Genomic actions of the androgen receptor are required for normal male sexual differentiation in a mouse model

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Abstract

Androgens mediate their effects in target cells via the androgen receptor (AR), which acts predominantly as a ligand-dependent transcription factor. In addition, androgens induce rapid activation of second messenger signal transduction cascades, and this is thought to occur via non-genomic mechanisms. We have used the Cre/loxP system to generate an AR knockout (ARKO) mouse targeting exon 3, which encodes the second zinc finger of the DNA-binding domain. To generate universal ARKO mice, floxed AR mice were mated with CMV-Cre mice, which express Cre recombinase ubiquitously. Deletion of the floxed allele in our mice does not disrupt the reading frame, and has been designed so that the mutant AR can bind ligand but not target genes. ARKO males displayed a complete androgen insensitivity phenotype, with female external genitalia and a reduction in body weight compared with wild-type males ($P<0.001$). Testes of ARKO males were smaller than control males ($P<0.0001$) and were located intra-abdominally. We have demonstrated that genotypically XY mice lacking the second zinc finger of the AR have a female phenotype, and we conclude that the genomic actions of the AR (mediated by DNA binding) are indispensable for normal male sexual differentiation.

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Introduction

Androgens (testosterone and 5α-dihydrotestosterone) play an essential role in male sexual differentiation and development. They mediate their effects in target cells predominantly by interacting with the androgen receptor (AR). Like other steroid hormone receptors, the AR consists of an N-terminal hypervariable domain, a DNA-binding domain (DBD) and a ligand-binding domain (LBD) (MacLean et al. 1997). Inside target cells, androgens bind to the LBD, resulting in dissociation of inhibitory receptor-associated proteins. Once activated, the AR undergoes dimerisation, phosphorylation, nuclear translocation and DNA binding to androgen-response elements located within target genes to activate or repress transcription (Brinkmann et al. 1999). We define this action of the AR as genomic. Androgens can also rapidly induce second messengers, such as kinase signalling cascades. Some effects of androgens have been observed in the presence of inhibitors of transcription and translation in vitro. These responses, however, occur too rapidly to involve changes in target gene transcription and are therefore regarded as non-genomic (Kousteni et al. 2001, Heinlein & Chang 2002). It is not clear whether non-genomic signalling of androgens occurs through cell-surface receptors or the classical AR.

The AR gene is a single copy gene located on the long arm of the X chromosome. Mutations in this gene result in failure of AR-mediated signalling, leading to end organ resistance to androgens, otherwise known as the androgen insensitivity syndrome (AIS). Affected individuals with a 46,XY karyotype typically present with incompletely descended testes that secrete normal or even high levels of testosterone and female external genitalia (MacLean et al. 1995, Quigley et al. 1995).

The first murine model of AIS was described in 1970 and is known as the testicular feminised male ($Tfm$) mouse (Lyon & Hawkes 1970). XY $Tfm$ mice have female external genitalia due to a naturally occurring, single-point mutation in the N-terminal domain of the AR gene, resulting in a non-functional AR protein (Gaspar et al. 1990, Charest et al. 1991). These mice are totally insensitive to androgens. $Tfm$ mice are infertile; therefore, it is not possible to produce a genetically female (XX) AR-null mouse.

Conventional gene targeting in mice involves generating null mutations in a gene of interest and studying the consequences of gene inactivation. The major limitation associated with this approach is that the targeted gene is knocked out in all cells and during all stages of development, as occurs with the AR in the $Tfm$ mouse. This limitation may be overcome by using a conditional gene-targeting approach such as the Cre/loxP system, which allows deletion of genes in a cell-, tissue- and/or time-specific manner (Cohen-Tannoudji & Babinet 1998, Sauer 1998).
We have generated an AR knockout (ARKO) mouse model with the Cre/loxP system. Two mouse lines are required for this system, a loxP-flanked (floxed) target line and a Cre recombinase-expressing line. Here we describe the generation of an AR floxed line and a universal ARKO line, using a mouse that expresses Cre ubiquitously. We have targeted exon 3 of the AR gene, which is highly conserved among species and encodes the second zinc finger of the DBD (Faber et al. 1991). The deletion has been designed in order to retain the normal reading frame and so that the mutant AR is able to bind ligand but unable to bind DNA and regulate target gene transcription. The rationale for this design is based on the existence of human AIS patients with an in-frame deletion of the third exon of the AR gene (Quigley et al. 1992). We predict that the mutant AR will retain the ability to elicit any non-genomic effects mediated via the AR.

Materials and methods

Floxed AR targeting vector

A genomic DNA fragment containing the third exon of the AR was cloned from a 129 StJ mouse genomic library (Stratagene, La Jolla, CA, USA). The clone was characterised by restriction mapping and sequencing. Two fragments of this clone, a 6·6 kb EcoRI-HindIII fragment and a 5·9 kb BamHI-KpnI fragment that contained the third exon, were subcloned into pBluescript KS+ (Stratagene). An oligonuclease containing a 34 bp loxP site was inserted into an EcoRI site 550 bp upstream of exon 3, and a 1·9 kb floxed neomycin resistance (Pgr-neo) cassette was cloned into a KpnI site 466 bp downstream of the same exon. The Pgr-neo cassette was flanked by loxP sites, to allow removal by Cre prior to blastocyst injection, if required. Finally, a 5·5 kb thymidine kinase (TK) cassette was cloned downstream of the 3’-flanking arm, to allow negative selection of non-homologous recombinants. The plasmid containing the Pgr-neo cassette was a kind gift from Dr Shelley Ross (Department of Medicine, Monash Medical Centre, Clayton, Australia).

ES cell targeting and PCR screening

The targeting vector was linearised at the 5’-end with NotI and electroporated into 129 StJ mouse embryonic stem (ES) cells. ES cells were subjected to positive and negative selection with 175 µg/ml Geneticin (Gibco Invitrogen, Grand Island, NY, USA) and 200 nM gancyclovir (Roche) respectively. Approximately 200 ES colonies were isolated and screened for homologous recombination events by PCR, using primer pairs consisting of one vector-specific primer and one endogenous AR-specific primer. PCR was performed to ensure correct integration of the targeting vector at both the 5’- and 3’-ends. The primer pair sequences are as follows: 5’-end screening, 5’-GGCCTCATGTGACCTT CCATTAT-3’ and 5’-CGCATGCTCCAGACTGC CTT-3’; 3’-end screening: 5’-GCTTGAAGAACGAGA TCAGCA-3’ and 5’-CTAGCATGGTCTTGGGAC AAT-3’. PCR conditions are available upon request.

Floxed AR mouse line

One targeted ES cell clone was expanded and injected into C57BL/6J-derived blastocysts by IngenKO Pty Ltd (Monash Institute of Reproduction and Development, Monash Medical Centre, Clayton, Australia). Three high-percentage male chimeras were identified, and two were backcrossed to C57BL/6J female mice, resulting in germline transmission. As the phenotype of knockout mice can vary depending on their genetic strain (Linder 2001), we backcrossed our floxed AR mice onto a C57BL/6J background for at least six generations (>98% C57BL/6J) prior to use in experiments. All C57BL/6J mice were obtained from the Animal Resources Centre, Canning Vale, WA, Australia. All mice had ad libitum access to water and standard chow (Barastoc GR2 rat and mouse breeder ration, Ridley Agriproducts, Packenham, Australia), and were housed at 22 °C with a 12-h light/dark cycle in standard cages. All procedures involving animals were approved by the Austin Health Animal Ethics Committee.

Universal ARKO mouse line

To generate universal ARKO mice, we obtained CMV-Cre transgenic mice on a C57BL/6J background with permission from Dr Ursula Lichtenberg (Institute for Genetics, University of Cologne, Germany). These mice express Cre recombinase ubiquitously under the control of the CMV promoter (Schwenk et al. 1995). The CMV-Cre transgene is X-linked; therefore, two breeding steps were required to generate ARKO male mice. The first step involved breeding floxed AR mice with CMV-Cre mice to generate heterozygous ARKO females. To generate ARKO males, it was necessary to breed heterozygous ARKO females with wild-type males.

Genotyping

Approximately 100 ng genomic DNA isolated from tail biopsy was used as a template for PCR genotyping. The primer pair sequences used for genotyping mice were as follows: mouse SRY coding sequence (cds) 5’-AGAGAT CAGCAAGCGAGCTGG-3’ and 5’-TCTTTGCTGTA TGTTAGTGGC-3’; Neo cds 5’-GGCGCCCGGTTTCT TTGTTGCAA-3’ and 5’-GGCTTGGCGGAGCCCC TGAT-3’; Cre cds 5’-GGGCCATGCTGCAAAGTT
GAAT-3′ and 5′-ACCCCAAGGCTAAGTGCCCTT-3′; mouse exon 3 5′-CTTCTTCTAGGGAAACAGAAGT-3′ and 5′-CTCCCAAGTCATCCCCGTGCT-3′; and primers flanking floxed AR region 5′-GGGAGACACAGGATAGGAATT-3′ and 5′-GAGTGGGACCACATCTTCATTTC-3′. PCR conditions are available upon request.

**RNA isolation and RT–PCR**

Total RNA was isolated from the kidneys of three ARKO and three wild-type male mice at 8 weeks of age by standard methods. mRNA was reverse transcribed with M-MLV reverse transcriptase according to the manufacturer’s instructions (Promega). The resulting cDNA was subjected to PCR with the following AR-specific primer pairs: exon 2, 5′-GACAGTACC AGGACCATGTT-3′ and exon 4, 5′-CTCAATGGGCTTCCAGGACGGT-3′.

**Western blot analysis**

Kidneys were lysed with RIPA buffer (1% Nonidet P-40, 0·5% sodium deoxycholate, 0·1% SDS in 1 PBS) containing complete protease inhibitor cocktail (Roche). An amount of 60 µg protein lysates was fractionated on a 7% SDS–PAGE gel and transferred to an Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA). Membranes were first probed with a polyclonal rabbit anti-human AR antibody (1:400, C-19, Santa Cruz Biotechnology), Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA, USA) and then with a goat anti-rabbit IgG–HRP-conjugated secondary antibody (1:3000, Santa Cruz, CA, USA) and visualised using发光成像系统 (BioSciences, Model 8200). Bands were quantified using Multi Gauge software (FujiFilm, Tokyo, Japan).

**Serum testosterone measurements**

At 9 weeks of age, mice were anaesthetised (ketamine 80 mg/kg; xylazine 80 mg/kg i.p.), and blood was collected by cardiac puncture. Serum testosterone was measured in duplicate by radioimmunoassay as described previously (Singh et al. 1995).

**Histology**

Both testes from 8-week-old males were excised and weighed. Wild-type and ARKO male testes were fixed in 4% paraformaldehyde for 24 h at 4 °C and embedded in paraffin wax, and 4 µm sections were cut for histology. Sections were stained with hematoxylin and eosin.

**Statistical analysis**

Total body, testes and seminal vesicle weight was analysed by one-way ANOVA. A value of *P*<0·05 was considered significant. Tukey’s post hoc test was employed to identify significant differences between mean values. If, however, Levene’s test of equality of error variances was less than 0·1, indicating that the variance was unequal, Tamhane’s post hoc test was used. All tests were performed with SPSS 11 for Mac OS X software (SPSS Inc., Chicago, IL, USA).

**Results**

**Generation of universal ARKO mice**

After transfection of the targeting vector (Fig. 1A) into ES cells, 16 ES cell clones were identified as positive for homologous recombination by PCR at both the 5′- and 3′-ends (Fig. 1B). Blastocyst injection of targeted ES cells resulted in the generation of three high-percentage chimeras, which were backcrossed to female mice to achieve germline transmission. Female offspring heterozygous for the floxed AR allele were bred with male C57BL/6J mice, resulting in fertile male mice hemizygous for the floxed AR allele. To generate universal ARKO mice, floxed AR mice were bred with X-linked CMV-Cre mice, which express Cre recombinase ubiquitously (Schwenk et al. 1995). Offspring included heterozygous ARKO female mice, which were bred with C57BL/6J mice to produce ARKO males.

**Genotyping of ARKO mice**

All phenotypically female offspring were screened for the presence of the Y-linked SRY gene by PCR. SRY-positive females were confirmed positive for Cre-mediated deletion of the floxed AR allele in genomic DNA isolated from tail (Fig. 2A), kidney, skeletal muscle, liver, heart and spleen (data not shown) with intronic primers that flank the floxed AR region (represented schematically in Fig. 2B). We were unable to amplify a band corresponding to exon 3 (Fig. 2C) or the Neo cds (Fig. 2D) in ARKO male mice, verifying that Cre-mediated deletion of the floxed allele was complete.

**Molecular analysis of mutant AR**

RT–PCR on total kidney cDNA with exonic primers which flank the floxed region of the AR demonstrated a PCR product approximately 117 bp smaller than the wild-type band (Fig. 3A). Western analysis of total kidney protein lysates confirmed expression of AR protein in wild-type band (Fig. 3A). If, however, Levene’s test of equality of error variances was less than 0·1, indicating that the variance was unequal, Tamhane’s post hoc test was used. All tests were performed with SPSS 11 for Mac OS X software (SPSS Inc., Chicago, IL, USA).

**Phenotypic characterisation of ARKO male mice**

AKO male mice displayed a complete AIS (cAIS) phenotype, with female external genitalia (Fig. 4A).
Eight-week-old ARKO males had a lower body weight than wild-type or floxed AR male controls \((P<0.001)\) and a higher body weight than CMV-Cre female controls \((P<0.05)\) (Fig. 4B). The mean body weight of ARKO males was lower than wild-type males at 10–25 weeks of age \((P<0.05)\) (Table 1).

Gross anatomical internal examination revealed that ARKO male mice had small, intra-abdominal testes (Fig. 5A and B) and no detectable prostate or seminal vesicles (Fig. 5A). At 8 weeks of age, ARKO males had significantly reduced testis mass compared with wild-type and floxed AR male controls \((P<0.0001)\) (Fig. 5C). The testis masses of wild-type and floxed AR males were not significantly different. ARKO males had no detectable seminal vesicles; however, seminal vesicle mass was the same in wild-type and floxed AR males (wild-type: 185±0 ± 10.4 mg; floxed AR: 169±1 ± 10.4 mg) at 8 weeks of age. Serum testosterone levels...
were reduced in ARKO males compared with wild-type ($P<0.05$), floxed AR and CMV-Cre male controls; however, the last two did not reach statistical significance (Fig. 5D).

Histological analysis of the testis confirmed that testicular size was reduced in ARKO males compared with wild-type males. The seminiferous tubules in the ARKO testis were smaller in diameter with a substantial reduction in the total number of germ cells (Fig. 6). The number of spermatogonia appeared to be similar in

Figure 2 Characterisation of Cre-mediated deletion of floxed AR allele in universal ARKO male mouse. (A) Electrophoresis of PCR products using primers depicted in panel B. The floxed AR PCR product (LOX) is 3195 bp, the wild-type PCR product (WT) is 1295 bp and the ARKO PCR product is 178 bp. (B) Schematic diagram of WT, LOX and ARKO alleles, showing location of primer pairs used for detecting Cre-mediated recombination. (C) Electrophoresis of PCR products using exon 3 primers. (D) Electrophoresis of PCR products using Neo cds primers.

Figure 3 Characterisation of mutant AR mRNA and protein in universal ARKO male mouse. (A) RT–PCR using kidney cDNA from wild-type (WT) ($n=3$) and ARKO males ($n=3$) with AR-specific primers located in exons 2 and 4. The WT PCR product is 426 bp; the ARKO PCR product is 309 bp. (B) Western blot analysis of kidney protein lysates (60 µg loaded per lane) from WT and ARKO males ($n=3$), with a specific C-terminal anti-AR antibody.

Figure 4 Phenotype analysis of universal ARKO male mice. (A) External genitalia of wild-type (WT) male, ARKO male and WT female mice. (B) Body weights of WT male, floxed AR (LOX) male, ARKO male and CMV-Cre (CRE) female mice. *$P<0.001$ versus WT and LOX male mice; *$P<0.05$ versus CRE female mice. Values are mean±S.E.M. ($n=8$).
ARKO and wild-type mice, although this was not formally counted. The tunica propria was more prominent in the ARKO testis, with two to three layers of cells sometimes visible compared with only one layer of cells visible in the wild-type testis. Spermatogenesis was incomplete in the ARKO testis and probably arrested at meiosis I, as primary spermatocytes were readily observed, but not secondary spermatocytes, spermatids or spermatozoa. Within the interstitium, Leydig cells were present in similar numbers in both the ARKO and wild-type testes, but interstitial connective tissue appeared to be more prominent in ARKO testes.

Discussion

Rationale for targeting exon 3

To generate our ARKO mouse model, we chose to target exon 3 of the AR gene. Exon 3 encodes the second zinc finger of the DBD of the AR protein and plays a critical role in orienting the receptor for DNA binding and stabilising protein–DNA interactions (Berg 1989). Furthermore, natural deletion of this exon in man causes cAIS (Quigley et al. 1992), indicating that the second zinc finger is vital for the normal function of the AR. Mutant AR from human subjects with deletion of this exon showed supranormal levels of androgen binding in genital skin fibroblasts, localised to the nucleus, but had markedly reduced DNA-binding affinity. When monkey kidney CV1 cells were cotransfected with a plasmid containing mutant AR cDNA and a reporter plasmid containing androgen-response elements and the chloramphenicol acetyl transferase (CAT) gene, the mutant AR was unable to induce CAT activation and was therefore deemed to be transcriptionally inactive (Quigley et al. 1992).

Steroid hormone receptors are also capable of ligand-dependent negative regulation of transcription, although the mechanisms involved are not as well understood as the mechanisms of ligand-dependent activation. A number of distinct modes of action have been proposed to explain negative regulation of target gene transcription by the steroid hormone receptors.

One mechanism involves ligand-bound receptor binding to DNA sequences known as negative hormone-response elements. This is followed by recruitment of corepressors and histone deacetylases and results in reduced levels of...
transcription (Sasaki et al. 1999). Another proposed mechanism involves ligand-bound receptor binding to other transcription factors (that is, protein–protein interaction) without binding DNA (Gottlicher et al. 1998). This interaction causes the removal of coactivators which normally act to enhance target gene transcription and also results in negative gene regulation.

The importance of DNA-binding-independent transcriptional activities of the glucocorticoid receptor (GR) have been demonstrated in a DNA-binding-defective mutant version of the GR in a mouse model. In this model, the GR was unable to dimerise and therefore unable to activate target genes; however, it retained functions that require cross-talk with other transcription factors, such as repression of AP-1-driven genes (Reichardt et al. 1998). Similarly, our ARKO model will be useful in assessing the relative importance of AR DNA-binding-dependent and -independent mechanisms of AR function. To date, this has not been widely studied and remains poorly understood.

**Generation of universal ARKO mice**

The first step in the creation of the universal ARKO mouse model with the Cre/loxP system involved generating a floxed AR mouse line by embryonic stem cell technology. In theory, the introduction of loxP sites and the Pgk-neo cassette into intronic regions or untranslated regions (UTRs) of the genome of floxed mice should not interfere with normal gene function, and therefore floxed mice should appear to be phenotypically normal. Since a functional AR is required for fertility (Aiman et al. 1979), and our hemizygous floxed AR males are fertile, we are confident that our manipulation of the AR gene has not had a major effect on gene expression or protein function. In addition, we found that seminal vesicle mass (a biomarker of androgen activity) was the same in wild-type and floxed AR males at 8 weeks of age, providing further evidence that the floxed AR allele does not disrupt normal sexual differentiation. The second step in generating our ARKO model involved breeding floxed AR mice with CMV-Cre mice. Given that universal ARKO males were expected to have a female external phenotype, all female progeny were genotyped by PCR for the SRY gene. We confirmed deletion of exon 3 of the AR in SRY-positive female mice by PCR using primer pairs that amplify exon 3, part of the Neo ccds and the recombined AR allele.

**Molecular analysis of mutant AR**

RT–PCR of total kidney cDNA isolated from ARKO male mice resulted in a product that was approximately 117 bp smaller than the expected wild-type band, indicating that exon 3 had been deleted and that the mutant AR transcript is not highly labile. Since deletion of exon 3 results in a predicted loss of 39 amino acids, wild-type and mutant AR protein could not be distinguished on the basis of size by western blot analysis. Our finding that the mutant AR protein is expressed in the kidneys of our ARKO model contrasts with other published universal ARKO mouse models, which have demonstrated significantly reduced (Holdcraft & Braun 2004) or complete absence of AR protein (Yeh et al. 2002, Kawano et al. 2003, De Gendt et al. 2004).

**Phenotypic characterisation of ARKO male mice**

We observed that the body weight of our ARKO male mice is intermediate between normal male and female control littersmates, and this result agrees with other studies that have examined the body weight of Tfm mice (Tanaka et al. 1994) and Tfm rats (Martha et al. 1989, Vanderschueren et al. 1994). Unlike another, previously published ARKO model (Sato et al. 2003), our ARKO males did not become heavier than wild-type control males with age. It is difficult to make direct comparisons between these studies, since the previously published model targeted exon 1 of the AR gene for deletion, resulting in the complete absence of AR protein. Furthermore, the previous study did not state the genetic background of the ARKO mouse line.

As expected, ARKO males had small testes, located intra-abdominally. Testis mass was reduced by approximately 95% in ARKO males compared with wild-type and floxed AR control males. This finding agrees with other ARKO models (Yeh et al. 2002, De Gendt et al. 2004).

We observed that serum testosterone levels were reduced in ARKO males compared with wild-type control males but were not statistically different from floxed AR or CMV-Cre males. It is difficult to measure accurately serum testosterone levels in mice, as levels fluctuate greatly, probably because adult mice do not express detectable levels of plasma sex hormone-binding globulin (SHBG) (Janne et al. 1998). In Tfm mice, serum testosterone levels are low due to a deficiency in the steroidogenic
enzyme 17α-hydroxylase (Murphy & O'Shaughnessy 1991). Loss of 17α-hydroxylase activity is thought to be due to the intra-abdominal location of the testes and also in part to the loss of receptor-mediated androgen action (Murphy & O'Shaughnessy 1991).

Histological analysis of the testis demonstrated that spermatogenesis was incomplete in ARKO male mice. These findings agree with previous studies showing that a functional AR in Sertoli cells is required for the maintenance of spermatogenesis (Chang et al. 2004, De Gendt et al. 2004, Holdcraft & Braun 2004). Furthermore, our results demonstrate that the genomic actions of the AR are required for the development of mature spermatids.

In conclusion, we have generated an ARKO mouse model in which only exon 3 of the AR has been deleted. We have demonstrated that this mutation does not affect AR transcript or protein stability, and the mutation has been designed so that the mutant AR retains the ability to bind ligand and translocate to the nucleus, but is unable to regulate the transcription of target genes. We have shown that deletion of the second zinc finger of the AR results in complete resistance to androgens; therefore, the genomic actions of the AR are crucial for male sexual differentiation. We plan to breed our floxed AR line with other Cre recombinase-expressing lines in order to generate cell-specific ARKO models. These models will be powerful tools for differentiating the DNA-binding-dependent and -independent activities of the AR in a wide variety of cells. We hypothesise that our mutant version of the AR may still retain the ability to elicit non-genomic actions of androgens, but this requires further investigation.

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