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# Androgen receptor functions from reverse genetic models☆

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**Abstract**

The androgen receptor (AR) is a ligand-dependent transcription factor involved in the regulation of many different physiological processes. AR dysfunction causes a diverse range of clinical conditions, including testicular feminization mutation (Tfm) syndrome, prostate cancer, and motor neuron disease (Kennedy's disease). However, due to lack of genetic models, the molecular basis of the AR in these disorders remains largely unknown. Using a conditional targeting technique based on the Cre-loxP system, we successfully generated null AR mutant (ARKO) mice. ARKO males exhibited normal healthy growth, but showed typical Tfm abnormalities. Hormonal assay of ARKO males revealed that while serum androgen levels were very low, estrogen levels were normal. Another hallmark of ARKO males was late-onset obesity, with marked accumulation of white adipose tissue. To clarify the role of human AR (hAR) mutants with expanded polyQ stretches as observed in neurodegenerative disease, we also established a *Dorsophila* model in which either wild-type or polyQ-expanded hAR were ectopically expressed. Although no overt phenotype was detected in adult fly-eye neurons expressing mutant hAR, the ingestion of androgen caused marked neurodegeneraton.


*Keywords:* Androgen receptor (AR); Androgen receptor knockout mouse (ARKO); Cre-loxP system; *Drosophila*-eye model; Kennedy's disease; PolyQ repeat; Testicular feminization mutation (Tfm)

## 1. Introduction

The androgen receptor (AR) plays an essential role in a variety of biological processes, not restricted to male reproductive functions such as Wolffian duct development and spermatogenesis [1,2]. Testosterone and its more potent metabolite, dihydrotestosterone, act as ligands for AR, and liganded AR activates the target gene expression at transcriptional level. Liganded AR form homodimers and bind specific DNA elements referred to as androgen responsive elements (*ARE*) in target gene promoters [3,4]. The *AR* gene comprises eight exons that encode a 110 kDa protein. Members of the steroid/thyroid hormone family share common structural features, with distinct functional domains referred to as domains A to E(F). The highly conserved middle region (C domain) acts as a DNA binding domain, while the ligand binding domain (LBD) is located in the C-terminal E/F domain. During ligand-induced transactivation, the N-terminal domains A/B and the steroid receptor LBD act as interacting regions for the co-activator complexes [5–7]. The autonomous activation function-1 (AF-1) within the A/B domain is ligand-independent, while AF-2 within the LBD is induced upon ligand binding [8]. While unliganded LBD appears to suppress the function of the A/B domain, ligand binding to the LBD is thought to evoke LDB function and restore A/B domain function through an, as yet undescribed, intramolecular alteration of the entire steroid receptor structure.

In contrast to the other members of the steroid receptor superfamily, a number of clinical disorders of the AR have been reported [9–14]. Classical AR functional abnormalities cause a spectrum of disorders of androgen insensitivity syndrome (AIS) or testicular feminization mutation (Tfm) [10,13,14]. AR mutations underlying these disorders include amino acid substitutions in the DNA or ligand binding domains, point mutations leading to premature stop codons, and deletions of the *AR* gene. In addition, expansion of a polyQ repeat region within AR has been implicated in the pathogenesis of a motor neuron disease called Kennedy's disease [9,12]. AR is a relatively large protein to other steroid receptors, due to the long N-terminal A/B domain that contains this polyQ repeat. However, the molecular basis of AR function underlying these AR-related disorders remains largely unknown due to the lack of stable genetic models. In this article, we present recent results of our studies into

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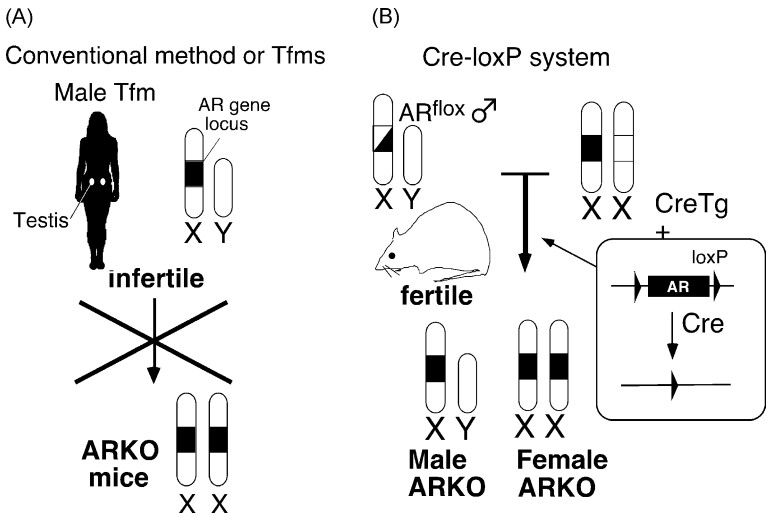


Fig. 1. Strategy for generating the ARKO mouse line. (A) The *AR* gene is located on the X chromosome. As male Tfm animals are infertile, the mutated *AR* gene cannot be transmitted to the next generation. (B) In the first step, floxed AR mice that carry a functional *AR* gene flanked by loxP sites are generated by the introduction of loxP sites into the first exon of the *AR* gene by homologous recombination in ES cells. By mating subsequent mice with CMV-Cre transgenic mice, the *AR* gene is disrupted during embryogenesis.

genetic models of loss of AR function in mice [15,16] and gain of AR function in *Drosophila* [17].

## 2. Androgen receptor inactivation by gene targeting using the Cre-loxP system in mice

As shown in Fig. 1A, there were both basic and technical difficulties in generating AR knockout (ARKO) mice. The *AR* gene is located on the X chromosome [18], and therefore exists as a single copy in 46, XY males, in which androgen exerts its most profound effects. As male mice lacking a functional *AR* gene would be expected to show Tfm abnormalities with complete infertility [10,13,14], successful targeted disruption of the *AR* gene, essential for reproduction, necessarily prohibits its transmission to the next generation. Thus, it was impossible to generate an ARKO mouse line by either breeding or conventional gene targeting methods. Furthermore, as all Tfm model animals are genetically male, it was impractical to generate genetically female animals homozygous for *AR* gene mutations.

To avoid this problem, we applied the Cre-loxP system [19] to establish an ARKO mice line (Fig. 1B). We first generated floxed AR mice, in which the *AR* gene locus was flanked by loxP sites. Floxed AR mice were fully fertile and showed normal expression of AR protein. We then crossed these mice with mice that expressed the Cre recombinase ubiquitously under the control of a CMV promoter, and obtained male and female ARKO mice at theoretical Mendellian frequencies.

## 3. Female-typical appearance of male ARKO mice

The appearance of male ARKO mice is shown in Fig. 2. ARKO males exhibited female-typical external appearance, such as a vagina with a blind end, and a clitoris-like phallus, instead of a penis and scrotum. Male reproductive organs, including seminal vesicles, vas deferens, epididymis and prostate were absent in ARKO males. However, no ovaries or uteri were observed, although small inguinal testes were present. Histological examination of the testes showed that spermatogenesis was severely arrested. From these results, it was clear that while AR was not required for the formation of testis, it was essential for the development of male reproductive organs and spermatogenesis.

Estimation of plasma hormone levels in ARKO males revealed markedly lowered androgens, but similar estradiol levels to that of wild-type males. This suggested that it was possible to investigate the effect of androgens independently in ARKO mice as only AR was disrupted, leaving estrogen receptors intact.

## 4. Late onset of obesity in male ARKO mice

A characteristic change was seen in the growth of ARKO males (Fig. 3). Until 10 weeks of age, ARKO males exhibited growth retardation with growth curves indistinguishable from that of wild-type female littermates. However, thereafter, the growth of ARKO males rapidly increased, such that at 12 weeks of age, male ARKO mouse body weights exceeded that of wild-type male littermates (Fig. 3A). This late onset of drastically increased ARKO male growth curve led to the clear development of obesity, with 30-week-old ARKO males, showing significantly increased wet tissue weights in subcutaneous, infrarenal and intraperitorial white adipose tissues (WATS) (Fig. 3B). Such clear increases were not detected in WATs of 8-week-old ARKO males. As no significant alterations in serum lipid parameters or food intake were observed, our results suggested that AR may serve

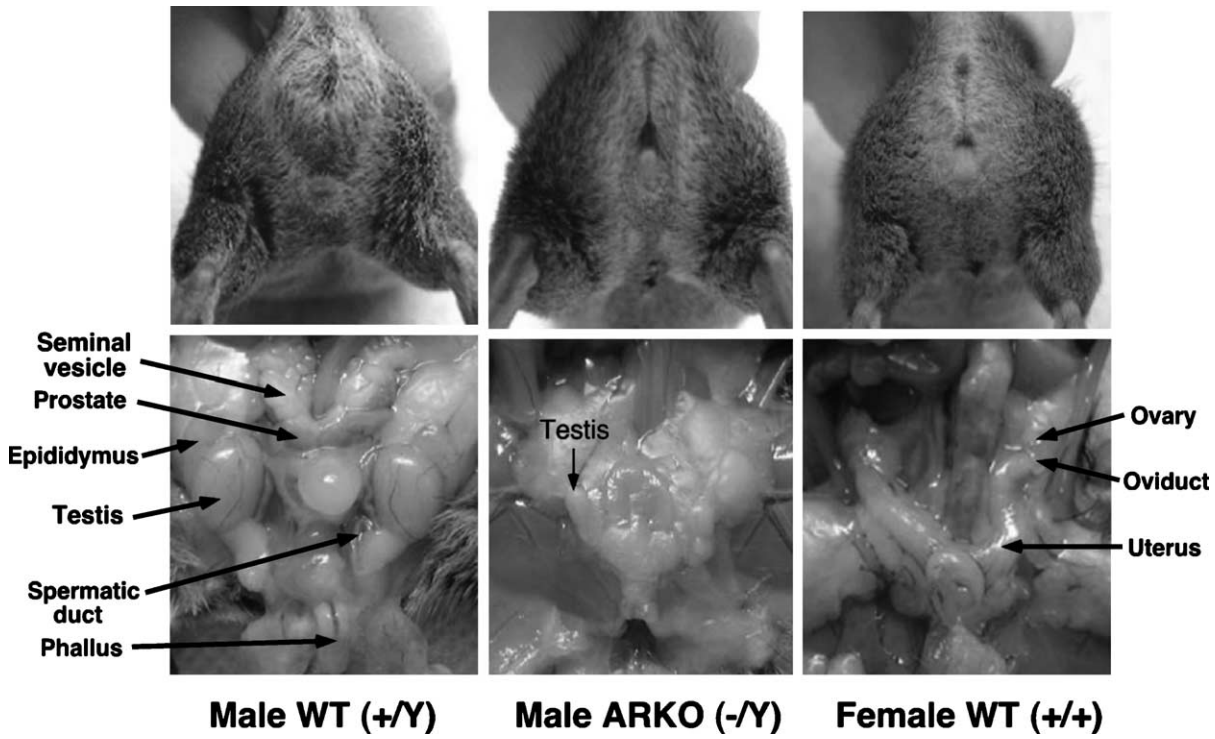


Fig. 2. Male ARKO mice are characterized by female-typical appearance, including a clitoris-like phallus and, a vagina with a blind end as well as the absence of internal male and female reproductive organs, except for the presence of atrophic testes.

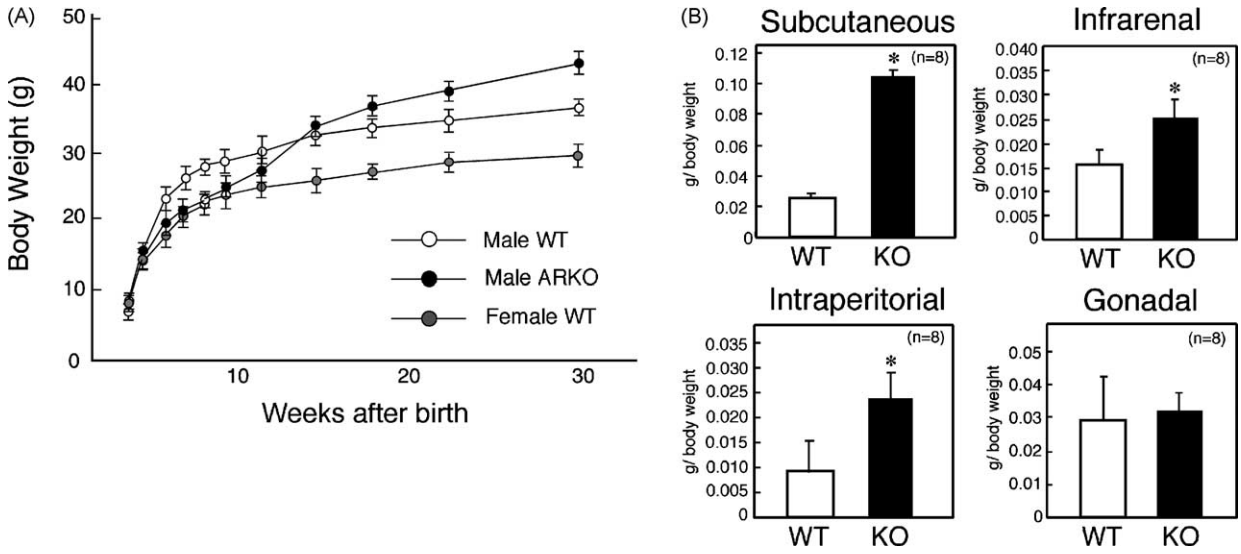


Fig. 3. Late onset of obesity in male ARKO mice. (A) Growth curves of wild-type male and female mice, and ARKO male mice. (B) Increased wet-weights of white adipose tissues in male ARKO mice.

as a negative regulator of adipocyte development in adult males.

## 5. Androgen-dependent neurodegeneration by polyQ-expanded human AR in *Drosophila*

A unique example of the tissue specific effects of an AR defect is Kennedy's disease. Kennedy's disease, or spinal and bulbar muscular atrophy (SBMA), is a rare degenerative disease of the motor neurons characterized by progressive muscle atrophy and weakness in male patients, usually beginning at 30–50 years of age [11]. Previous analyses of Kennedy's disease revealed expansions in the number of trinucleotide CAG repeats in the first exon of the *AR* gene, that generated expanded polyQ stretches in the A/B domain of the AR protein [9,12,20]. It was found that disease onset occurred when these repeat stretches encoded more than

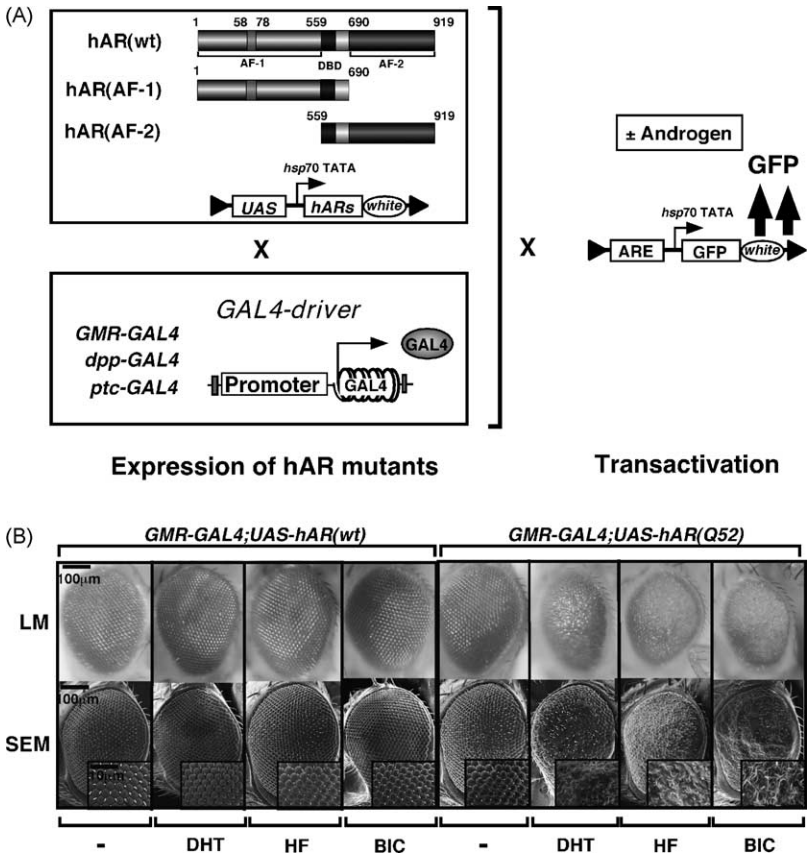


Fig. 4. Ligand-induced degeneration in photoreceptor neurons due to mutant hAR containing expanded polyQ stretches. (A) Expression of human AR constructs in *Drosophila* eyes using the GAL4-UAS system. To monitor the ligand-induced transactivation of hAR, hAR-expressing flies are further crossed to flies carrying a *GFP* reporter gene. Thus, GFP expression was induced by ligand-bound AR that recognized the consensus androgen response element (*ARE*) in the GFP promoter. Location of the polyglutamine (polyQ) region in relation to the DNA binding domain (DBD). Transactivation function-1 (AF-1) region is localized within the N-terminal A/B domain, and transactivation function-2 (AF-2) region is localized within the C-terminal E/F domain containing the ligand binding domain (LBD). (B) Rough-eye phenotype induced in hAR (Q52) lines by DHT or AR antagonist. Light microscopic (LM) and scanning electron microscopic (SEM) images of adult fly-eye tissue.

40 glutamine residues, compared to a range of 15–35 polyQ residues in normal individuals.

As the onset of Kennedy's disease occurred in adult men rather than women, even those women carrying AR mutations in the A/B domains, we reasoned that the binding of significant amounts of androgen and the subsequent structural alteration of mutant AR was likely to be a critical step in the onset of Kennedy's disease. To test this hypothesis, we investigated the role of human AR (hAR) mutants with expanded polyQ stretches in neurodegeneration. To this end, we established a *Drosophila* model that ectopically overexpressed a mutated AR in photoreceptor neurons (Fig. 4). We first expressed wild-type and mutated hAR in photoreceptor neurons in developing eye discs under the glass multimer reporter (*GMR*) gene promoter [21] using the *Drosophila melanogaster* GAL4-UAS system [22]. To monitor the ligand-induced transactivation function of hAR, hAR-expressing flies were further crossed to flies carrying a *GFP* reporter gene, such that GFP expression was induced by the binding of ligand-bound AR to the consensus *ARE* in the GFP promoter [23]. Expressed hAR proteins were then detected as red fluorescence in situ using an immunofluorescent antibody.

Although, eyes that expressed a mutant hAR containing an expanded 52-stretch polyQ (Q52) appeared normal, dietary ingestion of dihydroxytestosterone (DHT) or androgen antagonists induced marked degeneration and apoptosis of the photoreceptor neurons, despite the mutant hAR retaining only reduced transactivation function (Fig. 4). Ligand-independent toxicity was detected in fly eyes expressing truncated polyQ-expanded A/B domains alone, but this was abrogated by the co-expression of unliganded LBD domains. Thus, our results suggested that hormone binding and subsequent structural alteration of hAR mutants with nuclear localization appeared to be critical for Kennedy's disease onset, and that the fly-eye model may be useful for the development of novel therapeutic approaches to Kennedy's disease.

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