Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models

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Submitted on December 1, 2009; resubmitted on January 20, 2010; accepted on January 28, 2010

Background: Androgens and the androgen receptor (AR) have well known roles in male reproduction, and recent genetic mouse models inactivating the Ar gene have conclusively defined a role for androgens in female reproduction. In males, AR gene inactivation severely disrupts spermatogenesis by interrupting completion of meiosis, thereby eliminating production of mature sperm leading to male sterility. These effects have overshadowed the study of additional post-meiotic androgen effects required for the production of fully functional spermatozoa, as well as the production of females with complete androgen insensitivity which cannot be produced by natural breeding. However, these limitations have been overcome by the creation of global and cell-specific AR knockout (ARKO) mouse models using Cre–LoxP genetic engineering.

Methods: Pubmed searches were carried out using the following search terms: androgen receptor, knockout mouse and fertility. Articles published before the end of November 2009 were included.

Results: These experimental models have identified cell-specific AR-mediated androgen actions in testis and androgen actions in sex accessory glands independent of testicular effects which are crucial for sperm maturation, motion and fertilizing ability. The ability to produce homozygous ARKO females has revealed that AR-mediated androgen actions are important for normal female fertility. AR function is required for full functionality in follicle health, development and ovulation through both intra-ovarian and neuroendocrine mechanisms.

Conclusions: ARKO mouse models provide valuable tools to unravel novel roles of AR-mediated actions in male and female reproductive function, and new insights into the role of androgens in human reproductive function.

Key words: androgen receptor / testis / ovary / fertility
Introduction

Androgens act primarily via the androgen receptor (AR), a member of the nuclear receptor superfamily encoded by an X chromosomal gene (Quigley et al., 1995; Beato and Klug, 2000). In males, androgens mediate fetal sexual differentiation and pubertal sexual maturation, and maintain post-pubertal virilization and the anabolic status of virtually all non-reproductive tissues waning only with the accumulation of chronic, degenerative disorders towards the end of life. Despite the pivotal role of AR, some of its reproductive effects have remained obscure with research limited for technical reasons. In females, although the obligatory role of testosterone (T) as an estradiol (E2) precursor is well understood (Hillier et al., 1994; Simpson, 2002), there is no direct evidence for a physiological role of androgens in human female health. However, there is a strong pathological association of hyperandrogenism with polycystic ovary syndrome (PCOS) (Ehrmann, 2005), and androgens are implicated in the origins of premature ovarian failure (POF) (Kimura et al., 2007; Sugawa et al., 2008) and uterine hyperplasia (Terakawa et al., 1988; Tomic et al., 2004). Modern mouse genetic engineering Cre–LoxP techniques now allow the unravelling of AR’s actions and are revealing newly recognized aspects of AR-mediated androgen action in the reproductive system.

Lessons from spontaneous AR mutations in human and non-human mammals

Clinical genetic evidence for the pivotal role of androgens in male reproduction has come from detailed analysis of humans with androgen insensitivity syndrome (AIS) (Griffin, 1992; Quigley et al., 1995). AIS is caused by AR mutations involving virtually every type of DNA alteration known, all ultimately leading to expression of a suboptimal or non-functional AR (Quigley et al., 1995). AIS has a wide spectrum of severity with the most severe patients having totally inactive AR, manifesting as complete AIS (CAIS), which features a female phenotype with normal somatic and external genital features (but no female internal genitalia), despite a 46XY karyotype and undescended inguinal testes (Oakes et al., 2008). These features, once known as testicular feminization (tfm) for its conjunction of female external with male internal genitalia, were described in humans over 50 years ago (Morris, 1953) with similar findings subsequently reported in numerous mammalian species examined (Quigley et al., 1995; rodent: Bardin et al., 1970; Lyon and Hawkes, 1970; Allison et al., 1971; Olsen, 1979; Tsuji and Matsumoto, 1982; Mullen and Hawe, 1979; dog: Fentener van Vlissingen et al., 1988; Peter et al., 1993a; Wernham and Jerram, 2006; Nowacka-Woszuk et al., 2007; cat: Meyers-Wallen et al., 1989; Lawhorn, 1989; Bredal et al., 1997; cattle: Long and David, 1981; Peter et al., 1993b; mare: Kieffer, 1976; Crabbe et al., 1992; Howden, 2004; Switosliki et al., 2005; deer: Scanlon et al., 1975; pig: Lojda and Navratil, 1969; monkey: Pasello-Legrand and Mowat, 2004) as well as in several distinct mouse tfm mutations (Politch et al., 1988; Tanaka et al., 1994). Human AR mutations comprise a complete spectrum of effects, covering the range of male intersex development of external genitalia from minimally virilized females to mild under-virilization with near-normal male genital development (hypospasias being the most sensitive indicator), or even a normal male phenotype with only infertility due to non-functional but morphologically normal sperm (Quigley et al., 1995). These findings highlight that AR-mediated androgen action is fundamental for the development of a normal male phenotype, with structural and functional development of male sexual characteristics as well as initiation and maintenance of spermatogenesis and fertility (Handelsman, 2009).

Challenges in studying androgen action on reproductive function

Although much has been learned from spontaneous inactivating AR mutations causing the AIS phenotype, a variety of difficulties remain for studying additional facets of androgen action on reproductive function. First, both male and female gonads have complex and interdependent multicellular structure and functions. Neither the testis nor the ovary can be fully replicated by in vitro systems making it very difficult for decisive experimental studies of androgen action on intact gonads.

Second, despite the wide array of steroid receptor agonists and antagonists available, pharmacological approaches are hampered by the incomplete specificity and/or antagonism of steroid probes. For example, steroidal antagonists are usually not pure blockers with, typically, their steroid-blocking activity being most evident on a background of high endogenous steroid ligands. However, when endogenous ligand levels are relatively low, the steroid antagonists may have partial agonist effects unmasked, as shown in primary neuronal cultures with the antiandrogens flutamide and cyproterone acetate (Nguyen et al., 2007), and in analysis of selective estrogen receptor modulator actions as agonist and antagonists by gene expression profiling (Frasor et al., 2004). Furthermore, many androgens, notably T and androstenedione (A4), can be aromatized into corresponding estrogens, E2 and estrone (E1), causing ambiguity about whether AR and/or estrogen receptor (ER) mechanisms are involved in the precise molecular mechanism(s) of androgen actions. A conventional alternative is to use DHT, a non-aromatizable androgen; however, this approach is also flawed as DHT is irreversibly metabolized to 3β,5α-androstanediol (3βdiol) which, although biologically inactive as an androgen, interacts with ERβ allowing it to manifest ER-mediated estrogen-like effects (Kuiper et al., 1997; Steckelbroeck et al., 2004).

Finally, female mice homozygous for an inactivated AR cannot be produced by natural mating as the hemizygous male fathers with an inactive AR are sterile. This has long rendered impossible the systematic study of AR-mediated androgen action in females. Similarly, although naturally occurring mouse models of androgen insensitivity due to spontaneous inactivating AR mutations have provided fundamental knowledge on the role of androgens in the development of virilization and spermatogenesis (Lyon and Hawkes, 1970; Drews, 1998), the deficient spermatogenesis in these models has also made impossible any study of AR effects on the acquisition of mature sperm function.

Methods

A Pubmed search was carried out using the following search terms: androgen receptor, knockout mouse and fertility. Articles published before the end of November 2009 and papers cited in the primary references were included. This review provides a comprehensive review of research findings as well as clinical insights, implications and testable hypotheses arising from studies of both male and female AR knockout (ARKO) mouse models.
Results

Strategies used to generate AR knockout mice

The classical rodent models of CAIS [formerly known as testicular feminization (Tfm); Lyon and Hawkes, 1970] are ascribed to a naturally occurring single-base deletion in the mouse AR gene, generating a frameshift and an early stop codon (Gaspar et al., 1991; He et al., 1991), and a point mutation causing an amino acid substitution in the rat AR (Yarbrough et al., 1990) producing a non-functional AR. A recognized limitation of the mouse tfm is its cryptic internal translation site (He et al., 1994) with continued production of a truncated AR protein with some residual biochemical function. Nevertheless, such models have been extensively used to study androgen action in male reproductive physiology in mice (Drews, 1998) and rats (Chan et al., 1969; Allison et al., 1971).

As homozygous AR-deficient females cannot be produced by natural mating, the first research models for female androgen insensitivity utilized the naturally occurring but rare XO female mice where the X chromosome bore the ArTfm mutation (Ohno et al., 1973) or homozygous ArTfm/ArTfm female mice created by breeding males embryonically chimeric for hemizygous ArTfm/Y with females heterozygote for ArTfm (Lyon and Glenister, 1974). Data on these historical mouse models are limited presumably due to the difficulties in producing sustainable numbers of mice using the complex and laborious production methods.

A conditional gene-targeting approach, the Cre/loxP system, involving the bacterial Cre (cyclization recombination) enzyme that excises the DNA located between loxP sites, referred to as ‘floxed’ (Kuhn and Torres, 2002; Le and Sauer, 2000) allows generation of both global and cell-specific inactivation of AR in mice. The ARKO mouse models are created by crossing Cre-expressing transgenic mice, with mice harbouring a floxed (LoxP flanked) AR gene, to create cross-bred mice exhibiting a targeted deletion of the floxed exon(s) of the AR gene. To date, five distinct floxed Ar mouse models have been reported with loxP sites surrounding exon 1 (Kato, 2002; Holdcraft and Braun, 2004), exon 2 (Yeh et al., 2002; De Gendt et al., 2004) and exon 3 (Notini et al., 2005) (Table I). These floxed Ar mice have been crossed with different transgenic Cre lines to generate global ARKO models. Global CMV-Cre (ArEn1Ska denoted hereafter as ARKOEx1(CMV)) (Kato, 2002; Shima et al., 2006) and transgenic germline Sycp1-Cre (ArPm1/1Hab denoted hereafter as ARKOEx2Sycp1) (Holdcraft and Braun, 2004) have been used to excise exon 1; ACTB-Cre under the control of the universally expressed β-actin (ArPm1/1Oc denoted hereafter as ARKOEx3β) (Yeh et al., 2002; Hu et al., 2004) and PGK-Cre (ArPm1/1VeHab denoted hereafter as ARKOEx3PGK) (De Gendt et al., 2004) have been used to excise exon 2; whereas CMV-Cre has been used to drive the deletion of exon 3 (ArPm1/1dfz denoted hereafter as ARKOEx3) (Notini et al., 2005; Walters et al., 2007) of the Ar (Table I). The mouse models with a targeted deletion of exon 1 or 2 of the Ar have major loss of the AR protein due to insertion of premature stop codons resulting in the deletion of most (seven or six, respectively) of the eight exons. In contrast, the model generated by an in-frame excision of exon 3 alone, which encodes the second zinc finger essential for DNA binding, retains a minimally truncated mutant AR protein that is non-functional as a direct nuclear transcription factor. This is consistent with the critical requirement of exon 3 for AR action as shown by the effects of point mutations or deletion of exon 3 in humans, who then have an AR with normal androgen-binding affinity and nuclear localization, but a markedly deficient DNA-binding affinity and inability to transactivate androgen-responsive reporters in vitro (Quigley et al., 1992; Zoppi et al., 1992; Mowszowicz et al., 1993).

In all models, global ARKO males confirmed the abolition of classic genomic AR function by exhibiting the CAIS (Tfm) phenotype (Kato, 2002; Yeh et al., 2002; De Gendt et al., 2004; Holdcraft and Braun, 2004; Notini et al., 2005). The generation of cell-specific ARKO mouse models involves the use of Cre’s which are driven by a promoter that is cell specific. The specific strategies used will be described in the relevant sections below.

Reproductive phenotype of male ARKO mice

In males, AR is expressed in the testis in Sertoli cells, Leydig cells, peritubular myoid cells and vascular smooth muscle cells (Zhou et al., 2002; Collins et al., 2003), whereas germ cells are believed to be AR-negative (Grootegoed et al., 1977; Anthony et al., 1989; Sar et al., 1990; Zhou et al., 2002). Conflicting evidence indicating AR expression at various spermatogenic stages (Kimura et al., 1993;

<table>
<thead>
<tr>
<th>Ar mouse model</th>
<th>AR exon floxed</th>
<th>Promoter for Cre-recombinase</th>
<th>Resulting mRNA/protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARKOEx1CMV</td>
<td>Exon 1</td>
<td>CMV</td>
<td>Complete absence of protein Abundance of AR transcripts and protein in male brains. Loss of AR immunopositive staining in oocytes</td>
</tr>
<tr>
<td>ARKOEx1</td>
<td>Exon 1</td>
<td>Sycp1 and eIIa</td>
<td>Complete absence of protein Abundance of AR transcripts and protein in male brains. Loss of AR immunopositive staining in oocytes</td>
</tr>
<tr>
<td>ARKOEx2</td>
<td>Exon 2</td>
<td>β-Actin</td>
<td>Complete absence of protein RT–PCR demonstrates absence of wild-type Ar transcripts in testes</td>
</tr>
<tr>
<td>Yeh et al. (2002) (male); Hu et al. (2004) (female)</td>
<td></td>
<td></td>
<td>Complete absence of protein Loss of AR immunopositive staining in testes. Ovarian cDNA produce an excised exon 2 product</td>
</tr>
<tr>
<td>ARKOEx2PGK</td>
<td>Exon 2</td>
<td>PGK</td>
<td>Complete absence of protein Loss of AR immunopositive staining in testes. Ovarian cDNA produce an excised exon 2 product</td>
</tr>
<tr>
<td>ARKOEx3</td>
<td>Exon 3</td>
<td>CMV</td>
<td>Presence of non-functional protein Kidney cDNA from males produce an excised exon 3 product. Presence of AR immunopositive staining in kidneys from males and ovaries</td>
</tr>
<tr>
<td>Notini et al. (2005) (male); Walters et al. (2007) (female)</td>
<td></td>
<td></td>
<td>Presence of non-functional protein Kidney cDNA from males produce an excised exon 3 product. Presence of AR immunopositive staining in kidneys from males and ovaries</td>
</tr>
</tbody>
</table>
Zhou et al., 1996; Collins et al., 2003) probably reflects either ectopic AR expression or artefactual staining as AR has no obligatory function in germ cells. AR null germinai cells harbouring a genetically inactive AR gene develop normally in tubules containing Sertoli cells with a functional AR (Lyon et al., 1975; Johnston et al., 2001). AR is also highly expressed in secondary sex accessory glands including the epididymis, prostate and seminal vesicles (Ruzvey de Winter et al., 1991). The strong AR expression in the male reproductive tract has suggested a functional role for androgenic actions in the acquisition of mature sperm functions required for male fertility.

Global complete ARKO male mice

The classical tfm phenotype was indentified in the 1970s (Bardin et al., 1970) and demonstrated the indispensable role of androgens in male sexual differentiation, development and maintenance of virilization, spermatogenesis, fertility, as well as the anabolic status of non-reproductive tissues. Genetic tfm males exhibit a normal female somatic and external genital morphology but are sterile with small, intra-abdominal testes and absence of male (epididymis, vas deferens, seminal vesicles and prostate) or female (ovary, oviducts and uterus) internal genitalia (Lyon and Hawkes, 1970). The spermatogenesis in tfm testis is arrested at the late pachytene spermatocyte stage (Lyon and Hawkes, 1970). The tfm testis develops a normal fetal Leydig cell population and this population of cells is hormone-independent (O'Shaughnessy et al., 1998). However, after birth, the usual switch-over to the adult Leydig cell generation is defective with a progressive failure to develop the full complement of adult Leydig cells (O'Shaughnessy et al., 2002). The surviving adult Leydig cells are hypertrophic and appear relatively prominent due to underdeveloped seminiferous tubules, usually comprising most of the mature testis volume. The adult mouse tfm Leydig cells exhibit reduced T secretion (Jones et al., 2003), so that genetic male tfm mice are both androgen resistant and deficient.

Over the last decade, several studies have used modern genetic engineering approaches to produce global ARKO mice (Yeh et al., 2002; Notini et al., 2005; Kato, 2002; Holdcraft and Braun, 2004; De Gendt et al., 2004). These models consistently reproduce the tfm phenotype with high fidelity (Table II), and there appears to be no significant discrepancies in reproductive phenotypes between the classical spontaneous tfm and global ARKO males. Like the tfm testis, all the global ARKO features are comparable with the hypogonadal (hpg) mouse, which has a congenital deficiency in the synthesis of hypothalamic gonadotrophin-releasing hormone, with spermatogenesis arrested at the late pachytene stage (Singh et al., 1995), but full spermatogenesis inducible by T. The hpg model features a complete functional androgen-deficient state from birth onwards but experiences normal androgen secretion and action prior to birth (O'Shaughnessy et al., 1998). These findings indicate that the defect in androgen action responsible for all these models is primarily post-natal in timing.

Analysis of ARKO tests has revealed a number of other morphological features due to AR inactivation. For example, mature ARKO Sertoli cells are fewer (~30%) (Tan et al., 2005) and display fibrillary degeneration (Yeh et al., 2002). Mature ARKO Leydig cells are also less numerous (~20%) in number (Tan et al., 2005) but have a hypertrophic morphology (Kato, 2002; Yeh et al., 2002) particularly conspicuous in the more prominent interstitial space created by the lack of tubular development. Peritubular myoid cells in global ARKO models have received little attention apart from the observations that they form unusual multilayered formations in the ARKOEX2PGK mouse (De Gendt et al., 2004) similar to those observed in the classical tfm testes (Clark et al., 2000; Johnston et al., 2004). The significance of these observations is not clear.

Blood T levels are reduced in global ARKO as they are in classical tfm genetic male mice (Murphy and O'Shaughnessy, 1991; Kato, 2002; Yeh et al., 2002; Notini et al., 2005) making them both androgen resistant and deficient. This mouse-specific feature is due to the apparently extreme species specificity of the androgen dependence of 17α hydroxylase activity which has reduced expression and enzymatic activity in both ARKO and tfm mouse testes (Murphy and O'Shaughnessy, 1991; O'Shaughnessy et al., 2002; De Gendt et al.,

<table>
<thead>
<tr>
<th>Global ARKO mice</th>
<th>Sex</th>
<th>Fertility</th>
<th>Morphology of reproductive organs</th>
<th>Gamete development</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARKOEx1CMV (Kato, 2002)</td>
<td>Male</td>
<td>Infertile</td>
<td>Small, immature, intra-abdominal testes and lack of secondary reproductive organs</td>
<td>Spermatogenesis arrested at the pachytene stage</td>
</tr>
<tr>
<td>ARKOEx1CMV (Shiina et al., 2006)</td>
<td>Female</td>
<td>Subfertile</td>
<td>Ovary, oviducts and uterus appear normal</td>
<td>Growing follicle populations normal at 8 weeks of age. Total follicle depletion by 40 weeks. ↓ CL</td>
</tr>
<tr>
<td>ARKOEx1 (Holdcraft and Braun, 2004)</td>
<td>Male</td>
<td>Infertile</td>
<td>Small, immature, intra-abdominal testes and lack of secondary reproductive organs</td>
<td>Spermatogenesis arrested at the pachytene stage</td>
</tr>
<tr>
<td>ARKOEx2PGK (De Gendt et al., 2004)</td>
<td>Male</td>
<td>Infertile</td>
<td>Small, immature, intra-abdominal testes and lack of secondary reproductive organs</td>
<td>Spermatogenesis arrested at the pachytene stage</td>
</tr>
<tr>
<td>ARKOEx2 (Yeh et al., 2002)</td>
<td>Male</td>
<td>Infertile</td>
<td>Small, immature, intra-abdominal testes and lack of secondary reproductive organs</td>
<td>Spermatogenesis arrested at the pachytene stage</td>
</tr>
<tr>
<td>ARKOEx2 (Hu et al., 2004)</td>
<td>Female</td>
<td>Subfertile</td>
<td>Ovary, oviducts and uterus appear normal. ↓ uterus diameter</td>
<td>Growing follicle populations normal at 4 and 6 weeks of age. ↓ CL</td>
</tr>
<tr>
<td>ARKOEx3 (Notini et al., 2005)</td>
<td>Male</td>
<td>Infertile</td>
<td>Small, immature, intra-abdominal testes and lack of secondary reproductive organs</td>
<td>Spermatogenesis arrested at the pachytene stage</td>
</tr>
<tr>
<td>ARKOEx3 (Walters et al., 2007)</td>
<td>Female</td>
<td>Subfertile</td>
<td>Ovary and oviducts appear normal. ↓ uterus diameter and ↑ in uterine horn length</td>
<td>Growing follicle populations normal at 10–12, 26 and 52 weeks of age. ↓ CL and natural ovulation rates</td>
</tr>
</tbody>
</table>

CL, corpus luteum.
Androgen actions and fertility

2005; Eacker et al., 2008). However, mice with only partial inactivation of AR function (Holdcraft and Braun, 2004; Eacker et al., 2008) can exhibit elevated blood T levels more in keeping with other mammals including rats and man as would be expected for a state of pure global androgen resistance (Purvis et al., 1977; Quigley et al., 1995). In the more usual situation in other mammals reflecting pure androgen resistance, Leydig cell T secretion is strikingly increased responding to consistent and sustained increases in blood LH responding to the lack of negative androgenic feedback on the brain. Although blood E2 levels are reported to be similar in ARKO males as in wild-type mice (Kato, 2002), serum levels of LH and/or FSH are elevated in the global ARKO males (Kato, 2002; Chang et al., 2004), presumably due to disruption of androgen negative feedback to the hypothalamus.

In conclusion, the global male ARKO models reproduce with fidelity the classical tfm and thereby confirm that AR signalling is essential for the normal development of spermatogenesis, sperm, epididymis, vas deferens, seminal vesicles and prostate. However, they also verify that AR actions are not required for fetal sex determination or testis development, but are required for sexual maturation. AR actions are not required for Sertoli or Leydig cell development but play a role in maintaining their optimal development and numbers as well as their function.

**Cell-specific ARKO male mice**

The targeting of tissue or cell selective gene inactivation is achieved by using the Cre/LoxP method with Cre driven by a tissue or cell-specific promoter which allows analysis of the specific AR-mediated androgen actions in individual testicular cell types and/or in distinctive male reproductive tissues (Table III). The caveats on this technology include the influence of floxing on the functionality of the AR gene (Holdcraft and Braun, 2004; MacLean et al., 2008), completeness of excision and leakiness of Cre activation involving off-target cells and tissues.

Sertoli cell-specific ARKO male mice. Sertoli cells are located basally along the perimeter of the seminiferous tubules for which they form a cellular scaffold stabilized by tight intercellular junctions. This constitutes the diffusion-tight, blood–testis barrier which creates a highly specialized interior milieu in the seminiferous tubules (Russell and Griswold, 1993). These somatic cells are in intimate contact with every germ cell with each enshrouded by thin extensions of Sertoli cell cytoplasm. Sertoli cells regulate, nourish and shape the development of germ cells as they progress through spermatogenesis. Within the tubules, Sertoli cells are the locus of hormonal control of spermatogenesis as they express FSH receptor and AR, whereas germ cells do not (Walker and Cheng, 2005).

Five distinct Sertoli cell-specific ARKO mouse lines have been reported in male mice (Chang et al., 2004; De Gendt et al., 2004; Holdcraft and Braun, 2004; Lim et al., 2009) using two different Sertoli cell promoters aiming for AR inactivation in Sertoli cells but leaving AR functional in all other cells (Table IV). Four used the same AMH promoter driven Cre transgenic mice (Lecureuil et al., 2002) and another used the ABP promoter Cre line (Lim et al., 2009). AR function was not detected in any of the above mice.

### Table III In vivo effect of cell/tissue-specific androgen deficiency defined by distinct male ARKO mouse models.

<table>
<thead>
<tr>
<th>Cell-specific ARKO mice</th>
<th>Fertility</th>
<th>Macroscopic analysis of reproductive organs</th>
<th>Sperm development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sertoli cell-specific ARKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCARKO (De Gendt et al., 2004)</td>
<td>Infertile</td>
<td>↓ testis weight. Normal secondary reproductive organs</td>
<td>Spermatogenic arrest at meiosis (pachytene)</td>
</tr>
<tr>
<td>SARKO (Chang et al., 2004)</td>
<td>Infertile</td>
<td>↓ testis weight. Normal secondary reproductive organs</td>
<td>Spermatogenic arrest at meiosis (diplotene)</td>
</tr>
<tr>
<td>SCAR (Lim et al., 2009)</td>
<td>Infertile</td>
<td>↓ testis weight. Normal secondary reproductive organs</td>
<td>Spermatogenic arrest at meiosis</td>
</tr>
<tr>
<td>SHypoARKO (Holdcraft et al., 2004)</td>
<td>—</td>
<td>↓ testis and seminal vesicle weight</td>
<td>Spermatogenesis arrested at the round spermatid stage</td>
</tr>
<tr>
<td>Leydig cell-specific ARKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LARKO (Tsai et al., 2006)</td>
<td>Infertile</td>
<td>↓ testis and epididymis weight</td>
<td>Spermatogenesis arrested mainly at the round spermatid stage</td>
</tr>
<tr>
<td>Peritubular myoid cell-specific ARKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PMARKO (Zhang et al., 2006)</td>
<td>Fertile</td>
<td>↓ testis weight. Normal secondary reproductive organs</td>
<td>↓ total germ cell (testis) and sperm numbers (epididymis)</td>
</tr>
<tr>
<td>PTMARKO (Welsh et al., 2009)</td>
<td>Infertile</td>
<td>↓ testis, seminal vesicle and ventral prostate weights</td>
<td>↓ total germ cell (testis) and sperm numbers (epididymis)</td>
</tr>
<tr>
<td>Germ cell-specific ARKO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GARKO (Tsai et al., 2006)</td>
<td>Fertile</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Prostate epithelial-specific ARKO</td>
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<td></td>
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</tr>
<tr>
<td>PEARKO (Simanainen et al., 2007; 2008)</td>
<td>Subfertile</td>
<td>Normal testis. ↓ secondary reproductive organ and epididymis weights</td>
<td>Spermatogenesis normal but defects in sperm maturation and ability to undergo fertilization</td>
</tr>
<tr>
<td>pes-ARKO (Wu et al., 2007)</td>
<td>Fertile</td>
<td>Normal testis. Larger ventral prostate</td>
<td>Normal</td>
</tr>
</tbody>
</table>
abolished by deleting exon 1 (denoted SARKO; Holdcraft and Braun, 2004), exon 2 (denoted SCARKO; De Gendt et al., 2004; denoted SARKO; Chang et al., 2004) or by an in-frame deletion of only exon 3 (denoted SCAR; Lim et al., 2009).

In most models, testis size is reduced to a similar degree (Chang et al., 2004; De Gendt et al., 2004; Lim et al., 2009), although in the SHypoARKO model with a hypofunctional floxed exon 1 AR (Holdcraft and Braun, 2004) (where AR is deleted in Sertoli cells and hypomorphic in all other cells), the smaller testis size is probably proportional to the degree of AR inactivation, a phenomenon illustrated in a more recent study where the intensity of Cre expression influenced ultimate mature testis size most likely via the degree of AR inactivation (Lim et al., 2009).

Discrepancies between blood T levels between Sertoli cell ARKO models (Table IV) reflect the ‘leakiness’ of Cre activation. In theory, a pure Sertoli cell ARKO model should have unaffected blood LH and T levels to the extent that Leydig cell function is not affected by Sertoli cell dysfunction. However, if leaky Cre activation occurs in Leydig cells, T production will fall and blood LH levels will then increase due to reduced central negative feedback by T. Most of the five Sertoli cell ARKO models show at least mild reductions in blood T levels compared with controls, although the variation between models probably represents direct effects of mild Cre leakiness involving the Leydig cells although indirect, paracrine influence of Sertoli cells on Leydig cell function cannot be fully discounted (Table IV). The extreme increases in blood LH and T in the SHypoARKO model (Holdcraft and Braun, 2004) probably represents cerebral androgen resistance due to the influence on the brain of the partial inactivation of AR (‘hypomorphic allele’) due to exon 1 floxing. This resembles the increased LH and T secretion that occurs in other mammals with global AR inactivation (Quigley et al., 1995). The blood FSH levels are significantly but less prominently increased in models with normal cerebral AR sensitivity.

The spermatogenic phenotype is coherent for Sertoli cell ARKO models with small tubules lacking lumen and arrest of spermatogenesis at the pachytene spermatocytes stage with no sperm in the epididymis (De Gendt et al., 2004; Holdcraft and Braun, 2004; Lim et al., 2009) (Table IV). In contrast to the global ARKO testis which features decreased Sertoli cell numbers, Sertoli cell ARKO males exhibit minimal or no decrease in Sertoli cell numbers (Tan et al., 2005), indicating that maintenance of the mature Sertoli cell population depends partially on AR activity in cells other than Sertoli cells. Sertoli cell AR activity maintains mature Sertoli cell structure and function (Wang et al., 2006) including the cytoskeleton and intercellular junctions indicated by abnormal duplication of basal lamina of seminiferous tubules and abnormal mRNA levels of the Sertoli cell structural component vimentin and seminiferous tubule basement membrane component laminin α5 (Wang et al., 2006). This is also consistent with evidence for AR-dependent regulation of tubular structure- and cell junction-
related genes (Denolet et al., 2006) and maintenance of the integrity of the blood–testis barrier with decreased gene expression of claudin-11, occludin and gelsolin, components of the tight junction and anchoring junction complexes (Wang et al., 2006).

In conclusion, the five Sertoli cell ARKO models now provide a coherent picture confirming that AR activity in the Sertoli cell is essential to maintain mature Sertoli cell structure and function including intercellular junctions and blood–testis barrier integrity, to maintain normal testicular Leydig cell number and to support completion of post-meiotic germ cell development into spermatozoa capable of motion and fertilization. Conversely, AR activity in Sertoli cells is not required for sexual differentiation, testis development or descent, development of internal and external genitalia and accessory male reproductive organs.

**Leydig cell-specific ARKO male mice.** Leydig cells are located in the interstitial spaces between, and in close proximity to, the seminiferous tubules. Their primary function is the synthesis and secretion of T to bring about male sexual differentiation during fetal life, and during adult male life to support spermatogenesis locally as well as androgenization throughout the body (Payne et al., 1996).

Leydig cell-specific ARKO males (Amhr2<sup>m3 induce Cre line with the exon 2 floxed AR line (Tsai et al., 2007). The Amhr2 promoter was previously shown to drive Cre expression predominantly in the interstitial Leydig cells, but also to be responsible for significant leakage with scattered Cre expression also detected in the seminiferous tubules (Jeyasuria et al., 2004). Furthermore, AR inactivation in Leydig cells was not complete with some Leydig cells still expressing AR (Tsai et al., 2006).

LARKO males are infertile and exhibit normal testis descent but small testis and epididymis, without sperm present, compared with wild-type controls (Tsai et al., 2006; Xu et al., 2007). Histologically, LARKO testis features pachytene spermatogenic arrest and small testicular tubules with no lumen (Xu et al., 2007). Reduced blood T (Xu et al., 2007) is consistent with that reported in global ARKO and pmf models. However, surprisingly, despite the observed significant reduction in blood T levels and markedly elevated blood LH and FSH, the LARKO males demonstrate preserved mating behavior and copulatory plug formation as well as seminal vesicles and prostate weights that remained similar to wild-type controls (Xu et al., 2007).

The limitations on the specificity and completeness of the Leydig cell AR inactivation require confirmation of this phenotype using distinct complementary AR inactivation strategies. Nevertheless, the available findings do suggest that AR-mediated effects on the Leydig cells may have an influence on normal Leydig cell T production, spermatogenesis and male fertility.

**Peritubular myoid cell-specific ARKO male mice.** Peritubular myoid cells are mesenchymal cells that form the outer border of the seminiferous tubules and, in conjunction with Sertoli cells, maintain seminiferous tubule morphology and function (Maekawa et al., 1996). Although peritubular myoid cells occur in several layers in human testis, only a single layer is present in the mouse testis (Maekawa et al., 1996). Few studies have examined the functional significance of peritubular myoid cells in the testis, and the androgen dependence of peritubular myoid cells is unclear apart from the fact that peritubular myoid cells express AR.

Two peritubular myoid cell ARKO models have been created by crossing either the transgelin (Tagln or SM22) (denoted hereafter as PMARKO) (Zhang et al., 2006) or smooth muscle myosin heavy chain (smMHC) (denoted hereafter as PTMARKO) (Welsh et al., 2009) promoter driven Cre lines with the exon 2 floxed AR. These promoters drive Cre expression in smooth muscle cells (Holtwick et al., 2002; Xin et al., 2002) including peritubular myoid cells (Zhang et al., 2006; Welsh et al., 2009). However, a recent study suggests that SM22-Cre does not alter AR expression in testicular peritubular myoid cells, whereas smMHC-Cre deletes AR from a proportion of peritubular myoid cells (Welsh et al., 2009).

PMARKO males are fertile with normal gross morphology and external and internal male genital organ development (Zhang et al., 2006), whereas PTMARKO males are infertile (Welsh et al., 2009), exhibiting normal external sexual development and testis decent. However, testis weight is reduced (~70% of adult wild type) in both models and PTMARKO males also exhibit reduced seminal vesicle and ventral prostate weights (Welsh et al., 2009). Furthermore, PTMARKO seminiferous tubule volume is reduced and spermatogenesis severely impaired resulting in significantly reduced germ cell numbers in testis, with a progressive loss observed with age (Welsh et al., 2009). Epididymal sperm numbers are reduced in both models. Sertoli cell and Leydig cell size and numbers are normal (Welsh et al., 2009); however, the expression of androgen-dependent Sertoli cell-specific genes (Rhox5, Eppin and Tubb3) (Welsh et al., 2009) and Sertoli cell functional marker genes (transferrin, epidermal fatty acid-binding protein and androgen-binding protein) (Zhang et al., 2006) are reduced implying impaired Sertoli cell function. Serum T levels are normal in both models and LH and FSH are unchanged in PMARKO males; however, intra-testicular T and blood LH and FSH are elevated in PTMARKO males (Welsh et al., 2009). These findings raise the possibility of some leakiness of Cre activation in Sertoli and Leydig cells which cloud the interpretation of a strictly peritubular cell phenotype.

In conclusion, AR inactivation in the PMARKO model appears to have no major effect on overall fertility; however, this lack of phenotype appears to be due to an inefficiency of the model rather than a true reflection of the role of AR in peritubular myoid cells. The more recent PTMARKO model demonstrates that androgen actions via the peritubular myoid cells may play an important role in normal testis function, spermatogenesis and fertility. However, this model does not provide complete loss of AR function in all peritubular myoid cells; hence, further verification using a complementary but distinct strategy is required before a distinctive peritubular myoid cell function can be conclusively established.

**Germ cell-specific ARKO male mice.** Several studies have reported conflicting evidence as to whether germ cells express functional AR gene and/or protein or not (Zhou et al., 2002; Collins et al., 2003). Germ cell-specific ARKO (GARKO) males have been generated by crossing the Sycp-1 promoter driven Cre line with the exon 2 floxed AR (Tsai et al., 2006). The effectively complete inactivation of AR in postmitotic germ cells at a young reproductive age (<15 weeks) was proven by the finding that females sired from GARKO males carried...
the deleted AR allele (Tsai et al., 2006). GARKO males have unimpaired testis size and descent, with complete spermatogenesis and normal fertility as well as androgen status with normal blood T levels and virilization. These findings confirm the well-known functional redundance of AR expression in germ cells shown previously by studies of embryological chimeras (Lyon et al., 1975) and spermatogonial transplantation of germ cells harbouring an inactive AR (Johnston et al., 2001).

Reproductive accessory gland-specific ARKO male mice. The male reproductive accessory organs, comprising the epididymis, vas deferens, seminal vesicles and prostate, have important but poorly defined functions in establishing and maintaining mature sperm function and male fertility. These androgen-dependent organs are likely to have important roles in mediating androgen effects on the final structural development of sperm during post-meiotic spermiogenesis, the post-meiotic metamorphosis of round amorphous spermatids into specialized spermatozoa as well as the acquisition of sperm function in the efferent ducts of the post-testicular reproductive tract. Previous research has suggested androgen effects on the terminal stage of the structural and functional development of sperm involving the Wolffian duct derivatives (Hannema and Hughes, 2007) comprising the epididymis (Kirchoff et al., 1998; Robaire et al., 2005), vas deferens (Lafayette et al., 2008) and seminal vesicles (Aumuller and Riva, 1992). However, it has remained difficult to test decisively androgen effects on these tissues selectively in vivo using pharmacological (exogenous steroids or blockers) or genetic means (abrogating AR function causes defective spermatogenesis). The ability to distinguish androgen effects in vivo on the testis from those on the accessory glands is circumvented by the creation of post-testicular AR inactivation with the probasin promoter-driven Cre lines. Two mouse lines have been reported utilizing either the exon 2 floxed AR (denoted pes-ARKO) (Wu et al., 2007) or the exon 3 floxed AR (denoted hereafter as PEARKO) (Simanainen et al., 2007, 2008). Although these two models used nominally the same probasin-Cre mouse lines (Jin et al., 2003; Wu et al., 2001), they generated functionally distinct models of prostate epithelial ARKO due to unintended differences in tissue specificity of Cre activation. The pes-ARKO mouse features prostate epithelial AR inactivation restricted to the ventral and dorsolateral prostate, and a severe phenotype detected only in the ventral lobe (Wu et al., 2007). In the PEARKO model, all prostate lobes are equally affected, but AR inactivation is also evident in all the other accessory glands but not the testis (Simanainen et al., 2007, 2008). The different tissue pattern of AR inactivation in these two models is due to the small size and strong activity of the probasin promoter that can lead to unexpected but consistent variation in Cre expression patterns (Wu et al., 2001).

The pes-ARKO males with more restricted AR inactivation are fertile demonstrating that ventral prostate epithelial AR expression is not required for fertility (Wu et al., 2007). However, the PEARKO males with epithelial AR inactivation in all prostate lobes and reduced androgen action in seminal vesicles and epididymis exhibit severe subfertility (Simanainen et al., 2008). Despite normal sperm production in the unaffected testes, caput epididymal sperm numbers are reduced and kinetics of epididymal passage is impaired. Cauda epidymal PEARKO sperm have increased spontaneous acrosome reaction and abnormal flagellar morphology, and fertilize fewer oocytes recovered from the oviducts of wild-type females after natural matings. These data show that independent of androgen action on the testis or spermatogenesis, androgen action on accessory glands is essential to complete normal sperm structural and functional maturation. Furthermore, the PEARKO males produce smaller and less robust vaginal copulatory plugs due to reduced secretory function of seminal vesicles and prostate (Simanainen et al., 2008).

In conclusion, these data suggest that androgen actions on male reproductive accessory glands has an important influence on several aspects required for male fertility, including acquisition of mature sperm functions such as motion and fertilizing capability.

Reproductive phenotype of female ARKO mice

In females, AR is widely expressed in the ovary (Walters et al., 2008), uterus (Pelletier, 2000; Pelletier et al., 2004) and breast (Dimitrakakis et al., 2002; Somboonporn and Davis, 2004) consistent with a role for AR in ovarian and uterine reproductive function, and mammary gland development and growth.

Global complete ARKO female mice

The original homozygous female ARKO models, the XO female mice where the X chromosome bore the Ar<sup>Tm</sup> mutation (Ohno et al., 1973), and the homozygous Ar<sup>Tm/Ar<sup>Tm</sup> female (Lyon and Glenister, 1974) were subfertile (Lyon and Glenister, 1980) and exhibited increased follicle atresia and reduced follicle numbers. However, AR-mediated androgen action was not absolutely essential for ovulation, mating, pregnancy or lactation (Ohno et al., 1973; Lyon and Glenister, 1974, 1980).

More recently, ARKO female mice have been generated using the efficient Cre/LoxP system (Yeh et al., 2002, 2003; Hu et al., 2004; Shina et al., 2006; Walters et al., 2007). All ARKO female mouse models described to date are subfertile, with fewer pups/litter and decreased follicle health (Yeh et al., 2002; Hu et al., 2004; Shina et al., 2006; Walters et al., 2007) (Table II), but exhibit normal follicle populations at least up to 16 weeks of age (Hu et al., 2004; Shina et al., 2006; Walters et al., 2007). Older females with a complete loss of AR protein (ARKO<sup>Ex1CMV</sup>) exhibit accelerated follicle depletion (Shina et al., 2006), but this is not apparent in the ARKO<sup>Ex2</sup> model which maintains a minimally truncated but inactive AR protein.

Follicle diameters at different stages of development are not altered in the ARKO<sup>Ex3</sup> model, indicating that classical AR-mediated actions do not appear to play essential roles in follicle growth (Walters et al., 2007). However, ovarian Fsh and Igf1r expression is significantly reduced in ARKO<sup>Ex2</sup> mice (Hu et al., 2004), indicating that follicle growth may be impaired. Oocyte health is compromised in ARKO<sup>Ex2</sup> ovaries, with oocytes in pre-ovulatory follicles exhibiting a loss of cumulus cell contact during ovulation, and reduced expression of hyaluronan synthase 2 and tumor necrosis factor-a-stimulated gene 6 required for cumulus expansion (Hu et al., 2004). Moreover, microarray analysis of ARKO<sup>Ex1CMV</sup> ovaries indicated a reduced expression of several genes involved in the oocyte—granulosa cell regulatory loop (Shina et al., 2006), including KIT ligand, bone morphogenetic protein 15 and growth differentiation factor 9. In contrast, ARKO<sup>Ex2</sup> oocytes
exhibit no disassociation of cumulus cells in the pre-ovulatory follicles (Walters et al., 2008), normal fertilization rates and early embryonic development to the 2-cell stage (Walters et al., 2007). Conflicting results between the three models suggest an undefined AR activity, independent of direct DNA-binding-mediated transactivation influencing follicle and oocyte health and development.

In all ARKO models, dysfunctional ovulation has been identified as a key defect leading to the observed subfertility (Hu et al., 2004; Shiina et al., 2006; Walters et al., 2007) with defective follicle development during antral stages of follicular growth leading to reduced ovulation of oocytes and fewer corpora lutea (CL) formed (Hu et al., 2004; Shiina et al., 2006; Walters et al., 2007). Interestingly, in the ARKOEx3 model, the reduced ovulation rates are overcome by gonadotrophin hyperstimulation (Walters et al., 2007). This result taken together with delayed time to first litter (Walters et al., 2007) and abnormal estrous cycles (Hu et al., 2004; Walters et al., 2009) suggests a predominantly extra-ovarian defect in the hypothalamic–pituitary regulation, whereas decreased antral follicle health and increased ovarian T (Walters et al., 2007; McNamara et al., 2010) are consistent with an ovarian site of action. This hypothesis was supported by the findings that ovariectomized wild-type hosts with ARKOEx3 ovary transplants exhibit normal estrous cycling and overall fertility (percentage of females to produce a litter); however, ovariectomized ARKOEx3 hosts with wild-type ovary transplants display abnormal estrous cycles and a significant reduction in fertility, similar to those observed in intact ARKOEx3 females (Walters et al., 2009). Furthermore, AR has now been implicated as a key component of neuroendocrine mechanisms as ARKOEx3 females exhibit increased baseline FSH and E2 levels at estrus, as well as increased sensitivity to negative E2 feedback on LH secretion consistent with a neuroendocrine defect of negative feedback signalling (Walters et al., 2009).

The uterus has been implicated as a potential target for AR-mediated androgen action, yet a specific androgenic role in uterine reproductive function has yet to be firmly established. ARKOEx2 females exhibit a decrease in their uterine response to estrous and ovulation, which was more significant with age (Hu et al., 2004; Shiina et al., 2006; Walters et al., 2007). Interestingly, in the ARKOEx3 model, the reduced ovulation rates are overcome by gonadotrophin hyperstimulation (Walters et al., 2007). This result taken together with delayed time to first litter (Walters et al., 2007) and abnormal estrous cycles (Hu et al., 2004; Walters et al., 2009) suggests a predominantly extra-ovarian defect in the hypothalamic–pituitary regulation, whereas decreased antral follicle health and increased ovarian T (Walters et al., 2007; McNamara et al., 2010) are consistent with an ovarian site of action. This hypothesis was supported by the findings that ovariectomized wild-type hosts with ARKOEx3 ovary transplants exhibit normal estrous cycling and overall fertility (percentage of females to produce a litter); however, ovariectomized ARKOEx3 hosts with wild-type ovary transplants display abnormal estrous cycles and a significant reduction in fertility, similar to those observed in intact ARKOEx3 females (Walters et al., 2009). Furthermore, AR has now been implicated as a key component of neuroendocrine mechanisms as ARKOEx3 females exhibit increased baseline FSH and E2 levels at estrus, as well as increased sensitivity to negative E2 feedback on LH secretion consistent with a neuroendocrine defect of negative feedback signalling (Walters et al., 2009).

In summary, findings from the female global ARKO models have confirmed that androgens play an important role in maintaining female fertility. AR-mediated actions have been shown to influence follicle development, in particular ovulatory processes, via extra-ovarian neuroendocrine, as well as local intra-ovarian AR-mediated actions.

Cell-specific ARKO female mice
Although no publications exist describing cell-specific ARKO females, such mice would be very informative. Within the ovary, the role of AR present in the oocyte, granulosa cells and theca cells (Walters et al., 2008) could be studied using available Cre mouse lines to create granulosa cell (AMH promoter driven Cre transgenic mice; Lecureuil et al., 2002), theca cell (Cyp17Cre transgenic mice; Bridges et al., 2008) and oocyte (Zp3-Cre, Gdf9-Cre and Msv2-Cre transgenic mice; Lewandoski et al., 1997; Lan et al., 2004) specific ARKO females. Mammary gland epithelial cell-specific ARKO females could be created with the use of MMTV-Cre and WAP-Cre transgenic mice (Wagner et al., 1997); however, the stromal mammary gland–uterine-specific models remain to be established due to stromal heterogeneity (Jackson et al., 2008).

Haploinsufficient ARKO female mice
Although biologically informative, homozygous androgen-resistant females probably do not occur in humans, whereas the relatively high frequency of women heterozygous for AR mutations, such as obligate heterozygous mothers of CAIS children, renders the biology of heterozygous or haploinsufficient women of potential clinical significance. To date, heterozygote ARKO female mice from the exon 2 and exon 3 targeted deletion models have been analysed. Heterozygous Ar mice are fertile with no significant difference in estrous cycles (Walters et al., 2009) or pups/litter when compared with control wild-type females, up to 5 months of age (Hu et al., 2004; Walters et al., 2007). However, although heterozygote ARKOEx3 females initially display similar fertility to wild-type females, they exhibit an age-dependent significant reduction in pups/litter (Walters et al., 2007), distinct from the reproductive performance of complete ARKOEx3 mice. This indicates that quantitative variations in AR activity via gene dosage may play a role in determining female fertility. Heterozygous ARKOEx2 females show no disassociation of cumulus cells from ovulated oocytes collected from oviducts, implying normal oocyte–cumulus cell interactions (Hu et al., 2004). In contrast, heterozygote ARKOEx3, like the complete knockout, exhibit a reduction in CL numbers, indicative of the number of ovulations, suggesting that any alterations in AR activity can have a marked effect on ovulation rates (Walters et al., 2007).

In summary, heterozygote females exhibit a decrease in ovulation rates indicated by CL numbers and an age-dependent significant reduction in fertility. These findings suggest that even small alterations in AR activity can affect female fertility, notably via the control of ovulatory processes.

Human implications from ARKO mouse studies
Implications for male reproductive physiology
The mouse CAIS phenotype of either naturally occurring tfm or global ARKO genetic mice is very similar to that of humans with CAIS. These individuals lack all external male genitalia and their somatic and external genital phenotype is that of normal females but with complete absence of female internal genitalia or male reproductive accessory organs but small immature, inguinally located testes. The limitations on the availability of human tissues preclude much detailed investigations but opportunistic observations from surgery or tissue sampling
have provided much confirmatory information (Quigley et al., 1995). However, the resemblance between these models is not always exact. For example, in the testis, although the spermatogenic maturation is arrested at the pachytene (late meiotic) stage with well-developed pre-meiotic germ cell populations located within the small seminiferous tubules of undescended global ARKO mouse testes (Kato, 2002; Yeh et al., 2002; Notini et al., 2005), in human CAIS, the testis appears more severely depleted of germ cells with tubules lined by immature Sertoli cells which may develop pre-neoplastic tumours (Rutgers and Scully, 1991). However, information from humans is limited mainly to surgically excised specimens from individuals undergoing prophylactic orchidectomy to prevent gonadoblastoma, a procedure usually undertaken prior to the completion of testis development. Therefore, it is likely that the underlying pathogenesis may be consistent between species but more fully examined through the detailed experimental investigations feasible for a mouse model. This demonstrates the invaluable role of suitable genetic mouse models to overcome the ethical and practical limitations on scientific discovery about human genetic disorders of the highly conserved process of reproduction.

Another advantageous illustration is provided by the physiological insight into effects of a post-testicular ARKO provided serendipitously by the PEARKO mouse model (Simanainen et al., 2007, 2008). The strong dependence of spermatogenesis on AR function has limited the ability to examine post-testicular AR-mediated androgen effects on the development of sperm function. The PEARKO model’s dichotomy of reduced androgen-dependent sex accessory gland structure and functions with normal testis development and function provide a so-far unique opportunity to develop novel insight into the molecular determinants of androgen-dependent, post-testicular sperm functional maturation. This model could identify new inroads into the detection, diagnosis and treatment of unexplained male infertility as well as in developing new male-based hormonally targeted but non-hormonal contraceptives for both human and animal application.

Hormonal male contraceptives are the closest to clinical implementation (Meriggiola et al., 2003) following the demonstration of proof-of-principle for injectable (WHO studies: 1990, 1996) and for depot combination (Turner et al., 2003) male contraceptives, and evidence for their acceptability to men (Heinemann et al., 2005) and to their female partners (Glasier et al., 2000). However, industry developments have lagged for a variety of reasons including concerns about the systemic administration of hormones. The major alternative has been the possibility of targeting chemicals to the post-testicular environment where sperm could become exposed to chemicals during sperm storage and maturation in the distal epididymis. Prototype post-testicular non-hormonal chemical contraceptives [e.g. chlorinated sugars (Ford and Waite, 1980), miglустat (Amory et al., 2007)] have, however, yet to prove feasible. Rational approaches based on the profusion of genes influencing sperm production, function and male fertility (Matzuk and Lamb, 2002; Matzuk and Lamb, 2008) have opened numerous other possibilities. One novel, hybrid approach is the development of neo-hormonal male contraceptive methods which bridge between the hormonal and non-hormonal approaches but target the post-testicular ductular system. This concept has been considered previously during studies of cyproterone acetate, a mixed progesterin and anti-androgen, on male fertility regulation (Prasad and Rajalakshmi, 1976; Roy et al., 1976). These studies were based on the concept that CPA had predominant effects on post-testicular sperm functional maturation. However, clinical studies were inconclusive and experimental studies floundered on the inability to target the post-testicular environment including the epididymis selectively. The study using the PEARKO model has achieved this and confirmed the validity of this target for inhibition of sperm function (Simanainen et al., 2008). Furthermore, the PEARKO model supports the concept of contraceptive development based on coiled sperm (Cooper and Yeung, 1999; Cooper et al., 2004; Yeung et al., 2009) and provides an additional model to analyse the molecular mechanisms of sperm coiling. If this approach is coupled with the identification and development of non-steroidal paracrine mediators of androgen action on sperm via epididymal epithelium, this may constitute the basis for a neo-hormonal approach to male contraception.

The sensitivity of the post-testicular environment to inhibition of AR-mediated androgen action demonstrated in the PEARKO model (Simanainen et al., 2008) also raises the possibility of the existence of unrecognized chemical causes of male infertility. Chemicals that may be selectively concentrated by the epididymal epithelium and exert anti-androgenic action may represent an unrecognized cause of male infertility. Similarly, the mildest forms of AIS have minimal clinical features, the least intrusive of which may be isolated male infertility with preserved spermatogenesis. In this setting, the PEARKO mouse model findings raise the possibility that AR-mediated androgen action on the epididymal epithelium may be a sensitive site of androgen action sufficient to provide a basis for this hitherto unexplained mild phenotype.

Thus, such research findings on the physiological ramifications of AR action on the maintenance of male fertility provide valuable information for not only the improved diagnosis and treatment of male subfertility, most of which remains unexplained, but also for creating novel targets for neo-hormonal approaches to male contraceptive development.

Implications for female reproductive physiology

It has not been feasible to create androgen-resistant genetic female mice for scientific experimentation until recently. In biallelic genetic females, complete androgen resistance requires those individuals to be homozygous for AR null mutations. However, this cannot occur by natural mating (other than by parthenogenesis) because the fathers of the CAIS individual required for this outcome are sterile. Genetic engineering using Cre-Lox technology has overcome this obstacle by creating female ARKO models with mice homozygous for a completely inactive AR, rendering them totally androgen resistant. These have provided decisive evidence for an important and previously unsuspected role for androgens in female reproductive physiology. Despite some fundamental differences between humans and rodents in reproductive ageing, notably the shorter lifespan of rodents and their lack of any equivalent of menopause (ovarian failure in mid-adult life), with suitable caution for interpretation and caveats in extrapolating between species, the strong similarities in the evolutionarily conserved regulatory system of mammals are sufficient to support thoughtful interpretations between these species. This creates the opportunity to use the ARKO female mouse as a practical experimental model for experiments that are not feasible or ethical in women.
Women with PCOS exhibit arrested follicular development and anovulation, with excessive ovarian production of androgens, yet the mechanism(s) underlying the condition remains unclear (Ehrmann, 2005). Women with hyperandrogenic anovulation commonly exhibit an elevated GnRH drive and subsequently increased plasma LH, yet the mechanisms remain elusive. Recent findings have implemented androgenic actions in the altered gonadotrophin secretion (Daniels and Berga, 1997; Pastor et al., 1998), with flutamide (an AR antagonist) being shown to restore the sensitivity of the GnRH pulse generator to feedback inhibition by E2 and progesterone (Eagleson et al., 2000). Furthermore, the findings that human theca interna cells from the PCOS ovary produce 20 times more A4 than those from normal ovaries (Gilling-Smith et al., 1994), and these levels persist over a long-term culture (Nelson et al., 1999), and the observation that treatment with the flutamide improved fertility in some women with PCOS (Rittmaster, 1999; De et al., 1998) is consistent with, but cannot prove, a role for AR-mediated actions in human female reproductive function. Animal models (monkeys, sheep and rodents) with prenatal exposure to high doses of androgens develop many of the characteristic features of PCOS (Abbott et al., 2006), suggesting that the origins of PCOS occur during fetal life. Young ARKOEx3 mice exhibit transient elevated intra-ovarian T levels which are associated with poor follicle health and disrupted ovulation (Walters et al., 2007), raising the hypothesis that even transient abnormally high levels of ovarian androgens may initiate lasting effects on the developing follicle through an early-life androgen imprinting mechanism.

AR is strongly expressed in the mouse brain and regulated by T and E2 (Kumar and Thakur, 2004), thereby creating the potential for AR signalling to influence feedback mechanisms regulating the hypothalamic GnRH and pituitary LH and FSH secretion, including the LH surge that triggers ovulation. A role for AR actions in the control of hypothalamic–pituitary signalling is supported by the findings that reduced ovulation rates in global ARKOEx3 females (Walters et al., 2007, 2008) can be rescued by gonadotrophin hyperstimulation (Walters et al., 2007) together with recent neuroendocrine evidence of defective gonadotrophin regulation of ovarian function in ARKOEx3 (Walters et al., 2009). These findings suggest that unrecognized defects in central neuroendocrine mechanisms involving AR may play a part in the events causing unexplained anovulatory states in women. This unexpected finding may contribute to a better understanding towards explaining the relatively large proportion of women presently described as having idiopathic anovulation, still the most frequent single cause of female infertility (Hull and Cahill, 1998). Although the best-known relationship between androgen action and anovulation is the androgen excess disorder, PCOS, the present findings suggest that studies of AR function in women with unexplained or functional anovulation may be of interest.

The novel finding that heterozygous ARKOEx3 mice exhibit accelerated age-dependent reduction in fertility (Walters et al., 2007) may have implications for the reproductive performance of women who are obligate carriers of CAIS AR mutations. The heterozygous ARKOEx3 model predicts that these obligate heterozygous mothers of children with CAIS may have involuntarily impaired reproductive function, particularly at an older age. Furthermore, such obligate heterozygote mothers of children with CAIS presumably have partial androgen insensitivity whereby the insensitivity of their defective allele is balanced by the influence of a normal presumably normal second AR allele. The relatively high community frequency of AR mutations may also create the possibility of compound heterozygosity for different AR mutations. Hence, it may also be speculated that these women may be less sensitive to androgens than AR+/− women, and thereby less susceptible to the prevalence of androgen-associated diseases. Thus, whether they are protected from or even more sensitive to the adverse impact of hyperandrogenic disorders especially PCOS, but also possibly malignant (breast and uterine cancer) and benign (benign breast nodules, uterine fibroids) warrants further investigation.

ARKOEx1ICMV female mice with a loss of AR protein exhibit increased atresia leading to an early decline in follicle numbers and POF (Shina et al., 2006), suggesting that androgens may influence follicle atresia via effects on somatic cell apoptosis and/or oocyte degeneration. Some forms of POF are due to chromosomal (especially X) aneuploidies. However, these are usually related to defective ovarian development rather than premature ovarian depletion (Toniolo, 2006). Despite the experimental evidence in one mouse ARKO model for POF, this has not been confirmed by any excess of Ar mutations in women with POF (Kimura et al., 2007). Furthermore, the accelerated depletion of follicles in the ARKOEx1ICMV model is not apparent in the ARKOEx3 model, which retains a mutant but inactive AR protein. It is, therefore, possible that the excessive follicle depletion may not depend solely on AR activation but may be a secondary consequence of post-receptor mechanisms including disruption of common co-regulator proteins disrupting nuclear receptor-activated pathways beyond that of AR. Such differences between ARKO female models highlight the need to critically evaluate differences between mouse models and predictions for women.

AR expression patterns are similar in the rodent (Hirai et al., 1994; Pelletier et al., 2004) and human uterus (Kimura et al., 1993; Tuckerman et al., 2000). Androgens have a direct effect on endometrial function as A4 inhibits the growth of human endometrial cells in vitro and this is blocked by the addition of an anti-androgen, suggesting but not proving an AR-mediated effect (Tuckerman et al., 2000). Additionally, recently AR was found to regulate decidual gene expression suggesting an important but unrecognized role for AR in differentiation of human endometrial stromal cells into decidual cells, a process that critically controls embryo implantation (Cloke et al., 2008). Yet, the underlying mechanisms involved remain poorly understood. Recent findings from the ARKOEx3 model have shown that AR-mediated androgen action is not essential for murine uterine reproductive function (Walters et al., 2009); however, disruption of AR signalling creates altered uterine development (Walters et al., 2009), implying that androgens may play a role in the origins of uterine diseases associated with dysfunctional cell proliferation.

**Discussion**

Genetic studies have complemented and extended the observations from pharmacological approaches by establishing unequivocally a role for AR-mediated androgen actions and revealing novel insights into the specific roles of androgens in male and female reproduction. Male ARKO models have revealed that androgens play a pivotal role in the control of male fertility from spermatogenesis to the post-testicular sperm maturation through cellular and molecular
mechanisms. Future research efforts should focus on detailed characterization of the molecular mechanisms, paracrine mediators and cellular targets of AR in these models, which has the potential to lead to the identification of new effective diagnosis and treatment for male infertility, as well as providing novel neo-hormonal male contraceptive targets. In females, the study of ARKO mouse models has revealed that AR function is essential for maintaining female fertility, notably through optimizing the conditions for follicular growth, final follicle development and ovulation. Future research efforts should focus on a more detailed understanding of the role of androgens on follicle dynamics, via both intra- and extra-ovarian pathways, as well as AR targets within the uterus. These findings may point the way towards a better understanding and novel approaches to the treatment of age-related infertility as well as androgen-associated disorders, such as PCOS.

In conclusion, the genetically modified ARKO mouse models have clarified the classical concepts of the physiological role of androgens in reproductive function, while also highlighting novel and controversial concepts which require further detailed analysis to unravel the specific mechanisms. ARKO models continue to provide unique tools to further unravel the multitude of androgenic-dependent molecular mechanisms involved in regulating reproductive function, and hence in the future may lead to novel therapeutic targets for the treatment of various androgen-associated diseases and infertility.

Acknowledgements
The authors wish to thank Ms Jennifer Spaliviero for her help in the preparation of this manuscript.

Funding
The following agencies are acknowledged for their support: NHMRC of Australia, University of Sydney and Cancer Institute NSW.

References


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