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Study of Androgen Receptor Functions by Genetic Models

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Androgens exert most of their biological activities through binding to the androgen receptor (AR). The AR belongs to the nuclear receptor superfamily and acts as a ligand-inducible transcriptional factor. AR dysfunction causes a diverse range of clinical conditions, such as testicular mutation (Tfm) syndrome, prostate cancer, and spinal and bulbar muscular atrophy (SBMA). However, the molecular basis of the AR function underlying these AR-related disorders remains largely unknown due to the lack of stable genetic models. Here we review recent results of our studies into genetic models of the loss of AR function in mice and the gain of AR function in *Drosophila*.

Key words: androgen receptor (AR), androgen receptor knockout (ARKO), *Drosophila*-eye model, polyQ repeat, spinal and bulbar muscular atrophy (SBMA), testicular feminization mutation (Tfm).

Abbreviations: AR, androgen receptor; ARE, androgen response element; KO, knockout; SBMA, spinal and bulbar muscular atrophy; Tfm, testicular feminization mutation.

Androgens as male sex hormones have a critical role in wide rage biological processes (*1*, *2*). These include spermatogenesis, virilization of genitalia and brain functions. Most actions of androgens are mediated by the nuclear androgen receptor (AR), which acts as ligand-inducible transcription factor (*3*, *4*). Liganded AR forms homodimers and binds specific DNA elements in the target gene promoter. The DNA element that binds AR is the common sequence 5′-AGAACANNNTGTTCT-3′, referred to as the consensus androgen response element (*ARE*). To date, a number of clinical disorders of the AR have been reported. Classical AR functional abnormalities cause a spectrum of disorders of androgen insensitivity syndrome (AIS) or testicular feminization mutation (Tfm) (*5–9*). 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 (*6*, *8*, *9*). In addition, expansion of a polyQ repeat region within AR has been implicated in the pathogenesis of a motor neuron disease called spinal and bulbar muscular atrophy (SBMA) (*5*, *7*). AR is a relatively large protein compared to other steroid receptors, due to its 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 genetic models of loss of AR function in mice (*10–12*) and gain of AR function in *Drosophila* (*13*).

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Domain features of androgen receptor

In the late 1980s, the cloning the human AR opened an avenue for understanding the molecular mechanism of androgen actions (*14*). The AR gene comprises eight exons that encode a 110-kDa protein. In common with other members of the nuclear receptor superfamily, the AR contains distinct structural and functional domains referred to as domains A to E(F) (*3*, *4*). 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. The LBDs of most nuclear receptors, including AR, have been analyzed and are comprised of 12 α helixes that form a pocket to capture cognate ligands (*15*, *16*). Upon ligand binding, the C-terminal α helix 12 (H12) in the LBD shifts position to create a space, with helixes 3 to 5 serving as the key interface following dissociation of corepressor complexes and association of coactivator complexes (*3*, *17*). During ligand-induced transactivation, the N-terminal domains A/B and the steroid receptor LBD act as interacting regions for the co-activator complexes. 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 (*18*). 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.

Generation of AR-null mutant mice by gene targeting using the Cre-loxP system

Although androgens seem to exert beneficial effects in males, the physiological role of AR-mediated androgen signaling in male physiology and behaviors has not been established. This is because estrogens are locally converted from serum androgens by aromatase in target

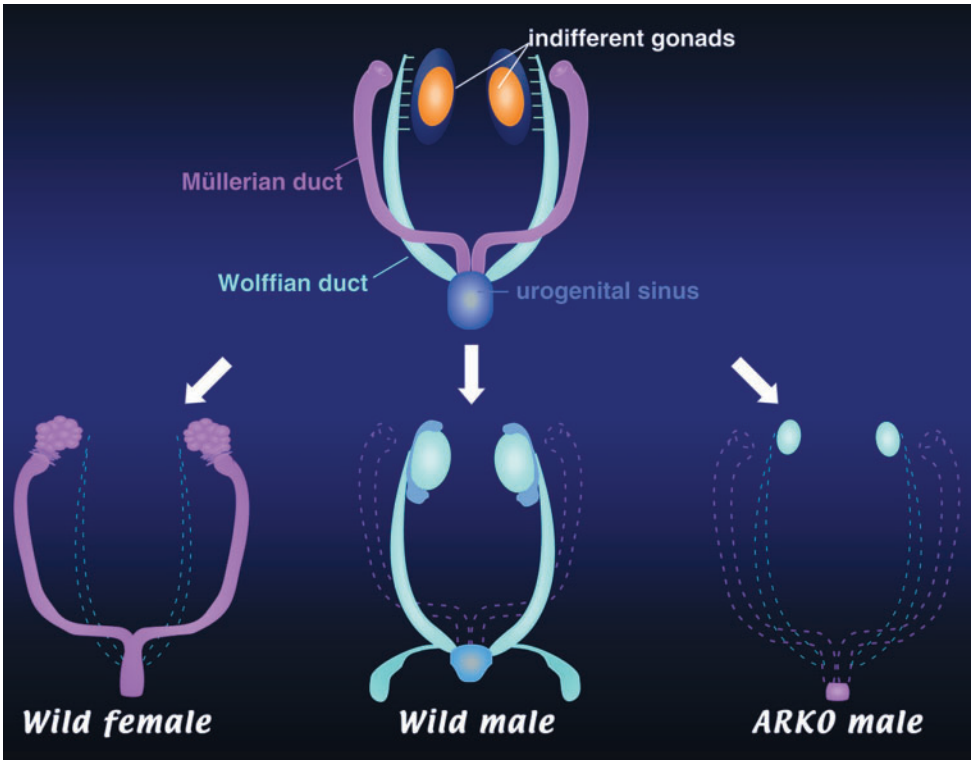


Fig. 1. **Schematic representation of reproductive organs in male ARKO mice.** Male ARKO mice are characterized by female-typical appearance, including a clitoris-like phallus and a vagina with a blind end, as well as the absence of internal male and female reproductive organs, except for the presence of atrophic testes.

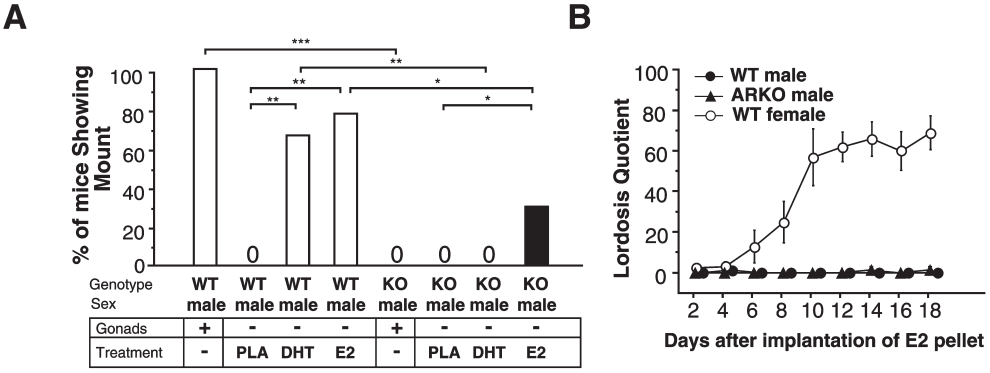
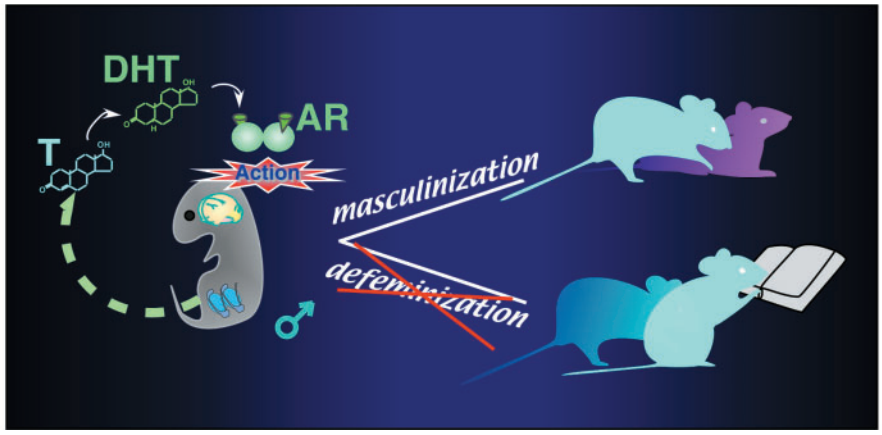


Fig. 2. **Ablation of AR in male mice resulted in the lack of both male and female sexual behaviors.** (A) Loss of all components of male sexual behavior in intact (Gonads: +) 10-week-old ARKO mice. (B) Lordosis was not induced in gonadectomized ARKO male mice after treatment with E2. (C) Schematic representation of AR function in perinatal brain masculinization and defeminization.



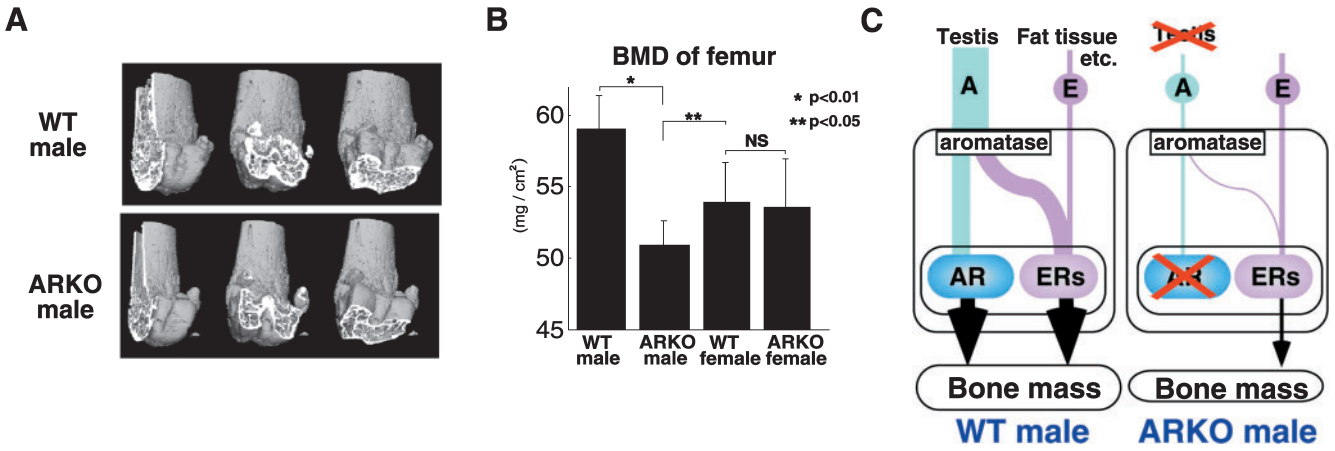
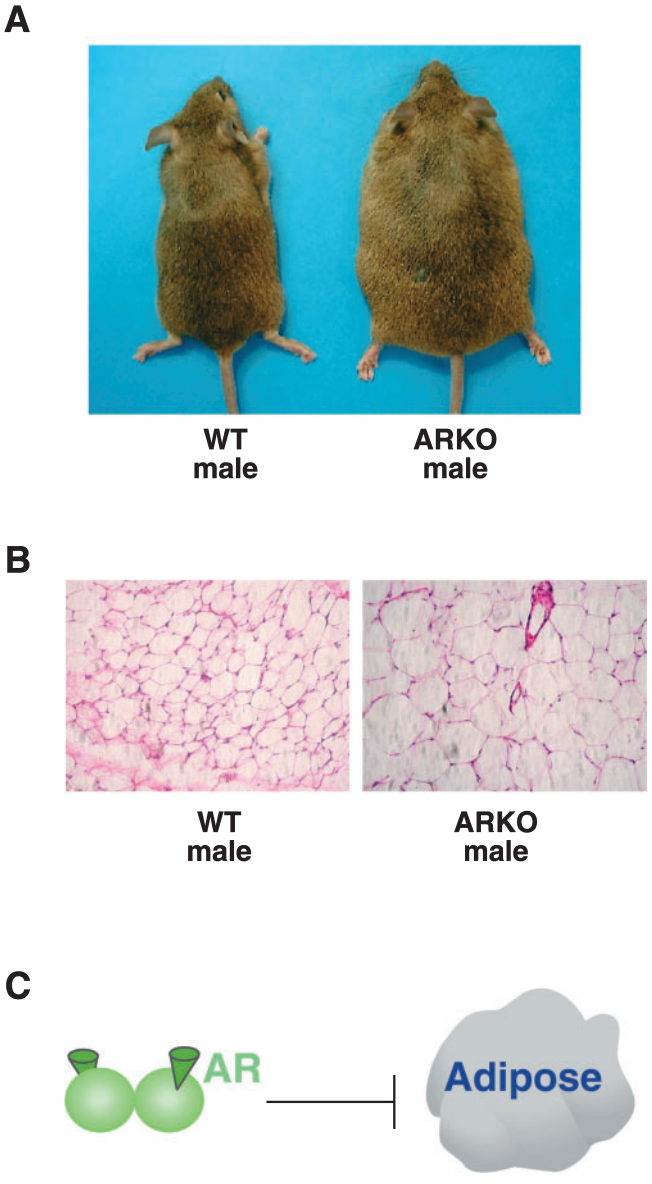


Fig. 3. **Osteopenia in male ARKO mice.** (A) Three-dimensional computed tomography images of distal femora from representative 8-week-old male ARKO mice. (B) Bone loss in femur of 8-week-old male ARKO mice by BMD analysis. (C) Schema of skeletal sex hormone action.



organs. The AR gene is located on the X chromosome, and therefore exists as a single copy in males. As male animals lacking a functional AR gene would be expected to show Tfm abnormalities with complete infertility, successful targeted disruption of the AR gene, essential for reproduction, necessarily prohibits its transmission to the next generation. Therefore, to define AR function, an AR-null mutant mouse line was generated by means of the Cre-loxP system (*19*), which was used to circumvent the problem of male infertility. 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. No AR transcript or protein was detected, which indicates that they were complete null mutant mice for the AR gene.

Tfm abnormalities in male ARKO mice

As expected, the AR-null mutation in males resulted in the ablation of masculinization of reproductive organs (*11*). ARKO mice exhibited female-typical external appearance, including a vagina with a blind end and a clitoris-like phallus, instead of a penis and scrotum (Fig. 1). Male reproductive organs, such as 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. These observations in ARKO males are similar to a human hereditary disorder, AIS or Tfm, in which mutations in the AR gene have been identified in several families. Testicular androgen levels were very low, whereas serum estrogen levels appeared normal in ARKO males.

Fig. 4. **Late onset of obesity in male ARKO mice.** (A) External appearance of 30-week-old male ARKO mice. (B) Subcutaneous white adipose tissues from 30-week-old male ARKO mice. (C) Schema of AR function in adipogenesis.

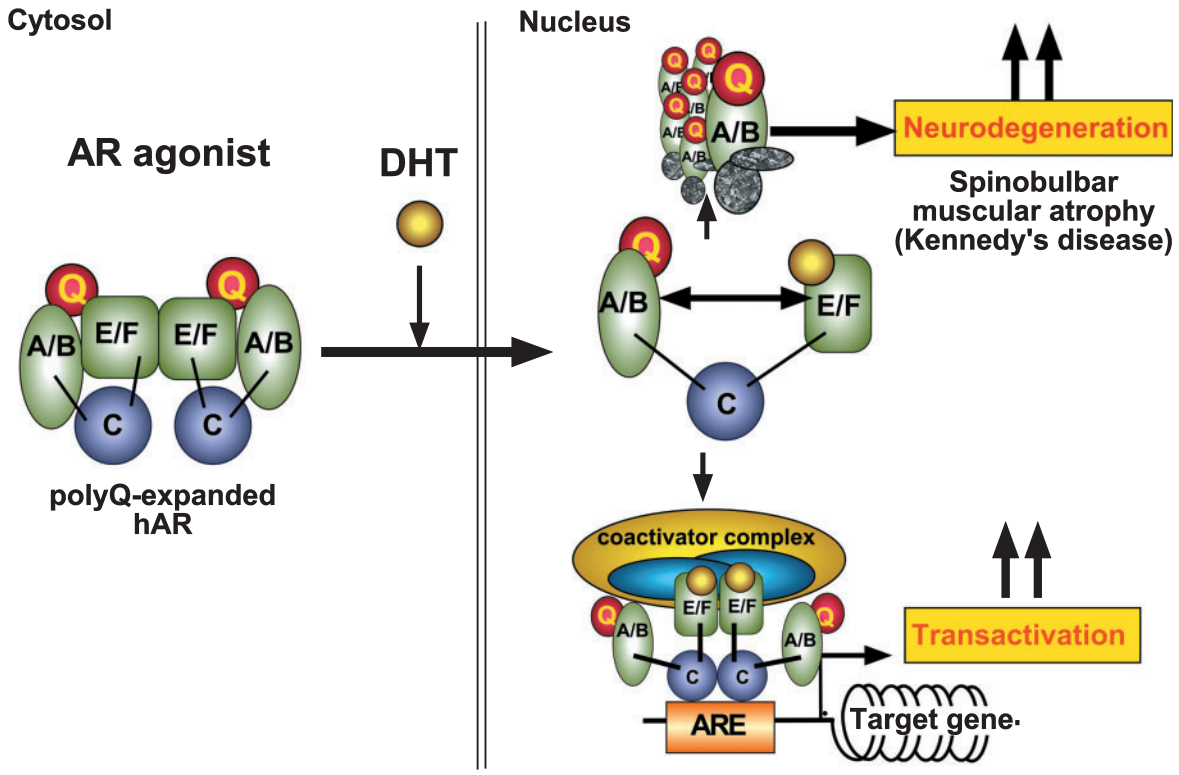


Fig. 5. **Androgen-dependent structural alteration by the polyQ-expanded human AR.** It is considered that the polyQ-expanded AR is inactive in the transactivation without the agonists (androgens); but in the presence of the agonists, it alters the molecular structure and also recruits the co-activators, while the polyQ repeat induces apoptosis by their aggregation.

Brain masculinization requires AR function

Next, we investigated the AR function in perinatal brain masculinization (*11*). Intact ARKO males showed no male sexual behaviors and impaired male aggressive behaviors (Fig. 2A). Treatment with the non-aromatizable androgen 5α-dihydrotestosterone (DHT) partially rescued impaired male aggressive behaviors in ARKO males, suggesting that DHT activity in male aggressive behaviors is mediated via both AR-independent and -dependent pathways in the adult. In contrast, no female sexual behavior was induced in ARKO males even when estrogen alone or both estrogen and progesterone was administered (Fig. 2B), suggesting that the brains of ARKO males were defeminized (Fig. 2C). The AR function in brain masculinization at a limited perinatal stage was further studied in ARKO females. While DHT-induced masculinization of female brain at the perinatal stage led to adult female mice sensitive to both E2 and DHT with the expression of male-typical behaviors, such responses were completely abolished in ARKO females. These data provide genetic evidence that once the brain is perinatally masculinized through liganded-AR, the sexually developed brain becomes sensitive to both androgen and estrogen with regard to the expression of male-typical behaviors in adulthood (Fig. 2C).

High turnover osteopenia in male ARKO mice

Beyond the reproductive physiology, sex steroid hormones are implicated in involvement of homeostatic processes, such as bone metabolism. Sex hormone status is reflected in bone mass, and hormonal deficiency is well known to lead to progressive bone loss. The most striking example, osteoporosis in postmenopausal women, is a state of estrogen deficiency coupled with imbalanced bone remodeling. However, the physiological role of the AR-mediated androgen signaling in the skeletal tissues has not yet been established. We performed in vivo analyses of the bone of 8-week-old ARKO males and found that the trabecular and cortical bone volumes were remarkably reduced (Fig. 3, A–B) (*12*). Bone loss in ARKO mice was only partially prevented by treatment with aromatizable testosterone (Fig. 3C). Histomorphometric analysis further showed that both bone formation and resorption were enhanced in ARKO males, but the increase in bone resorption exceeded that in formation. In view of these findings, we concluded that bone loss found in ARKO males was based on the high bone turnover osteopenia.

Late onset of obesity in male ARKO mice

Another hallmark of ARKO males was late-onset obesity (Fig. 4A) (*10*). From birth, ARKO male mice were externally indistinguishable from normal female WT littermates in terms of ano-genital distance and growth curve up to 10 weeks. However, thereafter, the growth of ARKO males rapidly increased, and at 12 weeks of age, male ARKO mouse body weights exceeded that of WT male littermates. 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 white adipose tissues (Fig. 4B). Such clear increases were not detected in

white adipose tissues of 8-week-old ARKO males. As no significant alterations in food intake were observed, our results suggested that AR may serve as a negative regulator of adipocyte development in adult males (Fig. 4C).

Drosophila model to dissect human AR mutants with expanded polyQ stretches in neurodegeneration

Another characteristic clinical disorder with an AR mutation is spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease (*5*, *7*). SBMA is a rare degenerative disease of the motor neurons characterized by progressive muscle atrophy and weakness in male patients, usually beginning at 30 to 50 years of age. Previous analyses of SBMA revealed expansions in the number of trinucleotide CAG repeats in the first exon of the AR gene, which generated expanded polyQ stretches in the A/B domain of the AR protein (*5*, *7*, *20*). It was found that disease onset occurred when these repeat stretches encoded more than 40 glutamine residues, compared to a range of 15 to 35 polyQ residues in normal individuals.

To dissect the molecular mechanism of human AR (hAR) mutants with expanded polyQ stretches in neurodegeneration, we established a *Drosophila* model that ectopically overexpressed a mutated AR in photoreceptor neurons (*13*). We first expressed WT 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, with the result 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 dihydrotestosterone (DHT) or androgen antagonists induced marked degeneration and apoptosis of the photoreceptor neurons, despite the mutant hAR retaining only reduced transactivation function. 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 SBMA onset (Fig. 5), and that the fly-eye model may be useful for the development of novel therapeutic approaches to SBMA.

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