

REVIEW

Androgen receptor knockout and knock-in mouse models

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Abstract

Androgens play an important role in male reproductive development and function. These steroid hormones mediate their actions by binding to the androgen receptor (AR). Diseases such as androgen insensitivity syndrome, prostate cancer, Kennedy's disease, and infertility can be caused by mutations in the AR. To get a better insight into the molecular working mechanisms of the AR, several knockout and knock-in mouse models have been developed. These models are reviewed here and are compared with human diseases.

Journal of Molecular Endocrinology (2009) **42**, 11–17

Introduction

Androgens are steroid hormones, which play an important role in the development and maintenance of male characteristics. The two most important androgens are testosterone and 5 α -dihydrotestosterone (DHT). Each of them plays a distinct role during male sexual development. Testosterone is crucial for the development and differentiation of Wolffian duct derived structures such as the epididymis, seminal vesicles, and vas deferens, whereas 5 α -DHT triggers the development and differentiation of male urogenital structures (male external genitalia, urethra, and prostate; Wilson *et al.* 1993, Nef & Parada 2000).

To mediate their actions, testosterone, and DHT bind to the intracellular androgen receptor (AR), a ligand-inducible transcription factor belonging to the nuclear receptor superfamily. The gene encoding the AR is located at position q11-12 on the X-chromosome and comprises eight exons. It encodes a protein of 919 amino acids with a molecular mass of ~110 kDa (Lubahn *et al.* 1988). The AR possesses an overall structure similar to that of the other nuclear receptors. It consists of four functional domains: an amino-terminal transactivation domain (NTD, encoded by exon 1) containing stretches of glutamine, proline, and glycine, a highly conserved central DNA-binding domain (DBD, encoded by exons 2 and 3), a hinge region (encoded by the 5' part of exon 4) and a carboxy-terminal ligand-binding domain (encoded by the 3' part of exon 4 and exons 5–8; Tsai & O'Malley 1994, Lee & Chang 2003). Liganded AR binds as a homodimer to specific DNA motifs called androgen response elements (AREs) that are situated in or near

target genes (Wang *et al.* 2007). These AREs can be divided in two classes: the classical AREs also recognized by the other steroid receptors and the selective AREs, which display selectivity for the AR (Claessens *et al.* 2001, Shaffer *et al.* 2004).

Due to the location of the AR gene on the X-chromosome and its role in male fertility, mutant AR genes affect males, whereas females carry and transmit the gene to the next generation. Malfunctioning ARs can be associated with different diseases such as prostate cancer, androgen insensitivity syndrome (AIS), Kennedy's disease, and infertility. Initial studies on androgen action involved castration and transplantation experiments combined with hormone injection (Jost 1953). More recently, molecular biological analyses lead to a more detailed view on the AR mechanism of action and on the specifics of the physiological role of androgens. However, to further the *in vivo* studies, the development of AR knockout and knock-in mouse models are crucial. Here, we review the different models and compare them with human diseases.

Comparison of complete AIS with the AR knockout mouse model

AIS is a rare X-linked recessive disorder with a prevalence of 1:20 400 male births (Poletti *et al.* 2005). In most cases, cAIS is caused by a mutation of the AR gene (Quigley *et al.* 1995). To date, about 400 different mutations (single base mutations, nucleotide insertions or deletions, intronic mutations, and partial or complete gene deletions) have

been identified in cAIS (<http://androgendb.mcgill.ca/AR23C.pdf>). These mutations lead to androgen insensitivity via different mechanisms: incomplete synthesis of the AR protein, inability of androgen binding by the AR or abnormalities in binding of the androgen–AR complex to AREs (Quigley *et al.* 1995, Gottlieb *et al.* 1999, 2004, Poletti *et al.* 2005).

The differential effect of the AR mutations results in a wide clinical spectrum of phenotypes ranging from a fully female external phenotype (complete AIS, previously known as testicular feminization (Tfm) syndrome) to an undervirilized male phenotype (partial AIS) of 46 XY individuals (Quigley *et al.* 1995). Individuals with cAIS are genetically male but exhibit a female phenotype. They have female external genitalia, small or normal clitoris and a blind ending vagina. The internal female genitals (uterus and Fallopian tubes) are absent because the secretions of the AMH during fetal life by the Sertoli cells are normal. The Wolffian duct derivatives and the prostate are absent. The undescended testes are located in the abdomen, the inguinal canal or in sacs of bilateral hernias. Development of gynecomastia is the same as in XX females and in the majority of cases axillary and pubic hair is absent (Quigley *et al.* 1995, Brinkmann 2001, Poletti *et al.* 2005). LH and plasma testosterone concentrations are elevated at puberty and in adults (Larsen *et al.* 2003).

The first animal model of androgen insensitivity was described in 1970 by Lyon and Hawkes. They reported an X-linked gene for Tfm in the mouse (Lyon & Hawkes 1970). The Tfm/Y mice carry a single nucleotide deletion in exon 1 of the AR gene, which introduces a premature termination of AR translation (Gaspar *et al.* 1991). Tfm/Y mice are infertile and their testes are smaller compared with WT mice. The testes are located in the inguinal region, comparable with some patients with cAIS (Couse & Korach 1998). These and other studies revealed that testicular descent is a biphasic process in which the second phase, the so-called inguinoscrotal migration, is androgen dependent (Hutson *et al.* 1994).

Besides this Tfm model, there are several transgenic mice models with a ubiquitous knockout of the AR (ARKO). The generation of these ARKO mouse models involves two transgenic mouse strains: a Cre transgenic mouse that expresses the recombinase ubiquitously and a mouse strain in which part of the AR gene is flanked by loxP sites (Table 1; Sauer 1998). Two ARKO mouse models were developed in which exon 2 was flanked by loxP sites (Yeh *et al.* 2002, De Gendt *et al.* 2004). These floxed AR mice were crossed with mice carrying Cre recombinase under the control of β -actin (Yeh *et al.* 2002) or under the control of the PGK promoter (De Gendt *et al.* 2004). Two other groups floxed the first exon of the AR that codes the complete AR–NTD. Matsumoto *et al.* crossed these mice with mice expressing Cre under the control of a CMV promoter (Matsumoto *et al.* 2003). The other group placed the loxP sites in opposite directions

Table 1 Summary of the five ARKO mouse models

	Floxed exon	Promotor of Cre recombinase	T levels versus WT
ARKO 1, Yeh <i>et al.</i> (2002)	Exon 2	β -Actin	↓
ARKO 2, De Gendt <i>et al.</i> (2004)	Exon 2	PGK	=
ARKO 3, Matsumoto <i>et al.</i> (2003)	Exon 1	CMV	↓
ARKO 4, Holdcraft & Braun (2004a)	Exon 1	Sycp1 and ella	/
ARKO 5, Notini <i>et al.</i> (2005)	Exon 3	CMV	↓

around exon 1. These mice were mated with Sycp1-Cre and ella-Cre mice and this resulted in mice with an inverted exon 1 (Holdcraft & Braun 2004a). In the model, developed by Notini *et al.* (2005), exon 3 (encoding the second zinc finger of the DBD) of the AR gene is floxed. Subsequently, the floxed AR mice were bred with CMV-Cre mice to generate ARKO mice in which only the third exon of the AR has been deleted. A comparable mutation is found in a pair of siblings with cAIS (Quigley *et al.* 1992).

The phenotype of the different ARKO males is comparable with the phenotype of the male Tfm mouse. They all have a female-like external appearance, testes are reduced in size and located intra-abdominally, Wolffian duct derived structures are absent, there is no vaginal opening, Fallopian tubes or uterus and LH levels are increased. Only one of the five developed ARKO mice models showed late onset of obesity (Matsumoto *et al.* 2003).

In contrast to what is observed in cAIS patients, there are always spermatogonia and early spermatocytes present in testes of Tfm mice and these mice have low levels of circulating testosterone (Lyon & Hawkes 1970, Couse & Korach 1998). The serum testosterone concentrations in ARKO males are equal or even decreased compared with WT males (Table 1). In Tfm/Y mice, gonadotropins (LH and FSH) are increased, while testosterone levels are significantly lower than in WT mice (Naik *et al.* 1984). The germ cells of the Tfm/Y mice almost never proceed beyond the spermatocyte stage. It is not clear whether this spermatogenic phenotype is caused by a defective gene action in the germ cells or by an unspecific effect of cryptorchidism (Lyon & Hawkes 1970).

Cell-specific roles of AR in the testis

Spermatogenesis is dependent on gonadotropins and testosterone. FSH is needed for the stimulation of Sertoli

cell proliferation and the initiation of spermatogenesis and together with high levels of intratesticular testosterone (produced via LH action on Leydig cells), FSH is important for the maintenance of the spermatogenic process in humans as well as in mice (Dohle *et al.* 2003). However, in mice androgens alone (low intratesticular androgen levels) can initiate spermatogenesis in the complete absence of FSH (Singh *et al.* 1995). This has been confirmed in the FSH receptor and FSH β -subunit knockout mice, which display normal fertility, albeit with smaller testis and reduced germ cell numbers (Kumar *et al.* 1997, Huhtaniemi & Aittomaki 1998, Abel *et al.* 2000, Wreford *et al.* 2001). Mutations in the AR gene in humans can also lead to defective spermatogenesis (Yong *et al.* 2003, Zuccarello *et al.* 2008). Spermatogenesis in the ARKO mice is arrested at the pachytene spermatocyte stage, but it is unclear whether this is a direct effect of the knockout or of the abdominal location of the ARKO testes (Johnston *et al.* 2001, Holdcraft & Braun 2004b). In the testis, we can distinguish somatic cells like Sertoli cells, Leydig cells and peritubular myoid cells, which are all AR positive (Bremner *et al.* 1994, Vornberger *et al.* 1994, Zhou *et al.* 2002). Whether germ cells express the AR is still controversial but mice with a germ cell selective knockout of the AR have normal spermatogenesis (Tsai *et al.* 2006). Using the spermatogonial stem cell transplantation technique, Johnston *et al.* (2001) concluded that testicular germ cells from the Tfm mice can complete spermatogenesis when they are transplanted in the seminiferous tubules of azoospermic mice expressing functional ARs. Comparison of Tfm mice with wild-type mice, however, showed that gonocytes, the precursors of the germ cells, are AR positive, and their numbers seem to be controlled by androgens (Merlet *et al.* 2007).

Sertoli cells are generally believed to be the primary mediators of the androgen regulation of spermatogenesis because they provide physical and nutritional support to the developing germ cells (Griswold 1998). To examine this hypothesis, three mouse models in which the AR is knocked out selectively in Sertoli cells have been reported (Chang *et al.* 2004, De Gendt *et al.* 2004, Holdcraft & Braun 2004a). In the first study, mice with a floxed exon 2 of the AR were crossed with mice expressing the Cre recombinase driven by the AMH promoter (AMH-Cre), which is selectively expressed in Sertoli cells (De Gendt *et al.* 2004). In comparison with ARKO males, Sertoli cell selective AR knockout (SCARKO) males show a normal external male appearance and have normal internal male genital tracts. The SCARKO males have fully descended testes but the size of the testis is markedly reduced. Despite the normal Sertoli cell nuclear volume per SCARKO testis, spermatogenesis appears to be blocked in meiosis since spermatocytes, round spermatids, and elongated spermatids are reduced to 64, 3, 0% respectively (De Gendt *et al.* 2004, Tan *et al.* 2005). Because SCARKO testes

contain normal number of Sertoli cells we can conclude that the effect of androgens on the number of Sertoli cells is mediated by a mechanism that does not involve functional AR in Sertoli cells themselves (Tan *et al.* 2005). More detailed analysis of the SCARKO testes revealed the role of the AR in the maintenance of the cell-cell contacts between the Sertoli cells and the developing germ cells (Denolet *et al.* 2006). A second mouse model with a Sertoli cell specific ablation of the AR (S-AR^{-/-y}) was obtained with the same strategy as in De Gendt *et al.* (Chang *et al.* 2004, Tsai *et al.* 2006). The S-AR^{-/-y} phenotype is comparable with the SCARKO phenotype but spermatogenesis is arrested in the premeiotic diplotene spermatocyte stage in S-AR^{-/-y} and S-AR^{-/-y} mice have decreased testosterone and increased LH serum levels. By contrast, the concentration of these hormones in SCARKO males is comparable with WT males. In another model, the first exon of the AR is flanked with inverted loxP sites and a neomycin resistance cassette is introduced in intron 1 (Holdcraft & Braun 2004a). This cassette seems to create a hypomorphic allele of the AR gene (Meyers *et al.* 1998). These hypomorphic animals (Ar^{flox(ex1+neo)/Y}) were mated with a different AMH expressing mouse strain than used by the other two groups to generate a partial Sertoli cell specific ARKO (Ar^{flox(ex1+neo)/Y}; AMH-Cre). Spermatogenesis in Ar^{flox(ex1+neo)/Y}; AMH-Cre males is not blocked as in SCARKO and S-AR^{-/-y} but a defect occurs during the transition of round to elongated spermatids. Possibly, this discrepancy is caused by the flox-induced phenotype of the Ar^{flox(ex1+neo)/Y}.

A recent mouse model lacking FSHR and AR on Sertoli cells (FSHRKO-SCARKO) was developed to determine the compounded effects of these hormones on Sertoli cell function and spermatogenesis. Meiosis in these animals is initiated, but the completion of meiosis is absolutely dependent on the expression of the AR (Abel *et al.* 2008).

The AR is also expressed abundantly in the mature Leydig cells. Knocking out the AR selectively in Leydig cells by Amhr2 driven Cre strategy (L-AR^{-/-y}) results in mice with decreased size of testis and epididymis, reduced serum levels of testosterone and increased serum levels of LH and FSH. This indicates the feedback of testosterone synthesis and AR expression in the Leydig cells and FSH and LH expression in the pituitary. It is important to note that the AR knockout in Leydig cells in this model was not complete. LAR^{-/-y} mice are infertile and spermatogenesis is arrested predominantly at the round spermatid stage (Tsai *et al.* 2006, Xu *et al.* 2007). A selective ablation of the AR in Sertoli cells also has consequences for the development of normal Leydig cell numbers. Compared with controls, SCARKO mice have a normal or increased Leydig cell size, while in ARKO mice the size of the Leydig cells is reduced (De Gendt *et al.* 2005).

Peritubular myoid cells are the AR positive cells surrounding the seminiferous tubules in the testis. These cells may play an indirect role in spermatogenesis through the control and maintenance of the Sertoli cell function as well as in the transport of spermatozoa through the tubular lumen by contractions of the seminiferous tubules (Maekawa *et al.* 1996, Romano *et al.* 2005). Indeed, peritubular myoid cell selective AR knockout mouse have a decreased testis size and oligozoospermia, but the fertility is normal (Zhang *et al.* 2006).

From the results of the AR knockout models we can conclude that the AR has specific roles in different cell types of the testis. Activity of the AR plays an important role during at least three steps of spermatogenesis: first, the AR is necessary for progression through meiosis I, secondly, the transition from round to elongated spermatids requires AR activity, and finally AR is important during the terminal stages of spermiogenesis (Fig. 1; Holdcraft & Braun 2004b, Xu *et al.* 2007).

AR and knock-in mouse models

The *in vitro* molecular study of the AR has provided many new insights into its action mechanism in normal and pathological conditions that urgently need verifications in *in vivo* settings (Claessens *et al.* 2008). For this, several 'knock-in' strategies have been developed. This technique was used to develop a mouse model for Kennedy's disease. Kennedy's disease or spinal and bulbar muscular atrophy (SBMA) is caused by an abnormal expansion of a CAG triplet repeat in the first exon of the AR gene, corresponding to a polyglutamine stretch in the NTD. This disease is characterized by proximal muscle weakness, fasciculations, and atrophy. Some patients display additional clinical features like androgen insensitivity, severe oligozoospermia or azoospermia, testicular atrophy, feminized skin changes, and gynecomastia (Arbizu *et al.* 1983, Casella *et al.* 2001). Different mouse models are developed in which exon 1 of mouse AR was swapped with human exon 1 and during this process 12, 21, 48 or 113 CAG repeats were inserted (Albertelli *et al.* 2006, Yu *et al.* 2006b). The first three mouse models show comparable growth, behavior, reproductive tract morphology, and fertility as WT littermates. However, AR113Q mice develop hormone-dependent neuromuscular pathology. Mutant males, aged between 3–5 months, develop muscle pathology and only at 24 months the spinal cord pathology can be detected. Eighty percent of the males die at 2–4 months of age. This early death is dependent upon circulating testosterone and is probably caused by acute urinary tract obstruction. This obstruction is caused by altered muscle membrane excitability in the lower urinary tract of AR113Q males (Yu *et al.* 2006a). The weight of the testes of AR113Q males is reduced to

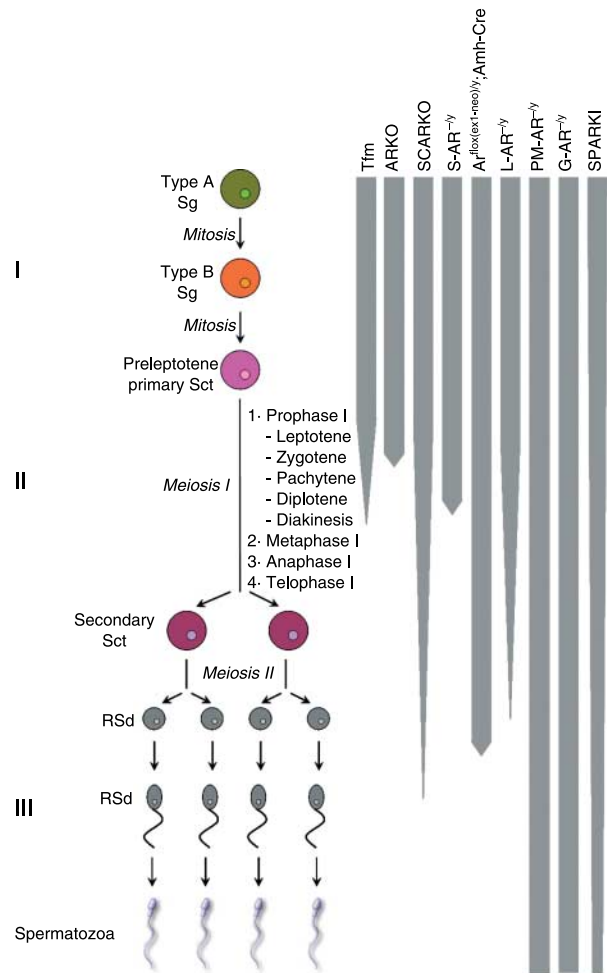


Figure 1 Schematic of spermatogenesis and the different knock-out and knock-in mouse models. Spermatogenesis can be divided into three phases: (I) proliferative phase, (II) meiotic phase, and (III) spermiogenesis (Sg, spermatogonia; Sct, spermatocytes; RSd, round spermatids; ESd, elongated spermatids).

50% of WT testes and they have decreased fertility. Serum testosterone levels are comparable with WT, serum LH levels are elevated and serum FSH levels are decreased. These observations show that AR113Q males display signs of partial androgen insensitivity that commonly occur in Kennedy's disease patients. The testicular pathology in the AR113Q male mice progresses with increasing age and is characterized by abnormal germ cell maturation (large multinucleated cells, decrease of epididymal sperm and increase of TUNEL-positive cells) and abnormalities of the Sertoli cell cytoskeleton. The solubility of the mutant AR in protein lysates is decreased and the mutant AR protein forms complexes in testes but these complexes could not be detected by conventional morphological methods. Yu *et al.* (2006b) concluded that the testicular phenotype is mediated by toxic effects of the mutant AR protein rather than a partial loss of AR

function, however; hypothalamic dysfunction or changed levels of estrogens or inhibin can also contribute to the testicular pathology.

AR knock-in mouse models are not only developed to get a better insight into diseases like SBMA but also to study the normal working mechanism of the AR. The androgen-AR complex can bind as a dimer to classical AREs (recognized by the other steroid receptors) or selective AREs (not recognized by the glucocorticoid receptor, GCR). To investigate, the *in vivo* relevance of these selective AREs, a knock-in mouse model, called specificity affecting AR knock-in (SPARKI), was developed in which the second zinc finger of the DBD and the first three amino acids of the carboxy terminal extension (encoded by exon 3 of the AR gene) are swapped with that of the GCR (Schauwaers *et al.* 2007). *In vitro* analysis showed that the SPARKI-AR lost its ability to bind to selective AREs, but still can bind classical AREs. Phenotypically, SPARKI males have normal body weights and the amount of body fat, muscle, and bone is comparable with WT males indicating that selective AREs are not essential for the anabolic effects of androgens. The reproductive organs of SPARKI males are reduced in size and weight and they are subfertile. They have normal levels of total testosterone and gonadotropic hormones, which suggest that the androgen responsiveness of the pituitary feedback system is unaffected. SPARKI testes have reduced Sertoli cell numbers, which is also seen in ARKO males but not in SCARKO males suggesting that androgen effects on Sertoli cell number are mediated by a process dependent on selective AREs but outside of the Sertoli cell. Meiosis in SPARKI testes is not completely blocked but the number of round and elongated spermatids are reduced by respectively 47 and 44%, an indication of an effect at the same stage as in ARKO (Fig. 1). As expected, androgen regulated genes are differentially affected by the SPARKI mutation. The expression of *Rhox5*, aquaporin-5, and *Rad9*, genes that contain selective AREs, is reduced in SPARKI testes, while the expression of the *Spinl1* gene, controlled by a classical ARE, is not affected (Schauwaers *et al.* 2007, Moehren *et al.* 2008). These results indicate that the *in vitro* classification of AREs based on *in vitro* data has *in vivo* relevance. Similarly, other AR-specific functions, such as the N/C interactions, the co-activator recruitment to its NTD should now be verified *in vivo* by similar knock-in mouse models.

Androgen reporter mice

Androgen reporter mice are mice with androgen-responsive enhancers cloned upstream of reporter genes inserted in their genome. Prostate-specific models e.g. have been made using *hK2*, PSA, and rPB

regulatory elements, which allow the study of prostate specific gene expression (Seethammagari *et al.* 2006). However, systemic analysis of the androgen response *in vivo*, analogous to the estrogen reporter mice, is hampered by the fact that AR, GCR, PR, and MR all recognize very similar response elements (Ciana *et al.* 2003, Lemmen *et al.* 2004). The more selective AREs might provide a solution (Claessens *et al.* 2001). Such androgen reporter mice would be very useful for the *in vivo* study of the androgen response, the role of co-activators, possible crosstalk with other signaling pathways and even pharmacological studies.

In the mean time, an AR activity indicator (ARAI) mouse was made (Ye *et al.* 2005). In the ARAI mice, exons 2 and 3 encoding the AR-DBD are swapped with a GAL4 DBD cassette. This modified AR can target a reporter gene driven by GAL4 upstream activation sequences. Cross-breeding of this transgenic mouse with knockout models of the steroid receptor co-activator 1 and 2 showed that AR activity in the testis was decreased by loss of steroid receptor co-activator (SRC)-2, while ablation of SRC-1 showed surprising increase of AR activity. Experiments like these are much needed for the *in vivo* study of the many AR action mechanisms that have been proposed mainly on the basis of *in vitro* data.

Declaration of interest

The authors have nothing to disclose. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This work was supported by grants from the 'Bijzonder Onderzoeksfonds' of the University of Leuven and the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen'.

Acknowledgements

We wish to thank all members of the Molecular Endocrinology Laboratory for interesting discussions and helpful suggestions. A H is a postdoctoral fellow of the 'Fonds voor Wetenschappelijk Onderzoek-Vlaanderen'.

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Received in final form 10 October 2008

Accepted 15 October 2008

Made available online as an Accepted Preprint 15 October 2008