Accepted Manuscript

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PII: S0303-7207(17)30353-2

DOI: 10.1016/j.mce.2017.06.026

Reference: MCE 9998

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 28 March 2017

Revised Date: 28 June 2017

Accepted Date: 30 June 2017

Please cite this article as: Walters, K.A., Handelsman, D.J., Role of androgens in the ovary, *Molecular and Cellular Endocrinology* (2017), doi: 10.1016/j.mce.2017.06.026.

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Short title

Androgen actions and the ovary

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Keywords

androgens, androgen receptor, ovary, PCOS

Abstract

It has been well established for decades that androgens, namely testosterone (T) plays an important role in female reproductive physiology as the precursor for oestradiol (E_2). However, in the last decade a direct role for androgens, acting via the androgen receptor (AR), in female reproductive function has been confirmed. Deciphering the specific roles of androgens in ovarian function has been hindered as complete androgen resistant females cannot be generated by natural breeding. In addition, androgens can be converted into estrogens which has caused confusion when interpreting findings from pharmacological studies, as observed effects could have been mediated via the AR or estrogen receptor. The creation and analysis of genetic mouse models with global and cell-specific disruption of the *Ar* gene, the sole mediator of pure androgenic action, has now allowed the elucidation of a role for AR-mediated androgen actions in the regulation of normal and pathological ovarian function. This review aims to summarize findings from clinical, animal, pharmacological and novel genetic AR mouse models to provide an understanding of the important roles androgens play in the ovary, as well as providing insights into the human implications of these roles.

Contents

1. Introduction

In women the major circulating androgen precursors and bioactive androgens, in descending order of serum concentrations, are dehydroepiandrosterone sulphate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione (A_4), T and dihydrotestosterone (DHT) (Davison & Davis 2003). T and DHT are the only potent bioactive androgens that bind directly to the AR, while DHEAS, DHEA and A_4 are pro-androgens which require conversion to T and/or DHT to exert androgenic effects (Burger 2002). In females, T and DHT are predominantly formed by peripheral conversion (in liver, adipose tissue and skin) of androgen precursors that are secreted from the adrenal glands and the ovaries. DHEA and DHEAS are largely derived from the adrenal glands (Abraham 1974), while T, DHT and A_4 levels originate equally from the ovary and adrenals (Davison & Davis 2003).

In the ovary androgen synthesis favours the Δ^5-pathway (Figure 1), which involves the conversion of cholesterol to pregnenolone by the enzyme P450 side chain cleavage (P450scc, CYP11A1). Pregnenolone is then metabolized to DHEA by P450c17 (CYP17A1) and then A_4 by 3βHSD. A_4 can then be converted to the bioactive androgen T by 17βHSD. Subsequently, T can then either be aromatized into oestradiol (E_2) by P450arom (CYP19) or

reduced to DHT by 5α-Reductase 1 (SRD5A1) or 2 (SRD5A2). DHT can be enzymatically reduced into 5α-androstanediols, reversibly into 3α-diol and irreversibly to 3β-diol (Longcope 1986; Miller & Auchus 2011). The production of androgens within the ovarian follicle is under the control of luteinizing hormone (LH), with LH acting via LH receptors on theca cells to stimulate the rate-limiting conversion of cholesterol to pregnenolone (Longcope 1986; Erickson *et al.* 1985). Within the ovarian follicle, androgen synthesis and then the subsequent conversion of androgens to estrogens is compartmentalized in a cell-specific manner, known as the two-cell, two gonadotrophin hypothesis (Hillier *et al.* 1994). A_4 and T are synthesized in the theca cells, before being diffused into the granulosa cells where they are converted into oestrone (E_1) or E_2, respectively (Ghayee & Auchus 2007; Burger 2002).

Androgens can exert direct effects by mediateing their action primarily via the AR, which is a member of the nuclear receptor superfamily encoded by a single copy X-chromosomal gene (Quigley *et al.* 1995; Lubahn *et al.* 1988). The classical androgenic response is termed genomic AR actions as it involves gene transcription. Current evidence demonstrates that AR expression is not a static mediator of tissue androgen action, but in fact can dynamically change and adapt to pathological changes. Examples of modified AR structure variants are AR splice variants present in prostate cancer, such as AR-V7 which has the ligand binding domain deleted by RNA splicing (Antonarakis *et al.* 2014). Modification of the AR structure potentially alters the function of AR and hence may play an active role in development of androgen-sensitive human pathologies. Interestingly, AR splice variants have been reported to occur in the ovaries of PCOS women, potentially linking them with the pathogenesis of PCOS (Wang *et al.* 2015; Walters & Handelsman 2016). ~~However, a~~ Androgens have also been reported to exert their effects via indirect mechanisms. These being the conversion of androgens to estrogens and subsequent effects mediated via the estrogen receptor (ER); the

production of factors of androgen regulated genes, such as FSH or IGF1; or non-genomic AR actions, where androgenic actions occur within seconds or minutes after ligand binding (Foradori *et al.* 2008). Interestingly, a zinc transporter protein, ZIP9, which is distinct from nuclear steroid receptors, has been identified in granulosa cells of Atlantic Croker ovaries (Berg *et al.* 2014). It functions as a high affinity, specific membrane receptor for T, mediating rapid activation of intracellular signal transduction pathways via a stimulatory G protein, including apoptosis and cell death pathways (Berg *et al.* 2014). Evidence to support a role for direct androgen actions in the ovary comes from clinical, animal, pharmacological and novel genetic AR mouse models which together have confirmed an important role for androgen in the regulation of normal ovarian function. This review will summarizes the key findings from these studies to provide an insight into the important roles androgens play in the ovary.

2. Androgen receptor expression in the hypothalamic-pituitary-gonadal (hpg) axis

The androgen receptor (protein and messenger RNA) is expressed throughout the hpg axis, with its expression identified in the brain, ovarian stroma, ovarian follicles and corpora lutea. Within ovarian follicles, AR is expressed at most stages of follicular development and exhibits distinct spatial and temporal patterns of expression at different developmental stages. This infers that at different follicular developmental stages there are changes in the specific roles AR-mediated actions play. These findings, together with the knowledge that there is evolutionary conservation of AR expression in the ovaries of numerous mammalian species (mouse, rat, sheep, pig, cow, primate and human), strongly supports a universal role for AR-mediated androgen actions in influencing ovarian function (Figure 2).

Primordial follicles in rat (Szoltys & Slomczynska 2000), bovine (Hampton *et al.* 2004), ovine (Juengel *et al.* 2006), primate (Hild-Petito *et al.* 1991) or human (Rice *et al.* 2007;

Suzuki *et al.* 1994) ovaries have been shown not to exhibit AR expression. However, AR expression is detectable in primary rat (Szoltys & Slomczynska 2000), bovine (Hampton *et al.* 2004; Salvetti *et al.* 2012), ovine (Juengel *et al.* 2006), primate (Hild-Petito *et al.* 1991) and human (Rice *et al.* 2007) follicles.

Within preantral follicles AR has been located in the oocyte, granulosa cells and theca cells of rat (Lenie & Smitz 2009; Szoltys & Slomczynska 2000) and primate (Hild-Petito *et al.* 1991) (Weil *et al.* 1998; Hillier *et al.* 1997) preantral follicles, and the granulosa and theca cells of bovine (Hampton *et al.* 2004; Salvetti *et al.* 2012), ovine (Juengel *et al.* 2006) and porcine (Slomczynska *et al.* 2001; Slomczynska & Tabarowski 2001) preantral follicles.

AR expression is present in granulosa and theca cells of bovine (Hampton *et al.* 2004; Salvetti *et al.* 2012), ovine (Juengel *et al.* 2006), porcine (Slomczynska *et al.* 2001; Slomczynska & Tabarowski 2001), primate (Hild-Petito *et al.* 1991; Hillier *et al.* 1997) and human (Suzuki *et al.* 1994; Nielsen *et al.* 2011) antral follicles. During antral to preovulatory follicle development a distinct pattern of AR expression has been described in porcine (Szoltys & Slomczynska 2000) and mouse (Lenie & Smitz 2009) antral follicles with a gradient of the intensity of AR developing, whereby expression progressively declines in the outer mural granulosa cells of late stage antral follicles, but the cumulus cells surrounding the oocyte maintain strong AR positive staining.

AR expression is also detected in ovine (Juengel *et al.* 2006), porcine (Slomczynska *et al.* 2001; Slomczynska & Tabarowski 2001), primate (Hild-Petito *et al.* 1991) and human (Suzuki *et al.* 1994) corpora lutea. AR's expression is present during the early luteal phase of a cycle but is dramatically reduced in fully regressing primate corpora lutea (Hild-Petito *et al.* 1991).

3. Clinical studies revealing a role for androgens in ovarian function

Clinical evidence supporting a direct role for androgen actions in regulating ovarian follicle development comes from the findings that women exposed to androgen excess due to congenital adrenal hyperplasia (Lucis *et al.* 1966; Hague *et al.* 1990), or exogenous testosterone treatment in female-to-male transsexuals (Becerra-Fernandez *et al.* 2014) exhibit polycystic ovaries. These findings imply that elevated levels of androgen stimulate early follicle development, but then lead to arrested follicle development in later stages. Hyperandrogenism is the major defining feature and most frequent trait of the female reproductive pathological disorder, polycystic ovary syndrome (PCOS). PCOS is a common condition characterised by numerous ovarian defects, including polycystic ovaries, reduced ovarian follicle health, ovulatory dysfunction and infertility as well as hyperandrogenism manifest as acne or hirsutism (Dumesic *et al.* 2015). Further support of a stimulatory role for androgens in follicle development comes from some, but not all (Yeung *et al.* 2014; Sipe *et al.* 2010), clinical studies where mainly older women who exhibit poor ovarian response to FSH during IVF have been treated with the androgens DHEA (Gleicher & Barad 2011) or T (Fabregues *et al.* 2009; Bosdou *et al.* 2012) in an attempt to improve ovarian response to stimulation (Gleicher & Barad 2011; Fabregues *et al.* 2009). Along with improving ovarian response, androgen pre-treatment has been reported to increase antral follicle, oocyte and embryo numbers, improve embryo quality and increased pregnancy and live births in IVF (Balasch *et al.* 2006; Wiser *et al.* 2010; Kim *et al.* 2011), inferring androgens can mediate their effects on various cells within the follicle, and at different stages of development.

4. Animal studies revealing a role for androgens in ovarian function

Animal studies assessing the effects of aromatisable (T and androstenedione A_4) and non-aromatisable (DHT) androgens have proven to be very informative in establishing the role of androgens on ovarian function (Figure ~~2~~3). At the earliest stage of follicle development, T and DHT in mice (Yang *et al.* 2010) and primates (Vendola *et al.* 1999) can stimulate primordial follicle initiation. This is despite the fact that AR expression is has been reported as not expressed in primordial follicles. Hence androgens must be mediating their effects via indirect mechanisms, such as upregulation of insulin-like growth factor 1 (IGF1) expression, as reported in the primate ovary (Vendola *et al.* 1999). *In vitro* culture of mouse preantral follicles with T, A_4, DHEA and DHT enhance follicle growth and development (Wang *et al.* 2001; Murray *et al.* 1998), with stimulatory effects blocked by a non-steroidal AR antagonist (bicalutamide) (Murray *et al.* 1998), confirming direct AR-mediated androgen actions. Similarly, while T and DHT increased the numbers of preantral and small antral follicles in primate ovaries (Vendola *et al.* 1998), *in vivo* DHEA treatment increased the proportion of antral follicles present in sheep ovaries (Narkwichean *et al.* 2014) although whether and to what extent this is due to conversion to potent androgens and estrogens remains unclear. Androgens have a stimulatory effect on genes involved in granulosa cell differentiation, as T increases granulosa cell expression of the two key steroidogenic enzymes Cyp19 and P450scc (Wu *et al.* 2011). The synergistic interaction between androgens and FSH appears to be important in the regulation of ovarian function. Treatment of primates with T increased FSH receptor mRNA expression in primary follicles (Weil *et al.* 1999), while DHT and T increased FSH receptor protein , but not mRNA, levels in mouse granulosa cells (Sen *et al.* 2014). Moreover, androgens synergise with FSH to stimulate follicle growth, as DHT enhances FSH-mediated mouse preantral to antral follicle development (Sen *et al.* 2014) and FSH-stimulated porcine cumulus cell proliferation (Hickey *et al.* 2004). Furthermore, mouse preantral follicle responsiveness is improved by T (Wang *et al.* 2001), and FSH-dependent E_2

secretion is increased in bovine granulosa cells in the presence of A_4 (Hamel *et al.* 2005). Local growth factors involved in regulating follicle development are also influenced by androgenic actions as stimulation of porcine granulosa cells proliferation by IGF1 alone or in the presence of GDF9 is enhanced by DHT (Hickey *et al.* 2004; Hickey *et al.* 2005). Importantly, these actions appear to be direct actions mediated via the AR, as these effects are reversed by the addition of an AR antagonist.

In addition to a stimulatory role for androgens in follicle growth, evidence also indicates a beneficial role for androgens in maintaining communication between follicular cells and thereby supporting follicle health. Evidence suggests that androgens are likely to regulate gap junctional communication, as expression levels of connexin 43, a gap junction protein, are reduced in human granulosa cells *in vitro* after treatment with DHT (Wu *et al.* 2010a). Androgens also indirectly maintain follicular health as they are the indispensable substrate for E_2 production, which is essential for follicle survival (Hillier *et al.* 1994). A direct role for androgens in influencing follicle atresia is also supported. Levels of apoptotic granulosa cells and follicle atresia are significantly decreased in growing primate follicles after systemic treatment with T or DHT (Vendola *et al.* 1998). Furthermore, T and DHT have been found to attenuate follicular atresia by increasing granulosa cell expression of microRNA125b, which supresses the expression of the proapoptotic proteins BAK1, BMX, BMF and TRP53 (Sen *et al.* 2014). However, in contrast, in an *in vitro* study, A_4 reportedly suppresses mouse preantral follicle growth and E_2 production (Almahbobi *et al.* 1995), potentially due to ER-mediated effects following the conversion of A4 to oestrone or another estrogen. Despite this finding, the overall conclusion from the body of work documenting the effects of exogenous androgens on follicle development, is that during the early stages of follicular development androgens exert a stimulatory effect on growth and maintain health.

Androgens are also implicated in regulating the final stages of follicle development and ovulation. For example, treatment of pigs with T or DHT during the late follicular phase increased preovulatory follicle and corpora lutea numbers (Cardenas & Pope 1994; Cardenas *et al.* 2002). This appears to be a direct AR-mediated effect as treatment of mice (Sen *et al.* 2014) and rats (Kumari *et al.* 1978) with the AR blocker, cyproterone acetate, decreased ovulations. However species differences exist, as T and DHT have no effect on primate preovulatory follicle numbers (Vendola *et al.* 1998). In response to DHT, rodent periovulatory granulosa cells exhibit an increase in expression levels of cyclo-oxygenase 2 and amphiregulin, both markers of follicular commitment to ovulation (Yazawa *et al.* 2013). Furthermore, an optimal level of androgens appear to be required to maintain normal ovulatory function as low but not high doses of DHT enhance ovulatory response to superovulation in rodents (Sen *et al.* 2014; Ware 1982). Similarly, a high but not low dose of DHT decreased ovulation rates in immature female rats primed with pregnant mare serum gonadotrophin (PMSG) (Conway *et al.* 1990). Evidence also supports a direct role for androgens in the process of oocyte maturation. T promotes *in vitro* germinal vesicle breakdown (GVBD) in murine (Gill *et al.* 2004) and porcine (Li *et al.* 2008) oocytes, which is supressed in the mouse by the addition of an AR blocker (flutamide). Similarly, a physiological role for androgens in the regulation of oocyte nuclear maturation in primates is supported by the finding that in even in the absence of an ovulatory surge, DHT treatment caused a significant percentage of oocyte to resume meiosis to the metaphase 1 (Borman *et al.* 2004). However, the level of androgens present appears to be crucial to the mediated effects. In mice oocyte meiotic maturation and embryonic development are inhibited by T in a dose dependent manner (Anderiesz & Trounson 1995), and oocyte meiotic competence is reduced by elevated levels of T and A_4 (Romero & Smitz 2010).

The conflicting results between some pharmacological studies appears to be, at least in part, due to the emerging theme that a balance in androgen actions is key for the maintenance of optimal ovarian function (Figure ~~3~~4). Besides the important positive effects of androgens on follicular growth and health, abnormal androgen levels disrupt the crucial balance required for normal follicular development, leading to negative androgenic effects on ovarian function. Support for this comes from animal studies that have used elevated androgen levels to induce characteristics of human PCOS in animal models. Pre-natal and post-natal elevated androgen exposure has been shown to induce ovarian PCOS characteristics in rodents (Walters *et al.* 2012a), sheep (Padmanabhan & Veiga-Lopez 2013) and primates (Abbott *et al.* 2005)

In rats and mice, exposure of offspring to elevated T or DHT levels late in gestation (days 16-19 of gestation) led in adult life to the development of irregular oestrous cycles, altered follicular development, and reduced follicular health and decreased corpus lutea populations, indicative of oligo-ovulations (Wu *et al.* 2010b; Caldwell *et al.* 2014). Likewise, long-term treatment (>11 weeks) of rodents with high dose DHT from ~3 weeks of age induced dysfunctional ovarian function with rats and mice displaying irregular oestrous cycles, oligo-ovulation and polycystic ovaries containing large atretic follicles with a thickened theca interna cell layer and a thin granulosa cell layer (Manneras *et al.* 2007; van Houten *et al.* 2012; Caldwell *et al.* 2014). The observation of dysfunctional ovulation was confirmed by the significant reduction in progesterone levels (Manneras *et al.* 2007; Caldwell *et al.* 2014).

This need for an appropriate balance in androgen actions to maintain normal ovarian function in rodents holds true for higher mammalian species. Prenatal exposure of ewes to excess

levels of T leads in adult life to irregular cycling and oligo- or anovulation (Clarke *et al.* 1976) and induces the PCOS ovarian characteristics of increased ovarian weight (West *et al.* 2001; Forsdike *et al.* 2007), polycystic ovaries (West *et al.* 2001; Forsdike *et al.* 2007), increased follicular recruitment (Clarke *et al.* 1977; West *et al.* 2001; Smith *et al.* 2009) and increased presence of large antral follicles (Manikkam *et al.* 2006; Steckler *et al.* 2007). Similarly, adult female rhesus monkeys exposed to excess levels of testosterone propionate during early-mid or late gestation display abnormal ovarian function with the presence of irregular cycles and polycystic ovaries (Abbott *et al.* 2005; Abbott *et al.* 2013). Oocyte development is also compromised by androgen excess. Prenatal exposure of female rhesus monkeys to elevated levels of T, in adulthood resulted in impaired oocyte competence with reduced percentages of zygotes developing to the blastocyst stage (Dumesic *et al.* 2002).

Despite the findings from these pharmacological animal studies proving to be very informative on the apparent effects of androgens on ovarian function, confusion still arises on the mechanism of actions as aromatisable androgens (T and A_4) can be converted into estrogens and DHT (a non-aromatisable androgen) can be reduced into 3β-diol, all of which have the potential to exert indirect actions via estrogen receptor (ER) (Figure ~~4~~5). This point is highlighted by the findings that while prenatal excess T increases follicle recruitment, prenatal DHT does not (Smith *et al.* 2009). Furthermore, excess prenatal exposure of ewes to T leads to an increase in the number of large antral follicles and follicular persistence, while excess prenatal DHT exposure only increases the number of small growing follicles, but not the number of large antral follicles (Steckler *et al.* 2007) with the discrepancy signifying an effect possibly due to aromatisation of T. These findings imply that both androgenic and estrogenic mechanisms are involved regulating follicular dynamics. Moreover, like all steroid blockers, the fact that AR antagonists are often mixed partial agonists/antagonists rather than

pure blockers, makes it difficult to conclusively elucidate the precise androgenic processes involved by purely pharmacological means. A different approach to reveal the direct role of androgens on ovarian function is to study female mice with an inactive AR. Several AR knockout mouse models (ARKO) have been generated and analysis of these models has extended and clarified the knowledge provided from pharmacological studies.

5. Androgen receptor knock out mouse models

It is not possible to generate female ARKO mice by natural breeding as hemizygous males bearing an inactive AR (the classical complete androgen insensitivity syndrome (CAIS), formerly known as testicular feminization syndrome (Tfm)) (Notini *et al.* 2005) are sterile. The first research models for female androgen insensitivity were the $X^{Tfm}O$ (Ohno *et al.* 1973) and homozygous Ar^{Tfm}/Ar^{Tfm} female mice (Lyon & Glenister 1974). $X^{Tfm}O$ females were found to exhibit ovarian degeneration from ~2 months of age (Ohno *et al.* 1973), but this was not the case in Ar^{Tfm}/Ar^{Tfm} females with follicles still present in their ovaries at 6 months of age. However, Ar^{Tfm}/Ar^{Tfm} females did display a reduced reproductive lifespan, and their ovaries exhibited a reduction in primordial follicles and increased follicle atresia (Lyon & Glenister 1974; Lyon & Glenister 1980). Overall, findings from these models inferred that AR-mediated actions are essential for normal ovarian function, however little data was available from these pioneering models presumably due to the complicated methods used to generate the mice that did not allow the production of sustainable lines for detailed analysis.

In more recent times global and cell specific AR knockout mouse models (ARKO) have been generated using the Cre/loxP system (Kuhn & Torres 2002). Each of the mouse models has been developed by crossing mice harbouring a floxed (LoxP flanked) AR gene with Cre-expressing transgenic mice. The Cre-expressing mouse lines have either global or cell

specific expression of Cre, creating a method for targeted deletion of the floxed region of the AR gene. This targeted loss of AR activity allows the analysis of the functional requirements for global and cell specific AR actions in the regulation of different physiological mechanisms (Walters *et al.* 2010).

To date three different global androgen insensitive female mouse models have been created with targeted deletions of exon 1 ($ARKO^{\Delta Ex1}$) (Shiina *et al.* 2006), exon 2 ($ARKO^{\Delta Ex2}$) (Hu *et al.* 2004) or exon 3 ($ARKO^{\Delta Ex3}$) (Walters *et al.* 2007) of the AR gene. In addition, more targeted ARKO models have been created with a specific deletion of the AR in the granulosa cells (GCARKO) (two distinct models with targeted deletions of exon 2 ($GCARKO^{\Delta Ex2}$) (Sen & Hammes 2010) or exon 3 ($GCARKO^{\Delta Ex3}$) (Walters *et al.* 2012b)), theca cells (TCARKO) (Ma *et al.* 2016), oocyte (OoARKO) (Sen & Hammes 2010), ~~or~~ pituitary (PitARKO) (Wu *et al.* 2014) or neurons (NeurARKO) (Caldwell *et al.* 2017) . The development of this array of ARKO mouse models has provided a unique insight into the role of androgen actions in the regulation of ovarian function (Table 1).

5.1. Global androgen receptor knockout mouse models (ARKO)

Sub-fertility is present in all of the global ARKO female mouse models, with females exhibiting fewer pups/litter (Yeh *et al.* 2002; Hu *et al.* 2004; Shiina *et al.* 2006; Walters *et al.* 2007). A key cause of this sub-fertility is dysfunctional ovarian follicle development, common to all ARKO female models. Elevated levels of follicular atresia are exhibited in ARKO ovaries (Yeh *et al.* 2002; Hu *et al.* 2004; Shiina *et al.* 2006; Walters *et al.* 2007) and impaired follicle health as evident by the presence of degenerate oocytes, significantly more pyknotic granulosa cells, and impaired antrum development in antral follicles (Walters *et al.* 2007; Cheng *et al.* 2013) in the $ARKO^{\Delta Ex3}$ model, and reduced granulosa cell thickness in

ARKO$^{\Delta Ex2}$ antral follicles (Hu *et al.* 2004). The ovarian expression of key regulators of follicle health, FSH and IGF1 receptors, are also significantly reduced (Hu *et al.* 2004), implying a wider alteration in normal signalling pathways has occurred. The maintenance of AR signalling during the later stages of follicle development is crucial as preovulatory follicle numbers within ARKO$^{\Delta Ex3}$ ovaries are significantly reduced (Cheng *et al.* 2013), oocytes within ARKO$^{\Delta Ex2}$ preovulatory follicles loose contact with the surrounding cumulus cell during ovulation, and all ARKO female models exhibited a significant reduction in corpora lutea numbers, confirming reduced ovulation rates (Hu *et al.* 2004; Shiina *et al.* 2006; Walters *et al.* 2007; Cheng *et al.* 2013). Regulatory pathways during ovulation are disrupted by the loss of AR signalling as ovarian expression of hyaluronan synthase 2 and tumor necrosis factor-α-stimulated gene 6, both of which are required for normal cumulus expansion, are reduced after hyperstimulation of ARKO$^{\Delta Ex2}$ females (Hu *et al.* 2004). Furthermore, ARKO$^{\Delta Ex1}$ ovarian expression levels of genes involved in the oocyte-granulosa cell regulatory loop (KIT ligand, bone morphogenetic protein 15 and growth differentiation factor 9) have been reported to all be reduced at the preovulatory stage (Shiina *et al.* 2006). Interestingly, the ARKO$^{\Delta Ex3}$ model, which retains non-functional AR protein, exhibits no disassociation of cumulus cells from oocytes within preovulatory follicles (Walters *et al.* 2008), and oocyte quality appears unaffected as ARKO$^{\Delta Ex3}$ embryo quality is unchanged with normal embryonic development to the blastocyst stage (Walters *et al.* 2007; Cheng *et al.* 2013). The discrepancies between these findings may potentially be explained by differences in the way the ARKO models were generated. The ARKO$^{\Delta Ex1}$ mouse model exhibits a major loss of the AR protein due to the insertion of a premature stop codon which results in the deletion of most of the 8 exons, and therefore the loss of all AR actions and interactions including with co-regulatory machinery. On the other hand, the ARKO$^{\Delta Ex3}$ model generated by an in-frame excision of exon 3, which encodes the second zinc finger essential for DNA-

binding, but maintains a minimally truncated mutant AR protein that is non-functional as a direct nuclear transcription factor. However, the mutant AR protein remaining in the ARKO$^{\Delta Ex3}$ model maintains interactions with co-regulators and other transcription factors which avoids possible secondary effects arising from deletion of the full protein. Support for non-genomic actions, such as manifest via ZIP9 gene product (Berg *et al.* 2014), playing an important role comes from the finding that T can induce *in vitro* germinal vesicle breakdown of mouse oocytes by transcription independent mechanisms (Gill *et al.* 2004).

The body of evidence from the ARKO models indicates that the observed sub-fertility is primarily due to dysfunctional late follicular dynamics. However there is also some evidence to support a possible role for androgens in the lifespan of the ovary. Loss of AR signalling in the ARKO$^{\Delta Ex1}$ model leads to an accelerated depletion of the ovarian follicular pool and a total loss of all follicles by 40 weeks of age (Shiina *et al.* 2006). As menopause is largely dictated by the rate of follicle atresia, this finding implies that AR signalling influences follicle atresia and lifespan. However, this loss is not observed in all ARKO models, with follicles still present at 52 weeks in ARKO$^{\Delta Ex3}$ ovaries (Walters *et al.* 2007). The reason for these conflicting results is unclear but presumably are due to the ability of the mutant AR protein present in the ARKO$^{\Delta Ex3}$ model to still interact with co-regulators and other transcription factors; and there is also the potential that AR non-genomic signalling is retained which may influence oocyte and follicle health via mechanisms independent of direct DNA-binding mediated transcription. Consequently, the premature loss of follicles in the ARKO$^{\Delta Ex2}$ mouse model may be due to the total loss of protein, which may have led to the disruption of other pathways beyond that of AR transcriptional activity.

While direct AR actions within the ovary are important in maintaining optimal follicle development, it is now clear that AR signalling across the hypothalamic-pituitary-gonadal axis is required to maintain normal ovarian function and female fertility. Several lines of evidence indicate that hypothalamic-pituitary-gonadal function is defective in the absence of normal AR signalling. ARKO females exhibit a delay in their 1st litter (Walters *et al.* 2007), abnormal oestrous cycles, which are longer and irregular (Walters *et al.* 2009; Hu *et al.* 2004), and reduced naturally ovulated oocyte numbers observed in ARKO$^{\Delta Ex3}$ females can be overcome by gonadotropin hyperstimulation (Walters *et al.* 2007). Additionally, transplantation of ARKO or control ovaries into ovariectomized control hosts, causes not change in oestrous cycles or fertility of the host. However in contrast, transplantation of control ovaries into ovariectomized ARKO hosts, leads the ARKO hosts to display abnormal oestrous cycles and reduced fertility (Walters *et al.* 2009). Together these findings support a role for extra-ovarian neuroendocrine AR-mediated actions in maintaining female fertility. The precise neuroendocrine AR signalling mechanisms involved remain to be fully elucidated however a role for AR actions in the control of the kisspeptin/GnRH/LH cascade is supported by the findings that ARKO females exhibit a decreased, and often mistimed, ovulatory LH surge with corresponding reductions in follicular steroidogenesis displayed by decreased E_2 and E_1 serum levels and *Kiss1* mRNA expression in the anteroventral periventricular nucleus at proestrus (preovulatory stage) (Cheng *et al.* 2013).

In summary, data from global ARKO mouse models has conclusively confirmed that androgens acting via the AR play important roles in maintaining normal ovarian function and female fertility. Data supports a positive role for androgens in follicle development, in particular during the later stages of follicle development where AR actions are involved in

maintaining follicle health, promoting preovulatory follicle development and ovulation priming by regulating appropriate gonadotropin secretion.

5.2. Granulosa cell specific androgen receptor knockout mouse model (GCARKO)

To date two distinct GCARKO female mouse models have been reported, and both are sub-fertile (Sen & Hammes 2010; Walters *et al.* 2012b), confirming that granulosa cells are a key site for androgenic actions regulating ovarian function. GCARKO$^{\Delta Ex2}$ females exhibit a reduction in pups per litter and total litters (Sen & Hammes 2010), while GCARKO$^{\Delta Ex3}$ females display an age-dependent reduction in total number of pups born and a reduction in total litters (Walters *et al.* 2012b). Hypothalamic-pituitary-gonadal feedback signalling appears to also be altered by a loss of AR granulosa cell AR actions as oestrous cycles in both GCARKO models were normal at 2 and 3 months of age but significantly longer by 6 months of age (Sen & Hammes 2010; Walters *et al.* 2012b). GCARKO ovaries exhibit defective follicle development. Preantral follicles numbers are increased, but antral follicles and corpora lutea numbers are decreased in GCARKO$^{\Delta Ex2}$ ovaries, while GCARKO$^{\Delta Ex3}$ ovaries display a reduction in large preantral and small antral follicles at 3 months of age (Walters *et al.* 2012b). The reduction in the growing follicle populations at later stages of development supports the concept of AR having a stimulatory role in normal follicle development. As was the case in the global ARKO$^{\Delta Ex1}$ females, GCARKO$^{\Delta Ex2}$ display accelerated follicle depletion and premature ovarian failure (Sen & Hammes 2010) although such effects were noticeably absent in any of the exon 3 deletion models which maintains a minimally truncated AR molecule (Walters *et al.* 2012b). Moreover, both GCARKO models displayed significant reductions in follicle health (Sen & Hammes 2010; Walters *et al.* 2012b). These findings support a role for AR in regulating granulosa cell survival and thus protecting the follicle from undergoing follicular atresia. GCARKO$^{\Delta Ex2}$ but not GCARKO$^{\Delta Ex3}$

females displayed reduced corpora lutea and naturally ovulated oocyte numbers (Sen & Hammes 2010) GCARKO$^{\Delta Ex3}$ females did exhibit reduced cumulus expansion and oocyte/embryo viability, displayed by decreased fertilization rates and progression to the two-cell stage (Walters *et al.* 2012b).

Difference in the observed reproductive phenotype between the two GCARKO models may be explained by the fact that while the GCARKO$^{\Delta Ex2}$ model has a complete loss of AR protein, the GCARKO$^{\Delta Ex3}$ model still maintains of a mutant AR protein which has the potential to maintain co-regulator machinery interactions. Alternatively, the observed differences may be explained by non-specificity of the Cre promoters used to generate the different mouse lines. Nonspecific expression of the Amhr2-Cre promoter has been detected in the uterus, oocyte and theca cells (Sen & Hammes 2010; Jorgez *et al.* 2004; Hernandez Gifford *et al.* 2009), inferring that in the GCARKO$^{\Delta Ex2}$ mouse model loss of AR action in other non-granulosa cells sites may also contribute to the phenotype. On the other hand, in the GCARKO$^{\Delta Ex3}$ mouse model while the excision of AR exon 3 was confirmed to only occur in the granulosa cells, not all granulosa cells exhibited the excised exon 3 AR, implying that the observed findings may be an underestimation of the importance of granulosa cell AR actions on ovarian function (Walters *et al.* 2012b). In conclusion, these findings have confirmed that within the ovary granulosa cells are an important site for AR actions, involved in maintaining normal follicle development and health.

5.3. Theca cell specific androgen receptor knockout mouse model (TCARKO)

Recently the first TCARKO model has been described which demonstrates that a loss of theca cell AR actions does not influence female fertility. Compared to controls, TCARKO females displayed comparable oestrous cycle patterns, total litter and pups per female fertility

and gonadotrophin and steroid levels (Ma *et al.* 2016). However, it should be noted that while a 4-fold reduction in AR mRNA expression is present in the theca-interstitial cells of TCARKO ovaries, compared to controls, some AR expression was rarely observed in the theca cells of TCARKO ovaries. This indicates that potentially the contribution of AR theca cell actions to ovarian function may be underestimated in this model. Interestingly, although AR signalling in the theca cells is not required for normal ovarian function, under conditions of elevated androgens, such as in women with PCOS, a loss of AR actions in theca cells was found to reduce the severity of the development of hyperandrogenemia-induced ovarian dysfunction. Unlike hyperandrogenised control mice, TCARKO females exposed to elevated androgen levels retain cyclicity, and displayed improved ovulation rates and fertility (Ma *et al.* 2016). These findings demonstrated that under conditions of abnormal androgen levels, sites of androgen mediated-AR actions not normally involved in regulating ovarian function may play a contributory role in the pathogenesis of hyperandrogenemic associated reproductive disorders, such as PCOS. Interestingly, this may be analogous to the alternative AR splice variants reported to be present in granulosa cells of most women with PCOS (Wang *et al.* 2015), which may represent an endogenous defensive response to the hyperandrogenic follicular environment (Walters & Handelsman 2016).

5.4. Oocyte cell specific androgen receptor knockout mouse model (OoARKO)

To date, one oocyte cell-specific ARKO model (OoARKO) has been generated (Sen & Hammes 2010). OoARKO denuded oocytes compared to control females display a significant reduction (~4-fold) in AR mRNA expression (Sen & Hammes 2010). However, low AR mRNA levels are still present, so observed findings may underestimate the contribution of oocyte AR actions in ovarian function. Analysis of this model implies that AR oocyte actions are not essential for overall ovarian function and female fertility as OoARKO females exhibit

normal fertility, oestrous cycles, follicle populations and CL numbers at 2 months of age (Sen & Hammes 2010). However, in the presence of hyperandrogenemic conditions, a key feature observed in women with PCOS, AR oocyte actions may play an important role in mediated effect of androgen excess in the ovary (Sen & Hammes 2010). Evidence to support this comes from the finding that oocyte maturation (germinal vesicle breakdown (GVBD)) induced in vitro by a high concentration of a non-aromatizable androgen (DHT) is significantly reduced in OoARKO oocytes (Sen & Hammes 2010).

5.5. Pituitary specific androgen receptor knockout mouse model (PitARKO)

A pituitary-directed ARKO model (PitARKO) was generated with the use of the α subunit of gonadotropins (αGSU)-Cre promoter driven Cre line (Wu *et al.* 2014). However, this pituitary glycoprotein alpha subunit is common to TSH as well as LH and FSH. Therefore by targeting this common alpha subunit, both thyrotrophes as well as gonadotrophes are involved and thus its inactivation produces hypothyroidism as well as gonadotrophin deficiency (Kendall *et al.* 1995). PitARKO pituitaries exhibited a 50% reduction in AR mRNA and protein levels, compared to control (Wu *et al.* 2014).

Analysis of the PitARKO model has confirmed a neuroendocrine role for AR-mediated actions in the regulation of female fertility as PitARKO females are sub-fertile producing fewer pups per litter (Wu *et al.* 2014). Late stage ovarian function is altered with PitARKO ovaries exhibiting reduced antral follicle health and fewer corpora lutea, indicative of reduced ovulation rates (Wu *et al.* 2014). These findings demonstrate that AR signalling in the pituitary plays an important role in optimizing ovulation.

5.6. Neuron specific androgen receptor knockout mouse model (NeurARKO)

Recently female NeurARKO mice, which a complete deletion of AR actions in the brain and pituitary, were created as a model to investigate the locus of androgen actions in the development of PCOS (Caldwell *et al.* 2017). While fertility has not been reported, the deletion of AR actions in both the brain and pituitary in this model did not significantly alter normal ovarian function. Compared to control females, NeurARKO females exhibited normal oestrous cycles and no change in growing follicle or corpora lutea populations. However, large antral follicle health was reduced (Caldwell *et al.* 2017). As with the global ARKO and GCARKO models, reasons for the difference between this model and the PitARKO, may be explained by the fact that while the PitARKO model has a complete loss of AR protein in the pituitary, the NeurARKO model still maintains a mutant AR protein in its brain and pituitary which has the potential to maintain co-regulator machinery interactions and AR non-genomic signalling.

6. Human implications of androgen actions in the ovary

The vast majority of studies on the role of androgens in follicle development support a stimulatory role for androgens in early follicle growth, a maintenance role in follicle health and an involvement of androgens in the priming of late stage follicle development. These finding support the current, but still unproven, concept adopted by some IVF clinics of androgen pre-treatment to enhance follicular response to FSH in women having previously exhibited a poor ovarian response to IVF hyperstimulation. Indeed clinical findings from mostly small or uncontrolled case series report improved antral follicle, oocyte and embryo numbers, embryo quality and pregnancy and live birth rates in some women following increased exposure to aromatisable pro-androgens (DHEA, testosterone) or an aromatase inhibitor (letrozole) (Garcia-Velasco *et al.* 2005; Balasch *et al.* 2006; Wiser *et al.* 2010; Kim *et al.* 2011; Meldrum *et al.* 2013). Further evidence to support this theory comes from PCOS

patients, who exhibit androgen excess and often display an increased sensitivity to gonadotrophins during IVF protocols. However, more critical, well-controlled clinical trials are required to fully evaluate the efficacy and safety of such androgen pre-treatments to augment IVF stimulation in women who are poor responders.

Evidence supports a role for androgens in the regulation of oocyte maturation (Borman *et al.* 2004; Gill *et al.* 2004), however an optimal level of androgens may exist to maintain normal processes as elevated levels of androgens can reduce mouse oocyte meiotic competence in a dose dependent manner (Anderiesz & Trounson 1995). Accordingly, this raises the question of the consequences of androgen excess on oocyte development and health, such as in the case of PCOS where altered oocyte competence has been put forward as a potential causative factor for the subfertility experience by PCOS women (Palomba *et al.* 2016). In addition, the presence of various androgens in follicular fluid (Kushnir *et al.* 2016), the strong expression of AR in the cumulus cells of preovulatory follicles (Lenie & Smitz 2009) and the findings that cumulus expansion and oocyte/embryo viability are impaired by a loss of granulosa AR signalling (Walters *et al.* 2012b) infers that the use potential use of androgens in *in vitro* maturation culture systems is an area that warrants investigation.

7. Conclusions

Data from clinical, pharmacological and genetic studies have now converged to conclusively demonstrate an important role for androgens in the regulation of ovarian function and female fertility. Indirectly, androgens are the obligatory precursor for E2 biosynthesis, which is essential for follicular development and more generally as a substrate for estrogen synthesis and action. A direct role for androgens has also been confirmed with their actions found to be important for optimising follicle growth, follicle health and ovulation. Within the ovary

granulosa cells appear to be an important site of action for AR signalling, and in addition, an unexpected role for AR-regulated neuroendocrine control of ovarian function has also been firmly established. Importantly, an optimal balance in the level of androgens present appears to be critical to maintaining normal ovarian function. A reduction in androgenic signalling, as observed in ARKO models, causes subfertility and defective ovarian function. On the other hand, androgen excess in animal models replicates human PCOS characteristics and there is strong evidence to support a direct pathological role for AR-mediated signalling in the development of PCOS (Caldwell *et al.* 2015; Walters 2015). Furthermore, recent evidence suggests that ectopic sites of AR signalling may be an important mediator in androgen induced reproductive dysfunction. Loss of theca cell AR signalling in mice has been shown to have no influence on normal ovarian function or female fertility, but it protects females from hyperandrogenemia-induced ovarian dysfunction and infertility (Ma *et al.* 2016). In conclusion, AR-mediated androgen actions clearly play an important role in regulating ovarian function and female fertility. However, a balance in these androgenic actions is key as evidence suggests that excessive androgen signalling is a major mediator in androgen associated reproductive disorders, as it alters the pathways regulating ovarian follicular dynamics.

Figure 1 Androgen biosynthesis

Androgen biosynthesis and metabolism. 3βHSD, 3-β-hydroxysteroid dehydrogenase; 17-HSD, 17β-hydroxysteroid dehydrogenase.

Figure 2 Androgen receptor expression

Androgen receptor expression is highly conserved across mammalian ovaries. AR expression is detected as follicles enter the growing pool and remains present throughout follicle

development. In general, a gradient of AR intensity has been observed as follicles grow, with AR expression increasing to the antral stage and then progressively declining in the outer mural granulosa cells of antral follicles, but remaining intense in the cumulus cells surrounding the oocyte.

Figure 3. Androgen effects on ovarian dynamics

IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; FSHR, follicle stimulating hormone receptor; COX-2, cyclo-oxygenase; HAS2, hyaluronan synthase 2; TSG-6, tumor necrosis factor-α-stimulated gene 6; KITL, Kit ligand; BMP15, Bone morphogenetic protein 15 ; GDF9, growth differentiation factor 9.

Figure 4. A balance in androgen actions is key in the regulation of ovarian function

Androgens have both positive and negative effects on follicular development and ovarian function depending on the levels present.

Figure 5. Mechanisms of direct and indirect androgen actions

Androgens can mediate their actions directly via the androgen receptor, or exert an indirect effect by conversion into estrogens or 3β-diol and activation of the estrogen receptor. DHT, dihydrotestosterone; 3β-diol, 5α-androstane-3β,17β-diol.

Table 1 Ovarian effect of a loss of AR a signalling

Key ovarian effects due to a global or cell specific loss of AR action as observed in ARKO mouse models.

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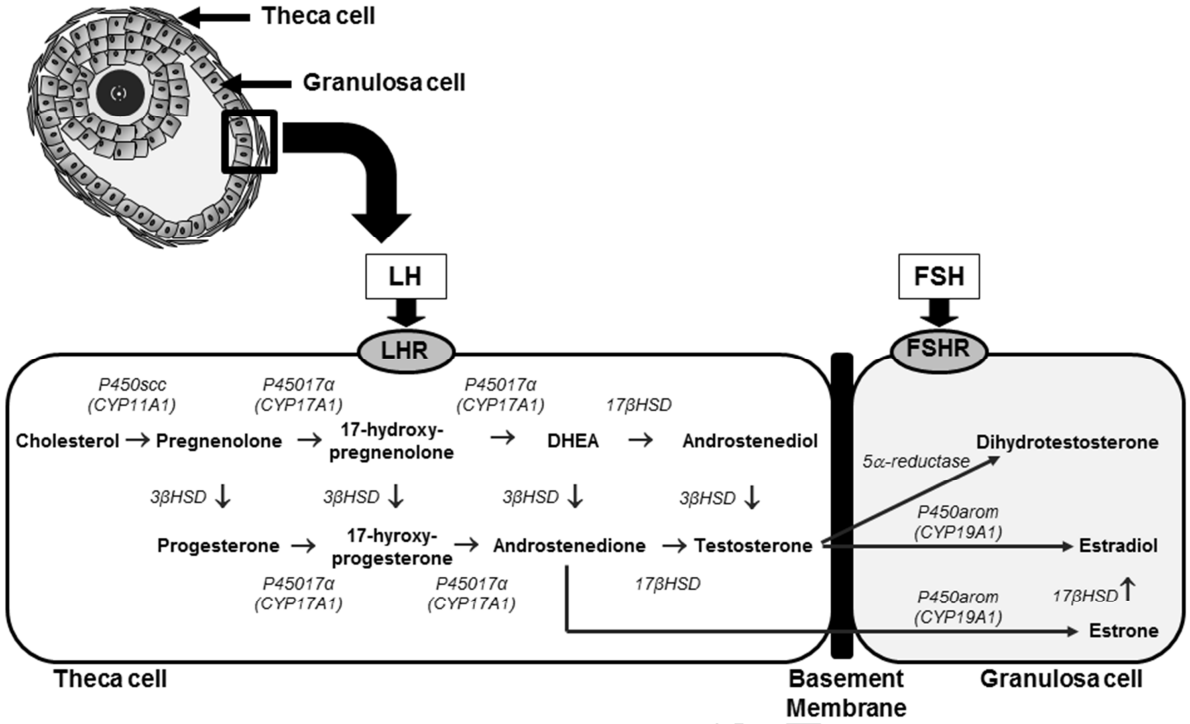
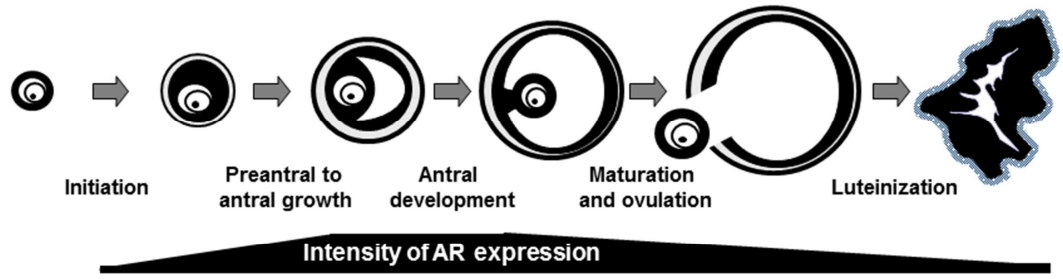
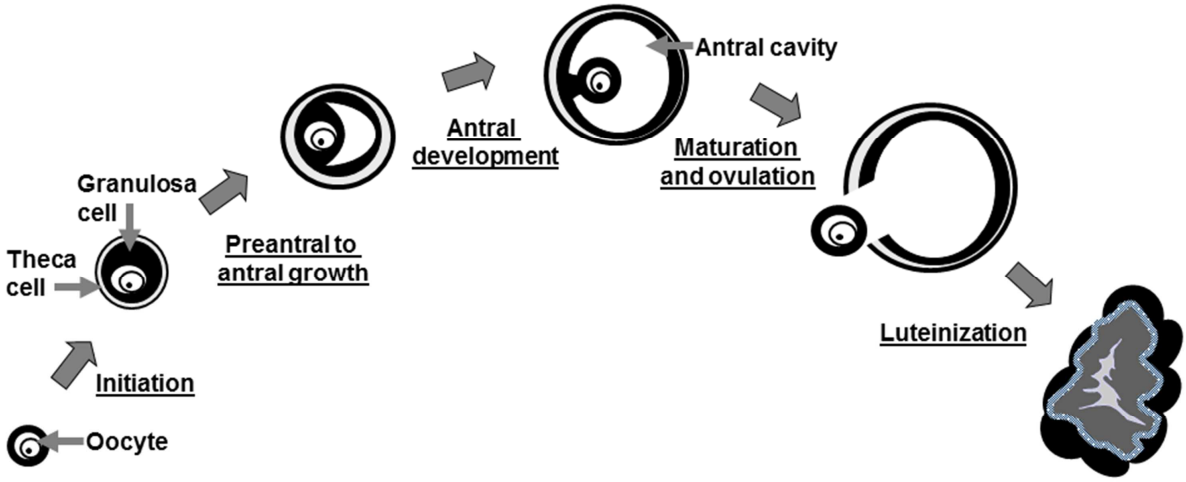
	ARKO^{Ex1} Shiina et al., 2006	ARKO^{Ex2} Yeh et al., 2002 & Hu et al., 2004	ARKO^{Ex3} Walters et al., 2007, 2009, 2013	GCARKO^{Ex2} Sen et al., 2010	GCARKO^{Ex3} Walters et al., 2012	TCARKO Ma et al., 2016	OoARKO Sen et al., 2010	PitARKO Wu et al., 2014	NeurARKO Caldwell et al., 2017
Fertility	↓ pups/litter	↓ pups/litter	↓ pups/litter	↓ pups/litter	↓ in cumulative pups/month from 6 mths	Normal fertility	Normal fertility	↓ pups/ litter	-
Estrous cycles	-	↑ estrous cycle length.	↑ estrous cycle length, irregular estrous cycles.	↑ estrous cycle length at 6mths but not 2 mth.	↑ estrous cycle length at 6mths but not 3 mths.	Normal estrous cycles.	Normal estrous cycles.	Trend to ↑ time at Estrus.	Normal estrous cycles.
Serum steroids and hormones	No change in FSH, LH, E2, T or P4 at proestrus.	-	No change in FSH, LH, E2, T at diestrus. ↓ LH, E2 and E1 at proestrus. ↓ LH after OVX. Normal LH response to GnRH and OVX+E2.	-	No change in FSH or LH at diestrus.	No change in FSH, LH, E2 or T at diestrus. Normal LH response to GnRH.	-	↓ FSH at all estrous cycle stages. ↓ LH but no change in E2 or T at proestrus. Normal LH response to GnRH. ↓ LH and FSH after OVX and OVX+E2.	No change in FSH or LH at diestrus.
Follicle populations	Growing follicle populations normal at 8wks. Total follicle exhaustion by 40wks. ↓ CL.	Growing follicle populations normal at 4 & 16wks. ↓ CL.	At diestrus growing follicles populations normal at 10-12, 26 and 52wks. ↓ CL. At proestrus ↓ preovulatory follicles.	Growing follicle populations normal at 4wks. At 2 & 6 mths ↑ preantral follicles, but ↓ antral follicles and CL, followed by premature ovarian failure.	↓ large preantral and small antral follicles at 3mths. No difference in follicle populations at 6mths.	Growing follicle populations and CL normal..	Growing follicle populations and CL normal.	At diestrus no difference in follicle populations. ↓ CL.	Growing follicle populations and CL normal.
Oocyte and follicle health	↑ atretic follicles.	↓ granulosa cell thickness in antral follicles. ↑ follicular atresia after hyperstimulation. Dissociation of cumulus cells from oocyte in preovulatory follicles.	↑ unhealthy antral follicles. No dissociation of cumulus cells from oocyte in preovulatory follicles.	↑ atretic follicles.	↑ unhealthy follicles and ZPR counts at 6mths.	-	↓ DHT-induced GVBD in vitro.	↑ pyknotic granulosa cells in antral follicles.	↑ unhealthy large antral follicles.
Ovulation	-	↓ superovulated oocytes.	↓ naturally ovulated oocytes. Superovulated ovulation rates normal.	↓ naturally ovulated oocytes. Superovulated ovulation rates normal at 2 mths but ↓ a 6 mths.	↓ cumulus expansion.	-	-	-	-
Embryo development	-	-	No change in fertilisation or progression to 2-cell stage.	-	↓ rate of fertilisation.	-	-	-	-
Ovarian gene expression	At proestrus ↓ Kitl, Bmp15, Gdf9, Hgf, but no change in Lhr, Fshr, Cyp11a1, Cyp17a1, Cyp19a1, Esr2, Ccnd2 or Igf1. No change in Ptgs2 or Pgr at estrus.	↓ Fshr and Igfr At 10 days of age. After hyperstimulation ↓ Pgr, Has2, Tsg6, p27, Cyp11a1 and ↑ Cyp17a1, but no change in Cyp19a1.	No change in Bax, Bcl2, Srd5a1, Srd5a2, Hsd3b1 and Akr1c14 at diestrus. At estrus Cyp19a1 ↓, but Star, Cyp11a1 and Cyp17a1 unchanged.	-	No change in Kitl, Igfr1 or Fshr at diestrus.	No change in Lhcgr, Fshr, Cyp17A1, Cyp19, StAR or Esr2.	-	No change in StAR, Cyp17A1 or Cyp19.	-





	Primordial	Primary	Preantral	Antral/Preovulatory	Corpus Luteum
Rodent	- protein	+ protein	+ protein	+ protein	+ protein
Porcine	-	-	+ mRNA + protein	+ mRNA + protein	+ mRNA + protein
Ovine	- mRNA	+ mRNA	+ mRNA	+ mRNA	+ mRNA
Bovine	- mRNA	+ mRNA + protein	+ mRNA + protein	+ mRNA + protein	-
Primate	- protein	+ protein	+ mRNA + protein	+ mRNA + protein	+ mRNA + protein
Human	- mRNA - protein	+ mRNA + protein	+ mRNA	+ mRNA + protein	+ protein



Primordial Follicle

↑ follicle initiation

↑ oocyte IGF1 and IGF1R

Preantral Follicle

↑ follicle diameter

↑ FSHR

↑ granulosa and theca cell IGF1 and IGF1R

↑ granulosa cell Cyp19 and P450scc

Antral/Preovulatory Follicle

↑ antral follicle numbers

↓ presence of apoptotic granulosa cells and follicle atresia

↑ preovulatory follicle numbers

Maturation and ovulation

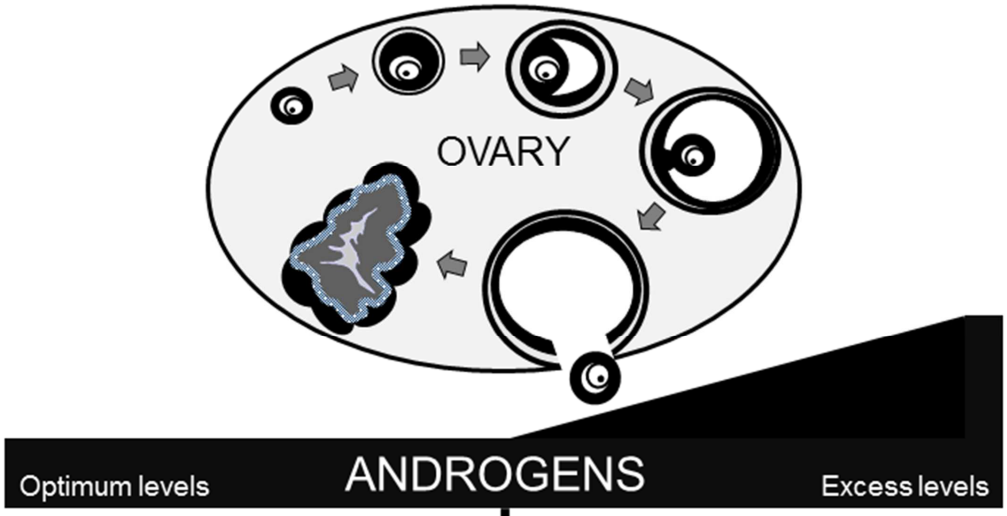
↑ granulosa cell COX-2 and amphiregulin

↑ follicle response to FSH

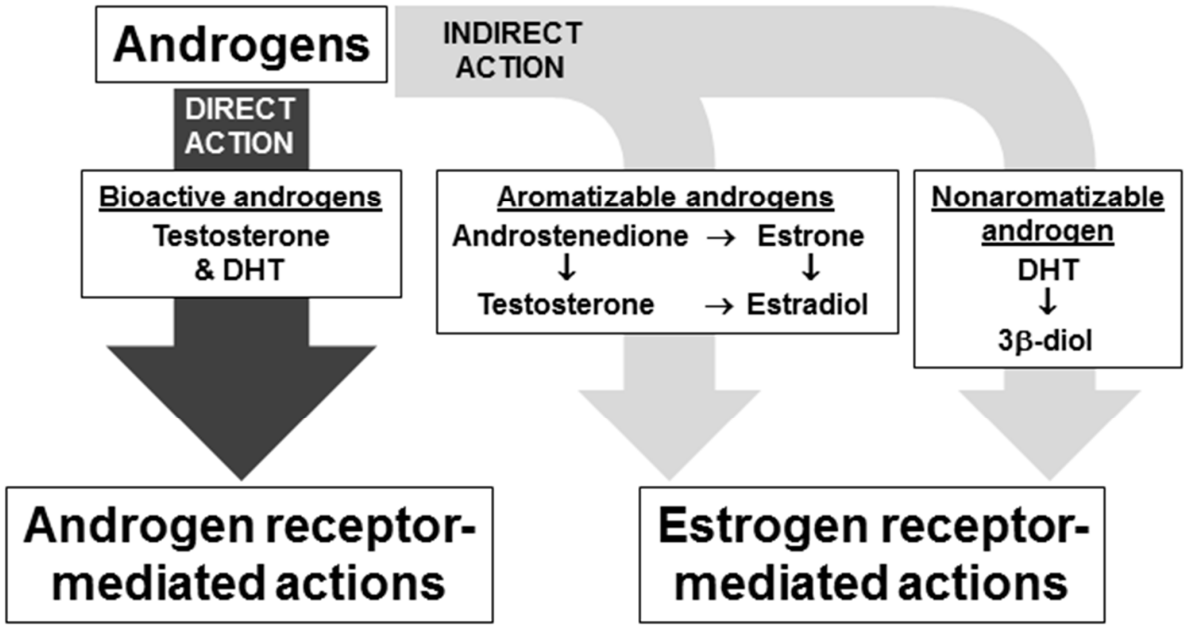
Stimulates oocyte maturation

↑ ovulation rates

↑ corpora lutea



Promote primordial follicle initiation	Increase follicular recruitment
Stimulate preantral/antral follicle growth	Arrest follicle development at antral stage
Maintain follicle health	Reduce follicle health
Optimize ovulatory processes	Reduce ovulation rates
Promote oocyte maturation	Impair oocyte development



Highlights

1. Androgens have a direct role in regulating female reproductive function.

2. Androgens actions are important for optimising ovarian follicle growth and health and ovulation.

3. An optimal balance in androgen actions is needed to maintain normal ovarian function.