyperplasia (3/10, 30%) were observed in ugePTENKO females. No uterine pathology was observed in WT (n=8) and ugeARKO (n=7) uteri. Uterine weight was increased in ugePTENARKO [374±97mg (mean±SE)] compared with WT (97±6mg), ugeARKO (94±12mg) and ugePTENKO (205±33mg) (p=0.002). ERα and P-AKT immunopositivity was modified by

uterine pathology but did not differ between ugePTENKO and ugePTENARKO suggesting ERα and P-AKT expressions are not directly affected by genotype. However, PR expression was decreased throughout ugePTENARKO uterus compared to ugePTENKO suggesting PR expression could be regulated by glandular epithelial AR. In conclusion, glandular epithelial AR inactivation (with persistent stromal AR action) enhanced PTEN deletion-induced uterine pathology possibly by down-regulating PR expression in the uterus.

## 5.2 Introduction

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 and Katzenellenbogen, 1979), while global AR inactivation in female mice resulted in thinner uterus with reduced total uterine area compared to wild-type (WT) controls (Walters et al., 2009). In chapter 3 of this thesis, we demonstrated that the effect of androgens (testosterone or DHT) on uterine growth is mediated via AR. In contrast, 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 which were reversed by administration of the steroidal antiandrogen and progestin, cyproterone acetate (Tuckerman et al., 2000). These finding suggest that androgens may have cell specific roles in the uterus, as demonstrated for prostate (Heinlein and 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, our study using global PTEN (a phosphatase and tensin homolog) knockout-induced experimental uterine cancer was enhanced when combined with global AR inactivation further suggested that AR mediated androgen action enhances PTEN inactivation-induced uterine cancer (Chapter 4).

*Pten* is a tumor suppressor gene located on chromosome 10 (Dahia, 2000). Physiologically, PTEN functions as a phosphatase which 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 (EMC) 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 (Chapter 3). The goal of the study was to investigate the role and potential mechanisms of androgen action in the susceptibility of the murine uterus to experimental cancer.

## 5.3 Experimental design

Experimental design for the current study is as follows:

i) The effect of glandular epithelial AR inactivation in PTEN inactivation induced uterine pathology as well as uterine morphology was compared between ugePTENKO and ugePTENARKO females at 20 weeks of age.

ii) To determine the molecular changes caused by glandular epithelial AR inactivation in PTEN inactivation induced uterine pathologies, AKT, P-AKT, ERα, PR and *Cox-2* uterine expressions were compared between WT, ugeARKO, ugePTENKO and ugePTENARKO females at 20 weeks of age.

**Uterine disorder classification**

All uteri from experimental mice were classified into three categories based on histological characteristics: normal glands (Figure 5.1A), hyperplastic glands (Figure 5.1B) and intraepithelial neoplasia (Figure 5.1C). Normal glands had widely separated tubular ductular glands embedded in a cellular stroma (Figure 5.1A). Hyperplastic glands had marked increase in the number of irregularly disorganized glands lined by enlarged glandular epithelial cells (Figure 5.1B). Intraepithelial neoplasia 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) (Figure 5.1C).

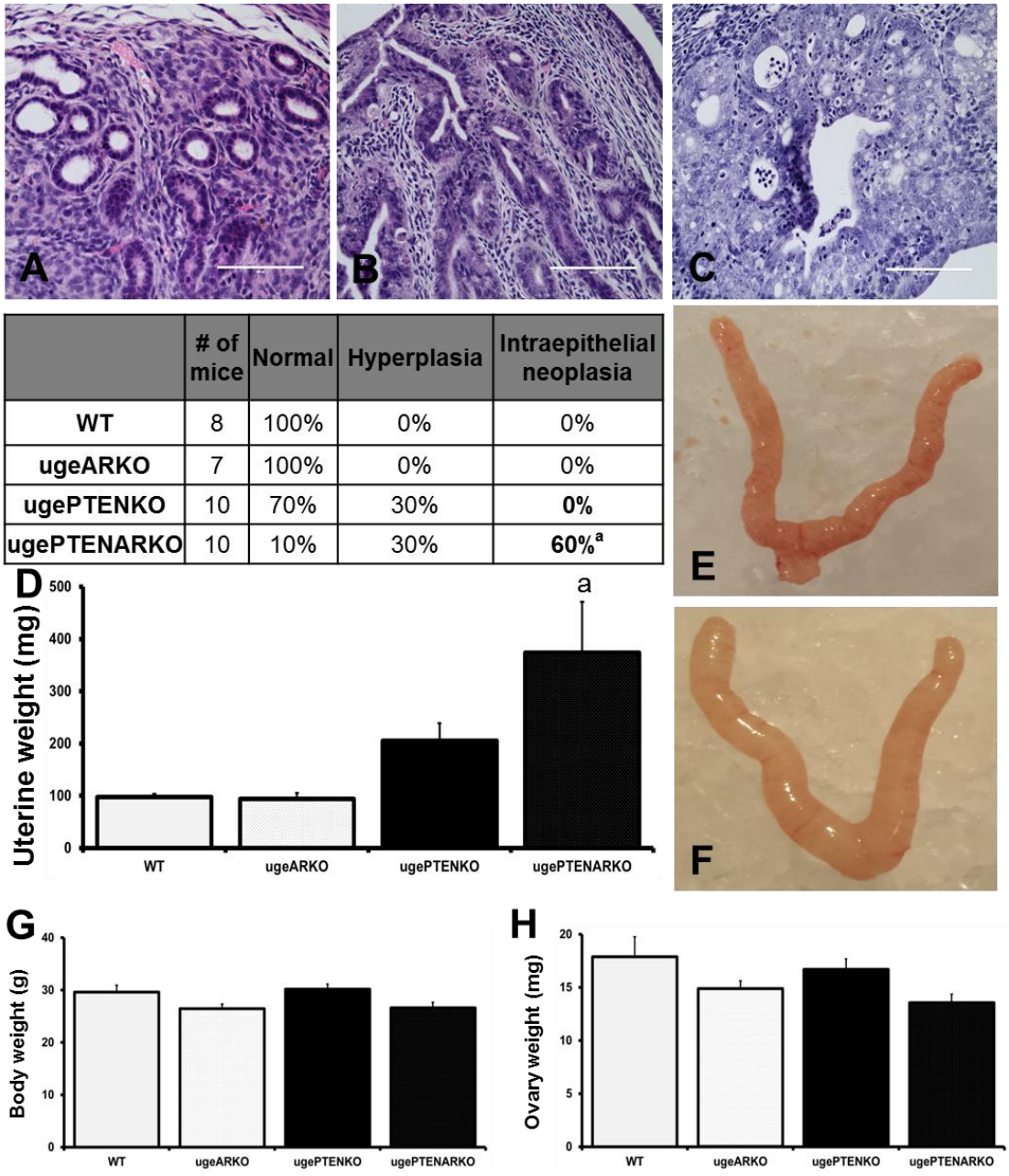
## 5.4 Results

### 5.4.1 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 (Figure 1A), hyperplastic glands (Figure 1B) or intraepithelial neoplasia (Figure 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 the present study, abnormal uteri were found solely in ugePTENKO and ugePTENARKO females (Figure 1: Table) 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 (Figure 1: Table). Intraepithelial neoplasia incidence was significantly increased by the AR inactivation ($p=0.008$; Fisher's exact test).

The uterine pathology in ugePTENKO and ugePTENARKO was also reflected in increased uterine weight (Figure 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, weight of ugePTENARKO uteri were significantly increased compared to other genotypes whereas uterine weight did not differ between WT and ugeARKO uteri (Figure 1D). Macroscopic anatomy of

ugePTENKO uterus (Figure 1E) and ugePTENARKO (Figure 1F) are shown. Body weights (Figure 1G) and ovarian weights (Figure 1H) were not affected by genotype.




| | # of mice | Normal | Hyperplasia | Intraepithelial neoplasia |
|---|---|---|---|---|
| **WT** | 8 | 100% | 0% | 0% |
| **ugeARKO** | 7 | 100% | 0% | 0% |
| **ugePTENKO** | 10 | 70% | 30% | **0%** |
| **ugePTENARKO** | 10 | 10% | 30% | **60%[a]** |

**Figure 5.1. Histological categorization of uterine phenotype and uterine weight.** A-C) 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=100µm). Table: Histological categorization of uterine abnormalities in WT, ugeARKO, ugePTENKO and ugePTENARKO females (a= significantly different to WT, ugeARKO and ugePTENARKO, $p<0.05$; Pearson Chi-Square). D) Uterus weights in WT,

ugeARKO, ugePTENKO and ugePTENARKO females at termination (a= significantly different to WT, ugeARKO and ugePTENARKO, $p<0.05$; one-way ANOVA, mean±SE; $n \geqslant 9$). E-F) Representative gross anatomy images of ugePTENKO (E) and ugePTENARKO (F) uterus. G-H) Body weight (G) and ovary weight (H) were compared among WT, ugeARKO, ugePTENKO and ugePTENARKO (mean±SE; $n \geqslant 9$).

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

As uterine weight was increased in ugePTENARKO uterus, we investigated whether different layers of the uterus were affected (Figure 2A-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 the 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 (Figure 2A) and endometrial (Figure 2B) cross-sectional areas were increased in ugePTENARKO compared to other genotypes. However, the myometrial areas were not significantly affected by either AR inactivation ($p=0.085$) or PTEN deletion ($p=0.486$) (Figure 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

(Figure 2D). The endometrial to myometrial ratios were significantly increased by both the AR inactivation (p=0.036) and PTEN deletion (p=0.009); interaction of AR inactivation and PTEN deletion was not significant.

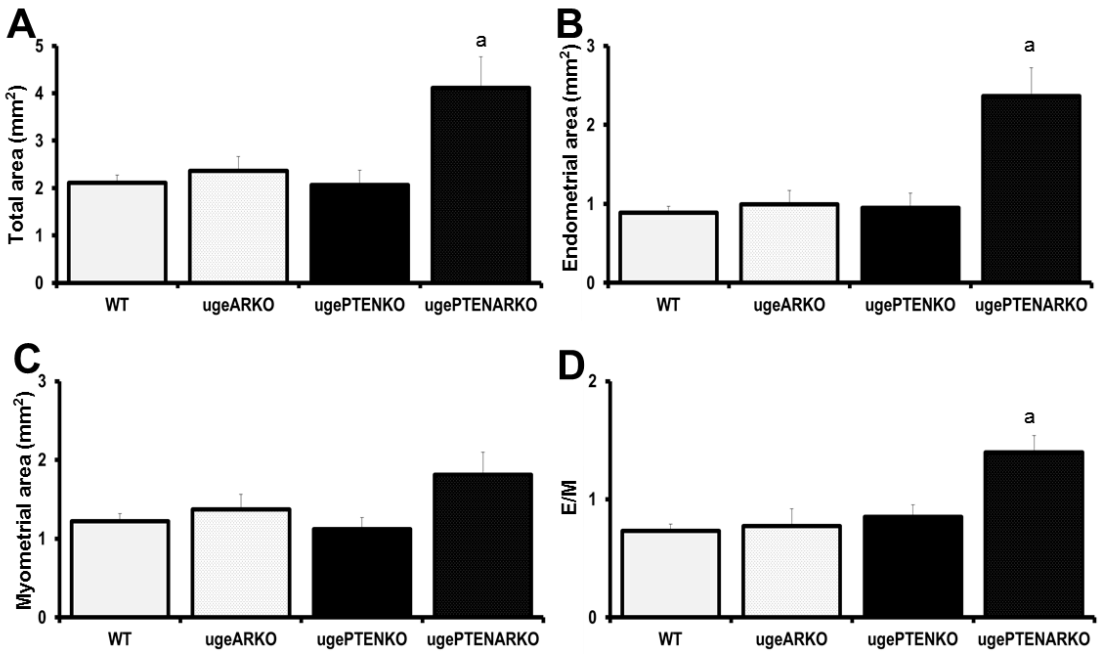



**Figure 5.2. Morphological analysis of the uterus.** Total (A), endometrial (B) and myometrial (C) uterine areas ($mm^2$) were measured in WT, ugeARKO, ugePTENKO and ugePTENARKO females. Endometrial to myometrial ratio (E/M) was calculated by dividing area of endometrium with area of myometrium (D) (a= significantly different to WT, ugeARKO and ugePTENKO, $p<0.05$; one-way ANOVA, mean±SE; $n \geqslant 5$).

### 5.4.3 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 when compared within the same histopathological categories (normal and hyperplastic) (Figure 3A-E). However, AKT immunopositivity was stronger in the epithelia with intraepithelial neoplasia compared to normal or hyperplastic epithelium (Figure 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 (Figure 3J) compared to normal (Figure 3F and G) or hyperplastic (Figure 3H and I) glandular epithelial cells. P-AKT expression appeared similar between ugePTENKO and ugePTENARKO within normal (Figure 3F and G) and hyperplastic (Figure 3H and I) glands.

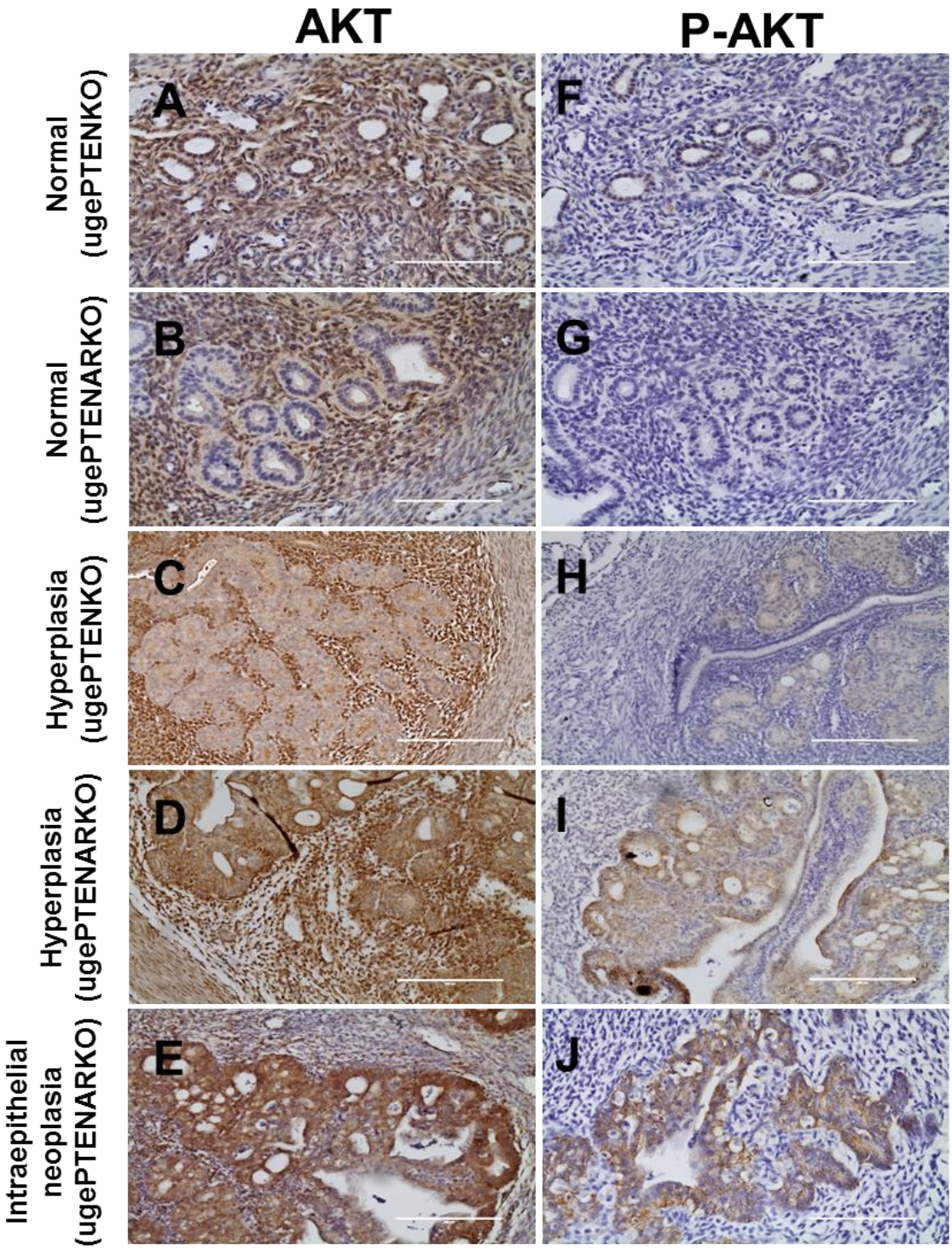



**Figure 5.3. Representative AKT and P-AKT immunohistochemistry in ugePTENKO and ugePTENARKO.** AKT expression (A-E) was compared between normal ugePTENKO (A) and ugePTENARKO (B), hyperplastic glands ugePTENKO (C) and ugePTENARKO (D) and intraepithelial neoplastic glands

(E). P-AKT expression (F-J) was compared between normal ugePTENKO (F) and ugePTENARKO (G), hyperplastic glands ugePTENKO (H) and ugePTENARKO (I) and intraepithelial neoplastic glands (J) (Scale bar=100μm for A-B, E-G and J & 200μm for C-D and H-I; n⩾4).

### 5.4.4 ERα expression was reduced in uterine stroma and myometrium with intraepithelial neoplastic glands when compared to the uteri with hyperplastic glands

As uterine pathology is highly hormone dependent and promoted by $E_2$ (Bender et al., 2011), we investigated ERα expression by immunohistochemistry. ERα expression was quantified according to percentage immunopositive (Figure 4A) while the intensity of staining (Figure 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 ERα expression (Choi et al., 2015a).

ERα immunopositivity and immunointensity were not signficantly different between histologically normal WT (Figure 4C) and ugeARKO (Figure 4D) uteri noting that ERα was relatively weakly expressed in all cells types (epithelia stroma, and myometrium). On the other hand, glandular epithelial PTEN deletion significantly increased ERα immunopositivity ($p \leq 0.028$) and immunointensity ($p \leq 0.028$) throughout the uterus in histologically normal appearing ugePTENKO (Figure 4E). The histologically normal ugePTENARKO (Figure 4F) uterus had a similar ERα 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) (Figure 4G and H), ERα immunopositivity ($p \leq 0.028$) and immunointensity ($p \leq 0.028$) were signficantly increased when compared to histologically normal WT and ugeARKO uterus (Figure 4C and D) regardless of cell type. However, when compared with histologically normal ugePTENKO and ugePTENARKO uterus (Figure 4E and F) there were no signficant changes. The ERα immunopositivity was similar in ugePTENKO (Figure 4G) and ugePTENARKO (Figure 4H) uterus with hyperplastic glands.

The intraepithelial neoplastic glands were only detected in ugePTENARKO females (Figure 4I and J). In these samples, the ERα 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 signficantly increased compared to histologically normal uterus in WT and ugeARKO females. However, in intraepithelial neoplastic glands detected in ugePTENARKO females, the ERα immunopositivity and immunointensity in stroma and myometrium were signficantly ($p=0.037$) decreased compared to ugePTENKO (normal), ugePTENKO (hyperplasia) and ugePTENARKO (hyperplasia). No signficant changes were observed in luminal epithelial ERα expression.

Collectively, these results suggest that the ERα expression is dependent on the histopathological stage as the glandular epithelial ERα expression was

increased in uterus with intraepithelial neoplastic glands compared to histologically normal uterus whereas stromal and myometrium ERα expression was decreased compared to hyperplastic uterus. In addition, glandular epithelial PTEN deletion upregulated ERα expression whereas glandular epithelial AR inactivation may not significantly affect ERα expression in uterus.

| 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] |

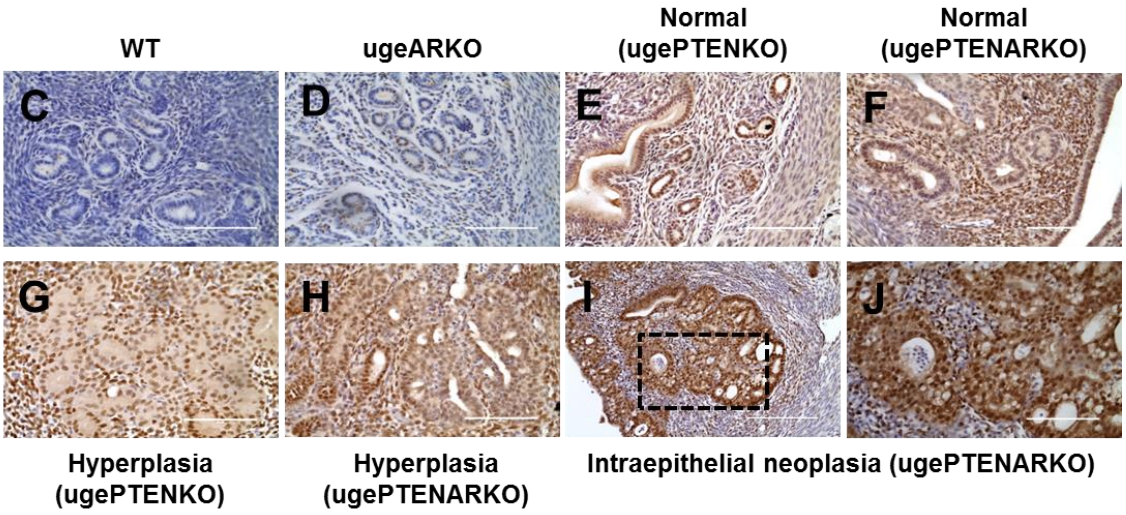



**Figure 5.4. ERα immunohistochemistry on uterus at 20 weeks old.** A-B) Percentage of ERα positive cells (A) and intensity (B) in different compartments of the uterus [a= significantly different to WT and ugeARKO and b=significantly different to ugePTENKO (normal), ugePTENKO (hyperplasia) and ugePTENARKO (hyperplasia) ($p \leqslant 0.037$; non-parametric test, mean±SE; $n \geqslant 3$)]. C-J) 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-J). (Scale bar=100µm for C-D, G-H and J & 200µm for E-F and I; n⩾4).

### 5.4.5 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 (Figure 5A) and immunointensity (Figure 5B) using H-Score method and further categorized based on histopathology.

PR was abundantly expressed all cell types (epithelial, stromal and smooth muscle cells) of WT uterus (Figure 5C). PR immunopositivity ($p \leq 0.021$) and immunointensity ($p \leq 0.021$) in ugeARKO uterus (Figure 5D) were significantly reduced compared to WT in all cell types suggesting glandular epithelial AR inactivation down regulates PR expression in uterus. On the other hand, in histologically normal uterus of ugePTENKO females (Figure 5E), PR immunopositivity ($p \leq 0.028$) and immunointensity ($p \leq 0.028$) were significantly increased in stroma and myometrium compared to WT and ugeARKO (Figure 5C, D). The PR expression in glandular epithelial and luminal epithelial cells was not affected by PTEN deletion. PR expression in ugePTENARKO (normal) uterus (Figure 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 uterus with hyperplastic glands (both ugePTENKO and ugePTENARKO) (Figure 5G and H), PR was strongly expressed throughout the uterus and PR immunopositivity ($p \leq 0.028$) and immunointensity ($p \leq 0.028$) was significantly increased compared to WT and ugeARKO uterus. 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 (Figure 5I-J). PR immunopositivity ($p=0.037$) and immunointensity ($p=0.037$) were significantly reduced in all cell types compared to hyperplastic uterus. Furthermore, stroma and myometrium PR expression were significantly ($p \leq 0.05$) reduced compared to WT and ugeARKO uterus as well. Hence, simultaneous glandular epithelial AR inactivation along with PTEN deletion significantly reduced PR expression in uterus.

| 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[acd] | 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[acd] |

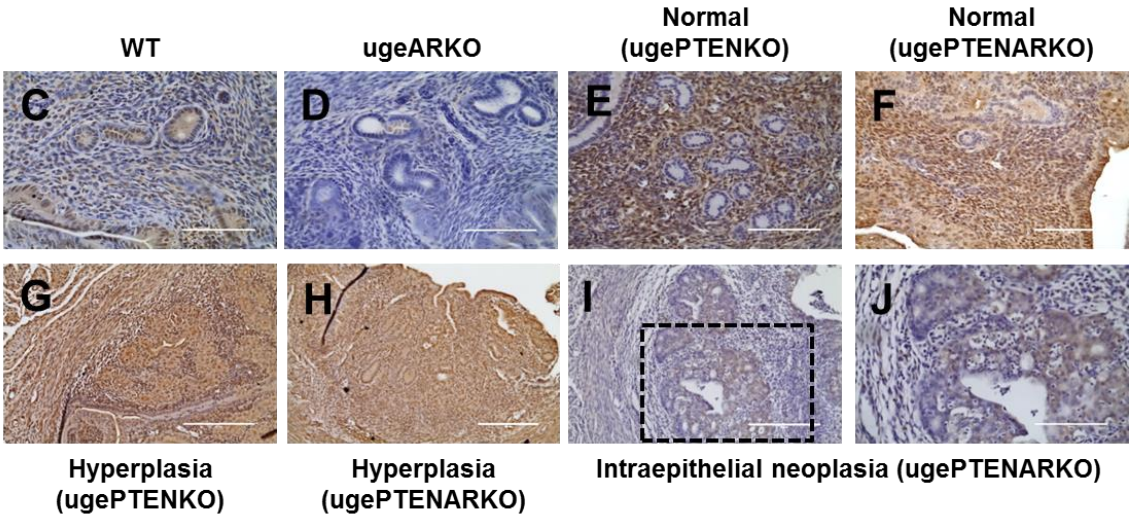



**Figure 5.5. PR immunohistochemistry on uterus at 20 weeks old.** A-B) Percentage of PR positive cells (A) and intensity (B) in different compartments of the uterus [a= significantly different to WT, and b=significantly different to ugeARKO, c=significantly different to ugePTENKO (normal) and d=significantly different to ugePTENKO (hyperplasia) and ugePTENARKO (hyperplasia) ($p \leqslant 0.05$; non-parametric test, mean±SE; $n \geqslant 3$)]. 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-J). (Scale bar=100μm for C-D and J & 200μm for E-F, G-H and I; n≥4).

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

Real-time RT-PCR was performed to determine the expression of *ERα* and *PR* and their dependent genes in uterus. No significant changes were observed in *ERα* gene expression as well as in expression of ERα dependent genes, *G6PDH* and *Complement C3* (Figure 6A-C). Although *PR* and its dependent genes, *Ihh* and *Il13ra2*, appeared to be increased by PTEN deletion (ugePTENKO) which was reversed by simultaneous AR inactivation (ugePTENARKO), the changes were not statistically significant (Figure 6D-F).

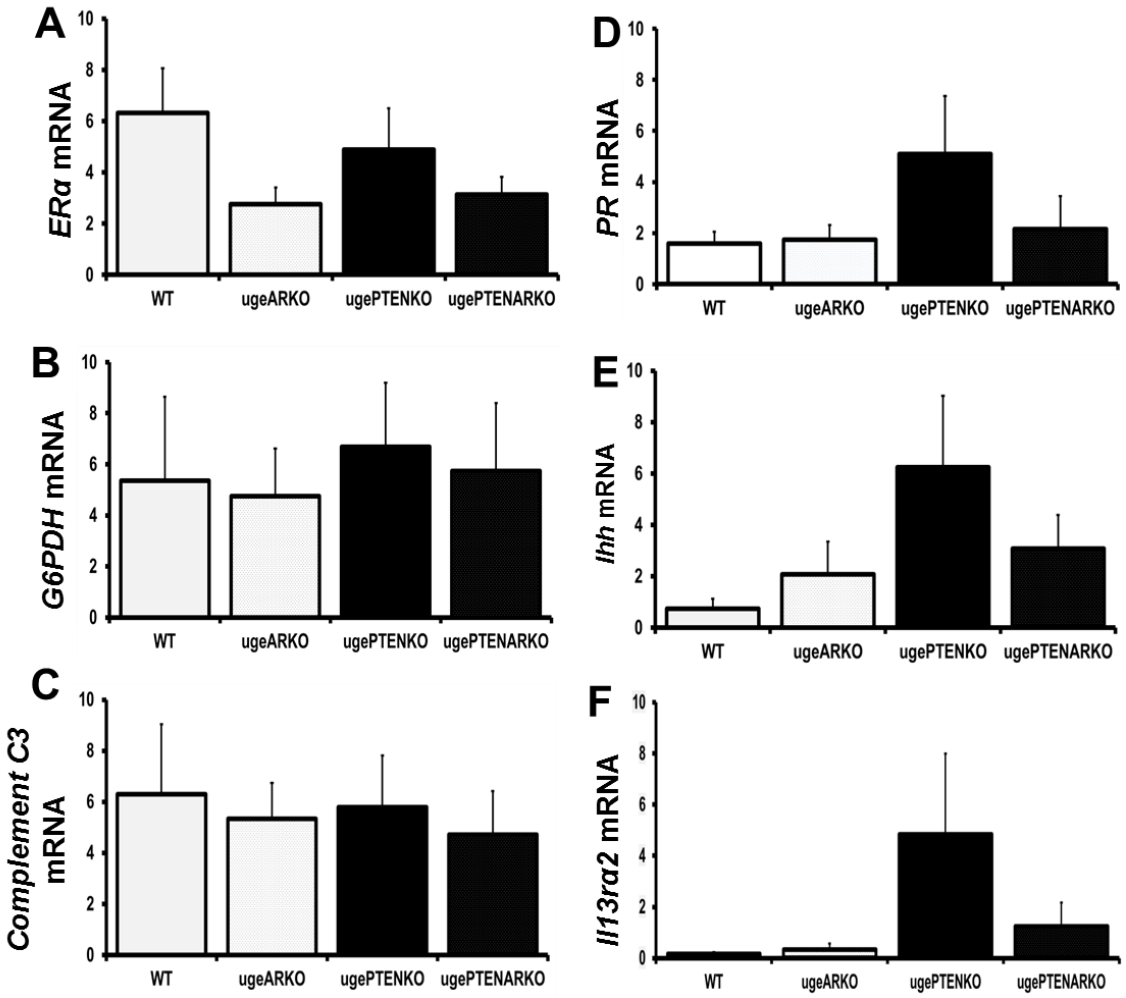



**Figure 5.6. mRNA expression of *ERα* and *PR* and its dependent genes.** *ERα* (A) and *ERα* dependent genes (B-C) and *PR* (D) and *PR* dependent genes (E-F) were measured in the uterus at 20 weeks of age. Gene expressions were relative to *18s rRNA* housekeeping gene.

### *5.4.7 Cox-2* gene expression was significantly increased in ugePTENARKO uterus

As modification of COX signaling is reported in PTEN knockout-induced uterine cancers where *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 the 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 whereas *Cox-2* expression was similar among WT, ugeARKO and ugePTENKO uterus (Figure 7A). *Cox-1* mRNA expression was not affected by either AR inactivation (p=0.76) or PTEN deletion (p=0.241) (Figure 7B).

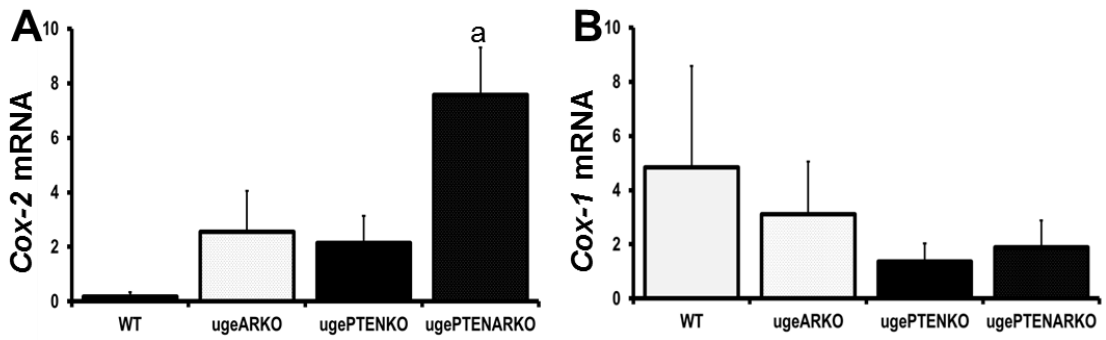



**Figure 5.7. Uterine cox-1 and cox-2 gene expression at 20 weeks.** Cox-2 (A) and cox-1 (B) mRNA expression was compared among experimental groups and gene expression was standardized using *rpl19* and *cyclophillin* (a= significantly different to WT and ugePTENKO; $p<0.03$; non-parametric test, mean$\pm$SE; n$\geqslant$5).

## 5.5 Discussion

We recently demonstrated that androgen action mediated via AR specifically exerted 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 (Walters et al., 2010, Somboonporn and Davis, 2004) 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. We demonstrate that AR mediated androgen action exerted in the same cells as having PTEN deletion-induced pathology, has a direct and significant role in the uterine carcinogenesis*. This is also consistent with a previous study proposing that endometrial cancers arise from endometrial glands* (Newbold et al., 1990). In our study, glandular epithelial AR inactivation significantly enhanced and accelerated progression of PTEN deletion-induced uterine pathology. We provide some evidence that this may be due to down-regulation 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 and 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 where 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, 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 (Choi et al., 2015a), the present study suggests that androgens acting via glandular epithelial AR decelerated progression of experimental, PTEN deletion-induced uterine pathology. Alternatively, the this observation 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 AR 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 model is outlined in table 5.1. In our previous report, AR and/or PTEN were knocked-out in all cells of the mouse including all uterine cells (glandular epithelium, luminal epithelium, stroma and myometrium). 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 PTEN are still functional in all other cells of the uterus (luminal epithelium, stroma and myometrium) and rest of the mouse. Furthermore, global knockout model has AR and/or PTEN knocked out in other hormone dependent organs such as ovaries and pituitary whereas uterine gland specific knockout model has functional AR and PTEN in these non-target organs (Choi

et al. 2015b). Furthermore, the heterozygous and homozygous loss of PTEN in inducing uterine pathology should be considered in future studies.

**Table 5.1. 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 & PTEN** | Non-functional | Non-functional |
| **Luminal epithelial AR & PTEN** | Functional | Non-functional |
| **Stromal AR & PTEN** | Functional | Non-functional |
| **Myometrial AR & 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 |

In previous studies, heterozygous PTEN deletion in uterus induced glandular hyperplasia (premalignant precursor to endometrial cancer) in the uterus (Daikoku et al., 2008). Previously, we demonstrated that PTEN deletion in uterine glands induced uterine hyperplasia (Choi et al., 2015b). In our current study, simultaneous glandular epithelial AR inactivation along with PTEN deletion significantly accelerated severity of uterine pathology from hyperplastic glands detected in both ugePTENKO and ugePTENARKO uterus 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 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 where androstenedione (an aromatizable pro-androgen) inhibited proliferation of human endometrial cells (Tuckerman et al., 2000). The effect was reversed by cyproterone acetate (antiandrogen) demonstrating AR mediated effects. Together with the results of the current 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 non-epithelial uterine cells, notably in the uterus, the stroma and other epithelial cells.

Our study has supported previous reports of PTEN negatively regulating P-AKT expression (Stambolic et al., 2000, Stambolic et al., 1998). P-AKT was only present in glandular epithelial cells of the uterus following complete deletion of PTEN, whereas P-AKT was absent in luminal epithelial cells, stroma and myometrium as well as in WT glandular epithelial cells where PTEN was present. Very low P-AKT levels in uterine cells expressing PTEN is supported by previous study (Stambolic et al., 2000). In uterus, AR does not appear to affect AKT signaling pathway as it has been suggested in breast and prostate cancer cells (Wang et al., 2011b). In the present study, 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. This suggests that glandular epithelial AR does have a significant affect the progression of uterine pathology; however, further investigation is required to determine whether AR has a direct role in increasing P-AKT pathway or increased P-AKT could be solely due to increased severity of the uterine pathology.

A previous study suggested that PTEN inactivation increased ERα expression in mouse uterus (Lian et al., 2006). Similarly, AR can also regulate ERα expression as shown by testosterone-induced suppression of ERα expression in mammary tissue (Zhou et al., 2000). Alternatively, AR could interact with PTEN to indirectly modify ERα expression in prostate and breast cancers (Wang et al., 2011b). Therefore, we explored the possibility whether glandular epithelial AR inactivation modified ERα expression in PTEN deleted uterus. Supporting the previous study, we observed increased ERα expression throughout the uterus upon PTEN deletion. However, in our study, glandular epithelial AR inactivation in uterus did not modify ERα expression, unlike in the prostate where inactivation of prostate epithelial AR cells causes marked up-regulation of ERα expression (Simanainen et al., 2011b). As the PTEN deleted uterus developed hyperplastic glands, uterine ERα expression was stronger than histologically normal PTEN deleted uterus. Furthermore, when hyperplastic glands progressed to intraepithelial neoplastic glands which were only found in ugePTENARKO uterus, ERα expression in the glandular epithelial cells increased but ERα expression in luminal epithelial cells, stroma and

myometrium decreased. This could be due to the increased P-AKT expression in glandular epithelial cells as it has been reported that P-AKT and ERα is positively correlated (Vilgelm et al., 2006). Collectively, these data suggest that the increased uterine gland pathology following PTEN deletion in glandular epithelial cells could be due to increased P-AKT and ERα expression as supported by our immunohistochemistry results. However, accelerated uterine pathology in ugePTENARKO uterus compared to ugePTENKO may not be due to altered P-AKT and ERα expression. Therefore, we have further investigated PR expression.

The protective effect of P4 on uterine cancer is known from using progestin (synthetic P4 analogs) (Mortel et al., 1990) where progestin actions mediated via PR (Mulac-Jericevic et al., 2000, Kurita et al., 1998) protect against uterine cancer (Martin et al., 1973a, Persson, 1996). Therefore we investigated PR expression in the uterus of our experimental female mice. In our study, 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 glandular epithelial AR action was maintains PR expression. Secondly, in glandular epithelial PTEN deleted uterus, we observed increased PR expression in luminal epithelial cells, stroma and myometrium but decreased PR expression in glandular epithelial cells of intraepithelial neoplastic ugePTENARKO uterus. The finding supports previous findings that PTEN down-regulation was correlated with PR down-regulation in breast cancers (Alen et al., 1999). Further, the present study demonstrates that a

uterus bearing intraepithelial neoplastic glands has further diminished PR expression in all cells when compared to a uterus with hyperplastic glands regardless of genotype. Whether this is due to the histopathological change or glandular AR inactivation is uncertain. 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. However, based on the findings in histologically normal uterus (reduced PR immunopositivity), the possibility of AR (specifically glandular epithelial AR) regulating PR expression in uterus should be further explored.

In addition, cell specific interaction of AR should be explored in future. As previously suggested, the different effects of P4 via PR in endometrial cancer (inhibitory) and breast cancer (stimulatory) may be due to the key difference between these tissues such as in the paracrine interactions of PR-expressing stroma and epithelium (Kim et al., 2013). Hence, the AR interaction between epithelial and stromal cells could also determine the role of AR in uterine pathogenesis. Our global AR knockout study where both the epithelial and stromal AR in uterus are inactivated, promoted uterine pathogenesis (Choi et al., 2015a). On the other hand, in current study, where only the glandular epithelial AR is inactivated (with persistent stromal AR activity), the AR activity appeared to inhibit uterine carcinogenic progression. Therefore, we suggest possible paracrine interaction of AR-expressing cells in the uterus (*i.e.* epithelial and stroma).

Furthermore, as the glandular epithelial PTEN deleted uterus developed hyperplastic glands, PR expression increased in glandular epithelial cells as well resulting in strong PR expression throughout the uterus. Increased PR expression in uterus with hyperplastic glands could be due to a compensatory mechanism to combat developing pathology in the glands as P4 is known to oppose uterine epithelial proliferation *(Mortel et al., 1990, Fang et al., 2004). A previous study observed that 90% of hyperplastic uteri had moderate to strong PR expression (Arnett-Mansfield et al., 2001).* However, when hyperplastic glands progressed to intraepithelial neoplasia as in ugePTENARKO alone, an adenocarcinoma precursor, uterine PR expression was very weak throughout the uterus including neoplastic glands. Furthermore, our finding was supported by the observations that PR expression decreased as uterine pathology advances to endometrial cancers (Kim et al., 2010, Arnett-Mansfield et al., 2001, Ehrlich et al., 1981). Therefore, accelerated uterine pathogenesis in ugePTENARKO compared to ugePTENKO could be due to the down-regulation of PR by glandular epithelial AR inactivation resulting in diminished protective role of P4 via PR. However, decreased PR expression in intraepithelial neoplastic glands could also be due to increased P-AKT expression in the glands as reported in breast cancers (Tokunaga et al., 2007).

Despite the PR immunohistochemistry results, we did not observe any significant changes in *PR* gene expression or expression of PR-dependent genes, *Ihh* and *Il13ra2*, expressions. A probable explanation for this is that

gene expression was quantified using RNA extracted from whole uterus whereas the AR and PTEN deletions are only in glandular epithelial cells constituting only a very small fraction of all uterine cells. Hence significant changes could have been diluted and lost. Nevertheless, we did observed similar, though not statistically significant, pattern where *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 due to variation in PTEN knockout induced pathology which varies in rate of progression between individuals, as shown in histopathology/immunostaining. Therefore, in the future, more specific cell retrieval and/or individual samples with comparable stage of histopathology should be analyzed.

As uterus is highly hormone dependent organ, changes in hormone profile could regulate its pathogenesis. However, we did not observe any changes in steroid hormone levels in our mouse model in the more radical global knockout study (Choi et al., 2015a) in which both PTEN and/or AR were inactivated (including in ovaries as well). Hence, we do not expect any significant hormonal changes in current study where the PTEN and/or AR inactivation is restricted to only the glandular epithelial cells in the uterus (Choi et al. 2015b). However, it would be interesting to measure serum and especially intrauterine steroid hormone levels as we observed very unusual PR immunohistochemistry expression despite all the uteri were collected at diestrus. Previous study has reported varying progesterone and testosterone levels in endometrium and endometriosis lesions (Huhtinen et al., 2014).

Cox enzymes are associated with development of many cancers (Mazhar et al., 2005) and its expression is regulated by androgens (Yazawa et al., 2013, Simanainen et al., 2015) and AKT (St-Germain et al., 2004, Leng et al., 2003). Therefore, we measured uterine *Cox-1* and *Cox-2* mRNA expressions. *Cox-2* is important in progression of uterine hyperplasia to carcinoma, where *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 mutation 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 endometrial carcinoma (Erkanli et al., 2007, Nasir et al., 2007). On the other hand, *Cox-1* which 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 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 ERα expression in glandular epithelial cells and resulting in increased uterine *Cox-2* expression. Furthermore, glandular epithelial AR inactivation enhanced PTEN inactivation-induced uterine gland pathology possibly by decreasing PR expression hence diminishing the protective role of $P_4$ in uterine pathogenesis. This suggests a different mechanism as to our previous global knockout study (Choi et al., 2015a), where global AR inactivation reduced uterine pathology incidence by decreasing ERα compared to 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.

# Chapter 6:

# Conclusions and future directions

The biological effects of AR-mediated androgen actions in male physiology and pathology of hormone dependent organs such as prostate has been well established. However, the direct role of androgens in females has only recently been recognised (Walters et al., 2009) and has not yet been fully understood. This is exemplified in uterus which has been shown to express AR (Pelletier et al., 2004, Hirai et al., 1994, Kimura et al., 1993, Ito et al., 2002) and respond to androgens (Armstrong and Papkoff, 1976, Schmidt and Katzenellenbogen, 1979, Nantermet et al., 2005, Walters et al., 2009), but there is only limited information on the specific role of androgens acting via AR during uterine development and pathology. Therefore, the aim of this thesis was to investigate the role of AR-mediated androgen actions in uterine development, function and pathogenesis using novel mouse models.

The first aim was to generate and validate uterine glandular epithelial specific AR knockout (ugeARKO) mouse model using Cre/LoxP strategy. Thereby investigate the role of AR in uterine growth and function (Chapter 3). This study have successfully generated and characterized the first endometrial gland specific knockout mouse model. Using the model and along with global ARKO mouse model, we have demonstrated that androgens acting solely via AR can support full uterine growth in the absence of E2. Furthermore, we have shown endometrial glandular AR mediates uterine (including myometrial) growth via AR-mediated androgen actions. As AR is expressed throughout the uterus, our finding warrants further studies of the role of cell-specific AR and cross-talk involving AR expression in different types of uterine cells such as luminal, stromal and myometrial cells. This could be achieved in future by generating

uterine cell-specific knockout models. However, currently there are no known models other than our endometrial gland specific knockout model which is the first mouse model to selectively knock out glandular epithelial cells. This would be important as our results suggest that AR in different uterine cell types exhibit different androgens effects in uterus. Better understanding of the relationship between different uterine cell-specific AR could be implicated to uterine development disorders and uterine cancers as uterine cancers also express AR in all cells.

Secondly, our aim was to investigate the role of global AR inactivation in PTEN deletion induced uterine cancer (Chapter 4). Importantly, our study is the first *in vivo* evidence that androgen actions via AR play a significant role in the uterine carcinogenesis where we have demonstrated that the global AR inactivation reduced its impact of PTEN inactivation-induced uterine carcinogenesis. This could be due to decreasing stromal ERa expression and thereby estrogen sensitivity. However, as the PTEN can directly interact with ERα, the haploinsufficiency of PTEN and involvement of stromal ERα requires further investigation possibly using complete PTEN deletion in the uterus (*i.e.* PR-Cre mouse model). Therefore, our finding raises the hypothesis that antiandrogen therapy which are readily available for breast and prostate cancer patients may also benefit uterine cancer patients. However, this would require further studies on the cell specific effect of AR as androgens appears to promote or suppress uterine cancers depending on the cell-specific expression of AR. Furthermore, the future studies could be directed to better understand how PTEN and AR separately or via interaction with each other can modify ERα expression (as

suggested by the present study) or other signalling pathways in uterus. Understanding the mechanisms involved in PTEN induced uterine cancers as well as modification of cancer incidences by AR-mediated androgen actions is important and would benefit in providing better prevention and targeting pathways for uterine cancer treatment, as many uterine cancers are AR positive and have PTEN mutations. In addition, we must note that there could be other important pathways involved in modifying uterine cancer incidences in our study, which further displays a complex molecular pathways involved in uterine physiology and pathology.

Lastly, our aim was to investigate the effect of glandular epithelial AR inactivation in PTEN inactivation induced uterine pathology (Chapter 5) using our novel endometrial gland specific knockout mouse model generated and characterized (Chapter 3). The study has demonstrated that the glandular epithelial PTEN deletion is likely to contribute uterine gland pathology by increasing P-AKT and ERα expression in glandular epithelial cells and resulting in increased uterine *Cox-2* expression. Furthermore, glandular epithelial AR inactivation enhanced PTEN inactivation-induced uterine gland pathology possibly by decreasing PR expression hence diminishing the protective role of $P_4$ in uterine pathogenesis. However, further investigations are required to understand the regulation of ERα and PR via AR in uterus. Nevertheless, in together with our global AR knockout study (Chapter 4); our findings suggest possible systemic or tissue-specific effects of androgens in uterus. Furthermore, supporting our uterine development study (Chapter 3), AR may also exhibit cell-specific effects in uterine carcinogenesis. These possibilities warrant further

studies on the role of AR in uterine carcinogenesis where the study is very much insufficient.

In conclusion, this thesis has provided multiple findings of novel *in vivo* evidence supporting the important role of androgens in uterine development and pathogenesis as well as providing mechanisms involved. Furthermore, we have generated the first endometrial gland specific knockout mouse model which could be used in other studies investigating the role of other important genes/protein in uterine research. However, as the study in this area is very much lacking, hence, the findings need to be validated in humans (*i.e.* cell culture studies). Nevertheless, our study provides wide platform and approaches to future uterine research which would lead to better prevention, biomarker and treatment of uterine disorders including endometrial cancers.

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# Appendix

# Appendix I: PCR procedures

## DNA extraction (Qiagen DNeasy Blood & Tissue Kit)

1. Cut tissue (≤10 mg spleen or ≤25 mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.
2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x *g*. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x *g*.
9. Optional: Repeat step 8 for increased DNA yield.

## Master Mix for genotyping PCR

All the PCR reagents are purchased from Bioline (Alexandria, Australia) and prepared in sterile 0.5ml PCR tubes (ThermoFisher Scientific, Melbourne, Australia).

| **Reaction mix** | **Cre** | **ARCUT** | **ARFLOX** | **PTEN** | **ARCUT RT** | **Conventional** |
|---|---|---|---|---|---|---|
| Autoclaved Milli-Q water | 12µl | 11.5µl | 12.875ul | 10µl | 12µl | 12µl |
| 5x MyTaq Red reaction buffer | 4µl | 4µl | NA | NA | 4µl | 4µl |
| 5x Mango Taq buffer | NA | NA | 5ul | 4µl | NA | NA |
| $MgCl_2$ (50µM) | NA | NA | 1ul | 0.8µl | NA | NA |
| dNTPs | NA | NA | 2ul | 2µl | NA | NA |
| Forward primer-1 (10µM) | 0.5µl | 0.5µl | 0.5µl | 0.5µl | 0.5µl | 0.5µl |
| Forward primer-2 (10µM) | NA | NA | 0.5µl | NA | NA | NA |

| | | | | | | |
|---|---|---|---|---|---|---|
| Reverse primer-1 (10µM) | 0.5µl | 1µl | 1ul | 0.25µl | 0.5µl | 0.5µl |
| Reverse primer-2 (10µM) | NA | NA | NA | 0.25µl | NA | NA |
| MyTaq Red DNA polymerase | 1µl | 1µl | NA | NA | 1µl | 1µl |
| Mango Taq | NA | NA | 0.125ul | 0.2µl | NA | NA |
| Template | 2µl | 2µl | 2µl | 2µl | 2µl | 2µl |
| Total volume | 20µl | 20µl | 25ul | 20µl | 20µl | 20µl |

## DNase treatment and cDNA conversion

DNase I, amplification grade (Invitrogen, CA, USA) was used for DNase treatment and SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, CA, USA) was used for cDNA conversion.

1. **DNase treatment**

**RNA** → 2µg (calculate volume to be added using RNA concentration)

**DNase 10x buffer** → 1µL

**DNase I** → 1µL

**RNase free water** → 10µL-(RNA, DNase 10x buffer and DNase I)

Total volume = 10µL

i) Incubate for 15 mins at Room temperature (RT).

ii) Add 1µL of EDTA (25mM)

iii) Incubate for 10 mins at 65°C (use PCR machine)

iv) Straight to ice for ~1 min.

v) Spin down the tube and transfer liquid to new PCR tube.

This is ready for cDNA conversion (reverse transcriptase treatment).

2. **cDNA conversion**

**2x RT Reaction Mix** → 10µL

**RT Enzyme Mix** → 2µL

**RNA (upto 1µg)** → XµL

**DEPC-treated water** → to 20µL

i) Gently mix tube contents and incubate at 25ºC for 10 minutes.

ii) Incubate tube at 50ºC for 30 minutes.

iii) Terminate the reaction at 85ºC at 5 minutes, and then chill on ice.

iv) Add 1µl of *E.coli* RNase H and incubate at 37ºC for 20 minutes.

v) Dilute cDNA with RNase free water (1 to 5) and store at -20ºC until use.

## Master Mix for real-time PCR

Reagent: SensiMix SYBR Hi-ROX Kit (Bioline, Alexandria, NSW, Australia)

| **Reaction mix** | **Real-time PCR** |
|---|---|
| RNase free water | 2.5µl |
| SensiMix (2x) | 5µl |
| Forward primer (10µM) | 0.25µl |
| Reverse primer (10µM) | 0.25µl |
| Template | 2µl |
| Total volume | 10µl |

# Appendix II: Solutions

## Tris-borate-EDTA (TBE) buffer (1x)

Dissolve 1 pack of TBE Buffer, 10x Ready-Pack (Amresco, Ohio, USA) in 1L of RO water. Dilute 100ml of 10x TEB buffer in 900ml of Reverse osmosis (RO) water (Millipore). Final concentration of 0.089M Tris base, 0.089M Borate, 0.002M EDTA).

## 0.9% Saline solution

Dissolve 9g of sodium chloride (NaCl) (Bacto Laboratories PTY LTD, NSW, Australia) in 1L of milli-Q water (Millipore, Milli-Rx 45 with Milli-Q academic).

## 0.5% toludine blue

Dissolve 500mg of Toludine blue powder (Amresco, Ohio, USA) in 100ml of RO water.

## 4% Paraformaldehyde (PFA)

Dissolve 4g PFA (MERCK, Darmstadt, Germany) in 96ml of phosphate buffered saline (PBS with 1-2 drops of 10M NaOH. 4% PFA was stored in 4ºC for up to a week.

## Phosphate buffered saline (PBS)

Dissolve 8g NaCl, 0.2g KCl, 1.44g $Na_2HPO_4$ and 0.24g $KH_2PO_4$ in 800ml of RO water. Adjust pH to 7.4 and add RO water to bring final volume to 1L. Stored at room temperature with final concentration of 0.137M NaCl, 0.0027M KCl, 0.01M $Na_2HPO_4$ and 0.0018M $KH_2PO_4$.

## LacZ staining soutions

**Fixing solution:** Mix 10µl of 100% NP40 (Sigma Aldrich, St Louis, USA), 0.5ml of 0.5M EDTA (pH=8) (Thermo-Fischer scientific, Waltham, USA), 0.4ml of 25% glutaraldehyde (4ºC) (Sigma Aldrich, St Louis, USA), 0.1ml of 1M $MgCl_2$ (Sigma Aldrich, St Louis, USA) and add PBS to make the final volume of 50ml.

**Washing buffer:** Mix 40µl of NP 40, 0.4ml of 1M MgCl2, 0.2ml of 10% Sodium Deoxycholate (Sigma Aldrich, St Louis, USA) and add PBS to make the final volume of 200ml.

**Staining solution:** Mix and dissolve 1ml of 50mg/ml X-gal in dimethylformamide (-20ºC) (Astral Scientific, Caringbar, Australia), 0.106g of potassium ferrocyanide (Sigma Aldrich, St Louis, USA), 0.082g of potassium ferricyanide (Sigma Aldrich, St Louis, USA), 1ml of 1M Tris (pH=7.4) (Sigma Aldrich, St Louis, USA) and add washing buffer to make the final volume of 50ml.

# Appendix III: Histology and staining

## Paraffin embedding

Tissues were processed at ANZAC Research Institute as the protocol below:

| Reagent | Duration |
|---|---|
| 75% Ethanol | 1 hour |
| 75% Ethanol | 1 hour |
| 100% Ethanol | 1 hour |
| 100% Ethanol | 1 hour |
| 100% Ethanol | 1 hour |
| 100% Ethanol | 1 hour |
| 100% Ethanol | 1 hour |
| Xylene | 1 hour |
| Xylene | 1 hour |
| Xylene | 1 hour |
| Wax | 1 hour 20 minutes |
| Wax | 1 hour 20 minutes |
| Wax | 1 hour 20 minutes |

## Haematoxylin and eosin (H&E)

H&E staining was performed as the protocol below:

| Reagent | Duration |
|---|---|
| Xylene | 5 minutes |
| Xylene | 5 minutes |
| 100% Ethanol | 3 minutes |
| 100% Ethanol | 3 minutes |
| 70% Ethanol | 3 minutes |
| Milli-Q water | 3 minutes |
| Harris Haematoxylin | 5 minutes |
| Tap water | 5 minutes |
| Acid alcohol* | Dip 1-2 times |
| Tap water | 5 minutes |
| Scott's bluing solution | 1 minute |
| Tap water | 5 minutes |
| Eosin | 30 seconds to 1 minute |
| Tap water | 5 minutes |
| 70% Ethanol | 3 minutes |
| 100% Ethanol | 3 minutes |
| 100% Ethanol | 3 minutes |
| Air Dry | Overnight |
| DPX coverslip | 1-2days |

*Acid alcohol: 175ml of 100% ethanol + 75ml of RO water + 2.5ml of 36% HCl

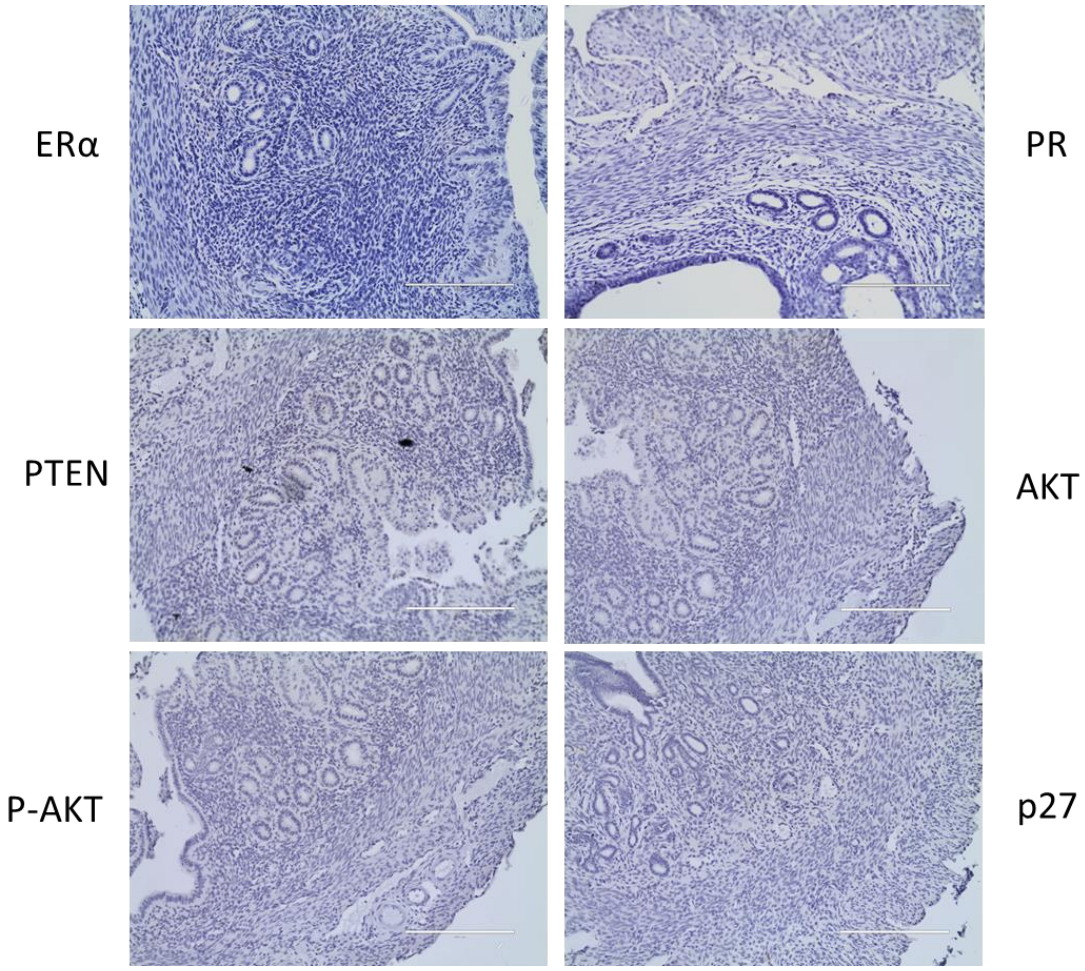
## Immunohistochemistry

Immunohistochemistry was performed as the protocol below:

1. Dewax paraffin sections through xylene and rehydrate through series of 100% ethanol to 70% ethanol and to Milli-Q water.
2. Perform antigen retrieval with 10mM citric acid (pH 6) in microwave for 10 minutes.
3. Block endogenous peroxidase with $H_20_2$ for 10 minutes at room temperature (in hood) to reduce non-specific background staining.
4. Wash PBS for 3 minutes.
5. Block unspecific binding sites with Pierce superblock (containing 0.5% Bovine serum albumin) for 1 hour at room temperature.
6. Without rinsing the slides, add primary antibody or PBS (negative control) and incubate for 1 hour at 37ºC.
7. Wash PBS for 3 minutes twice.
8. Add secondary biotinylated antibody and incubate for 30 minutes at room temperature. (Make up ABC reagent using Vectastain Kit and allow standing for formation of ABC complex).
9. Wash PBS for 3 minutes twice.
10. Add ABC reagent and incubate for 30 minutes at room temperature.
11. Wash PBS for 3 minutes twice. (Make up 3,3'-Diaminobenzidine (DAB) staining solution).
12. Apply DAB and observe colour (brown) development. Expose all sections to DAB for the same amount of time (15 seconds to 10 minutes).
13. Rinse DAB in Milli-Q water for 5 minutes
14. Counterstain slides in Harris haematoxylin for 15-30 seconds.

15. Rinse Harris haematoxylin by rinsing in running water for 5 minutes.
16. Dehydrate through a series of 70% ethanol to 100% ethanol.
17. Allow to air dry for overnight.
18. Coverslip using DPX and allow it to dry (1-2 days).

**Immunohistochemistry negative controls (scale bar=200µm):**



# Appendix IV: Primer sequences for real-time PCR

| Target gene | Annealing temperature (ºC) | Primer sequences |
|---|---|---|
| *18s rRNA* | 59 | Forward: 5-'AACTTTCGATGGTAGTCGCCG-3'<br>Reverse: 5'-AAACGGCTACCACATCCAAGG-3' |
| *Rpl 19* | 57 | Forward: 5'-GATCATCCGCAAGCCTGTGACTG-3'<br>Reverse: 5'-GTGCTTCCTTGGTCTTAGAC-3' |
| *B-actin* | 58 | Forward: 5-'AGCCATGTACGTAGCCATCC-3'<br>Reverse: 5'-GGAACCGCTCGTTGCCAATA-3' |
| *Hmbs* | 64 | Forward: 5-'GGCAATGCGGCTGCAA-3'<br>Reverse: 5-'GGGTACCCACGCGAATCAC-3' |
| *ERα* | 67 | Forward: 5'-ATGAAAGGCGGCATACGGAAAG-3'<br>Reverse: 5'-CACCCATTTCATTTCGGCCTTC-3' |
| *Cyclophillin* | 65 | Forward: 5'-ATCACGGCCGATGACGAGCC-3'<br>Reverse: 5'-TCTCTCCGTAGATGGACCTGC-3 |
| *Lactoferrin* | 59 | Forward: 5'-CTAACCAGACCAGATCCTGCA-3'<br>Reverse: 5'-CCTTCTCAGCCAGACACCTT-3' |
| *C3* | 57 | Forward: 5'-AACTGCTGGCCTCTGGAGTA-3'<br>Reverse: 5'-GCATGATTCCTCGAGGTTGT-3' |
| *G6PDH* | 61 | Forward: 5'-GCCTGGCATGTTCTTTAACC-3'<br>Reverse: 5'-CAATCTTGTGCAGCAGTGGT-3' |
| *Probasin* | 65 | Forward: 5'-GGAGGAGATGACGGAGTTCA-3'<br>Reverse: 5'-ACAGTTGTCCGTGTCCATGA-3' |
| *Cox-1* | 60 | Forward: 5'-ACCTACGTCTACGCCAAAGG-3'<br>Reverse: 5'-GTGGTTTCCAACCAAGATCA-3' |
| *Cox-2* | 60 | Forward: 5'-CCGTGCTGCTCTGTCTTAAC-3'<br>Reverse: 5'-TTGGGAACCCTTCTTTGTTC-3' |

# Appendix V: Additional PTEN western blots