

A floxed allele of the *androgen receptor* gene causes hyperandrogenization in male mice

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Submitted 6 November 2007; accepted in final form 28 December 2007

MacLean HE, Chiu WS, Ma C, McManus JF, Davey RA, Cameron R, Notini AJ, Zajac JD. A floxed allele of the *androgen receptor* gene causes hyperandrogenization in male mice. *Physiol Genomics* 33: 133–137, 2008. First published January 2, 2008; doi:10.1152/physiolgenomics.00260.2007.—We previously generated a conditional floxed mouse line to study androgen action, in which exon 3 of the *androgen receptor* (*AR*) gene is flanked by *loxP* sites, with the *neomycin* resistance gene present in intron 3. Deletion of exon 3 in global *AR* knockout mice causes androgen insensitivity syndrome, characterized by genotypic males lacking normal masculinization. We now report that male mice carrying the floxed allele (*AR*^{lox}) have the reverse phenotype, termed hyperandrogenization. *AR*^{lox} mice have increased mass of androgen-dependent tissues, including kidney, ($P < 0.001$), seminal vesicle ($P < 0.001$), levator ani muscle ($P = 0.001$), and heart ($P < 0.05$). Serum testosterone is not significantly different. Testis mass is normal, histology shows normal spermatogenesis, and *AR*^{lox} males are fertile. *AR*^{lox} males also have normal *AR* mRNA levels in kidney, brain, levator ani, liver, and testis. This study reaffirms the need to investigate the potential phenotypic effects of floxed alleles in the absence of *cre* in tissue-specific knockout studies. In addition, this androgen hypersensitivity model may be useful to further investigate the effects of subtle perturbations of androgen action in a range of androgen-responsive systems in the male.

androgen; testosterone; *cre/lox*; hypomorphic allele; androgen insensitivity

CRE/LOX TECHNOLOGY HAS REVOLUTIONIZED the approach to studying gene function *in vivo*, allowing the investigation of tissue-specific actions of genes via their targeted deletion in mice (5). A wide range of *cre/lox* studies have now been reported; however, unexpected phenotypes have sometimes been observed. Floxed mice are generated via homologous recombination in embryonic stem (ES) cells using a targeting vector with a selectable marker, usually the *neomycin* resistance gene cassette (*neo*). In many cases the *neo* cassette is left in the targeted locus, usually in an intron (2, 4, 27), to minimize the amount of ES cell manipulation and maximize the probability of germ line transmission in the host blastocyst (8). There is the presumption that the intronic *neo* cassette will not affect the floxed gene's function, but in some cases a hypomorphic allele is generated, where the floxed gene at the targeted locus is expressed at lower than normal levels (6, 12). The appropriate mouse controls are not always included in *cre/lox* studies, with

the phenotype of tissue-specific knockouts often compared only to wild-type littermates. Therefore, the possible effects of the floxed gene alone are not assessed, and misleading conclusions regarding the function of targeted genes may be reached because of confounding effects of the targeted allele in the absence of *cre*.

We have generated a conditional floxed mouse line to study androgen action, in which exon 3 of the *androgen receptor* (*AR*) gene is flanked by *loxP* sites and which retains a *neo* cassette in intron 3 (17). Excision of exon 3 leads to an in-frame deletion, producing an *AR* protein that lacks the second zinc finger of the DNA binding domain of the receptor (17). The *AR* gene is on the X chromosome, and male mice hemizygous for the floxed allele and heterozygous for a universal *cre* (*XAR*^{loxY}; *CMV-cre* het) have the classical androgen insensitivity syndrome. We now report that male mice carrying the conditionally targeted allele in the absence of *cre*, *XAR*^{loxY} (*AR*^{lox} males), have a phenotype of hyperandrogenization.

METHODS

Mice. *AR*^{lox} mice were generated as described previously (17), and targeted *AR*^{lox} offspring were backcrossed onto a C57BL/6 background for more than six generations. α -Actin *cre* mice (13) and muscle creatine kinase (MCK)-*cre* mice (2), used in matings to generate the *AR*^{lox} and wild-type males examined in this study, were also backcrossed onto a C57BL/6 background for more than six generations before use. Southern and PCR analysis of the targeted locus confirmed that no gene rearrangement or alternate splicing products are produced from the *AR*^{lox} locus (Ref. 17 and data not shown). Wild-type males were littermates of *AR*^{lox} males. Mice were housed in a conventional facility, with standard chow and water available *ad libitum*. All mouse studies were performed with the approval of the Austin Health Animal Ethics Committee.

Blood was collected by anesthetizing mice with inhaled isoflurane, transecting the cervical spine and carotid arteries, and collecting the blood from the cervical cavity. Serum was separated by centrifugation of blood in a microcentrifuge for 2×8 min at 4,200 rpm. After blood collection, tissues were immediately dissected and wet weight was determined. Tissues were snap-frozen in liquid nitrogen and stored at -80°C before extraction of DNA/RNA, which were isolated with standard methodologies.

PCR, cDNA synthesis, and quantitative real-time PCR. PCR using genomic DNA to amplify the targeted *AR* gene locus was performed as previously described (17). For cDNA synthesis, 2–5 μg of total RNA was treated with 2 U of DNase I according to manufacturer's instructions (DNA-Free Kit, Ambion, Austin, TX). One microgram of DNase-treated RNA was primed with 500 ng of random hexamers (Promega, Annandale, Australia) at 70°C for 5 min. cDNA was synthesized with 100 U of Moloney murine leukemia virus (MMLV) reverse transcriptase in $1 \times$ MMLV buffer containing 12.5 U of RNasin and 0.625 mM dNTPs (Promega) at 37°C for 1 h.

Article published online before print. See web site for date of publication (<http://physiolgenomics.physiology.org>).

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Quantitative real-time PCR was performed in duplicate on all samples, using 500 ng of cDNA in each 25- μ l reaction and the Applied Biosystems (Scoresby, Australia) TaqMan mouse *AR* gene expression assay (Assay ID: Mm00442688_m1), which amplifies a product spanning exon 2 to exon 3 and uses a probe against the exon 2/exon 3 boundary, and the *insulin-like growth factor 1 (Igf1)* (Eal liver type specific) gene expression assay (Mm00710307_m1). Relative expression was determined by the $\Delta\Delta C_T$ method (Ref. 15; C_T is threshold cycle), normalized against eukaryotic 18S rRNA (assay ID: 4310893E), and expressed relative to one reference cDNA sample. All values were graphed as a percentage of the mean of wild type. Four to seven independent samples/genotype were analyzed for each tissue.

Testosterone assay. Testosterone was assayed in mouse serum with the DPC Coat-A-Count Total Testosterone Assay (Siemens Medical Solutions Diagnostics, Doncaster, Australia) after extraction. Fifty microliters of mouse serum was diluted to 500 μ l with normal saline-Calibrator A (DPC Coat-A-Count Total Testosterone Assay) (1:3.5) and extracted with 2 ml of hexane-ethyl acetate (17:3) by vortexing for 1 min. After centrifugation for 5 min at 1,000 rpm the aqueous layer was frozen in a methanol-dry ice bath. The supernatants were transferred to clean tubes and dried under an airstream before reconstitution in 60 μ l of Calibrator A. The reconstituted extracts were assayed according to the manufacturer's instructions (Coat-A-Count assay). The sensitivity of the assay is 0.14 nM, the intra-assay coefficient of variation range is 4–11%, the interassay coefficient of variation range is 5.9–12%, and the assay is highly specific for testosterone, with the percent cross-reactivity with other androgens as follows: 0.5% with androstenedione, $\leq 3.4\%$ with 5 α -dihydrotestosterone, 2.0% with 19-hydroxyandrostenedione, and 0.8% with 11 β -hydroxytestosterone.

Histology. Testes were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed in PBS, and stored in 70% ethanol before paraffin processing and embedding. Six-micron serial sections were cut and stained with hematoxylin and eosin according to standard protocols.

Statistical analysis. Data were analyzed by two-tailed unpaired Student's *t*-tests with SPSS version 13 for Mac. Data were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

***AR^{lox}* mice.** Floxed *AR* mice were generated as described previously (17), with *loxP* sites flanking exon 3 and the *neo* cassette in intron 3, 466 bp downstream from the intron/exon boundary (Fig. 1A). We have demonstrated (17, 18) that this floxed allele is functional by generating global and tissue-specific *AR* knockout (ARKO) mice. Using primers flanking the floxed exon 3, PCR on genomic DNA from tissue-specific ARKO mice demonstrates that in the presence of cre deletion of exon 3 and the *neo* cassette occurs (Fig. 1B). Global ARKO males are completely androgen insensitive, with XY, SRY-positive males indistinguishable from their female littermates based on their external genitalia, with a lack of normal masculinization of the reproductive system (17, 18). As expected, androgen-insensitive global ARKO males also have a decrease in the mass of a number of androgen-dependent tissues, including testis, seminal vesicle, kidney, and muscle, and increased spleen mass (Ref. 17 and data not shown). Similarly, a previously described hypomorphic allele of the *AR* gene that decreases levels of AR protein causes decreased testis mass and seminal vesicle mass and disrupted spermatogenesis in male mice (12).

Altered mass in androgen-sensitive tissues. We examined the phenotype of the *AR^{lox}* males and wild-type male littermates aged 12 wk generated from crossing *AR^{lox}* heterozygous females with heterozygous males from two different cre trans-

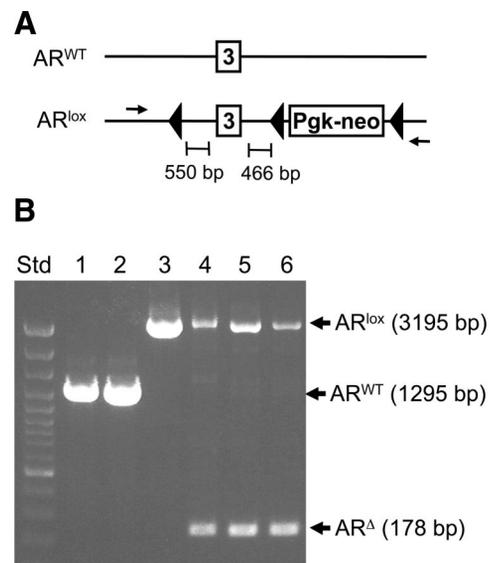


Fig. 1. Targeted region of the *androgen receptor (AR)* gene. **A:** structure of the wild-type and conditionally targeted *AR* alleles, showing exon 3 of the *AR* gene flanked by *loxP* sites (arrowheads) and the neomycin resistance gene (*Pgk-neo*). Arrows denote approximate location of PCR primers. **B:** PCR on genomic DNA amplifying across the targeted locus. Std, 100-bp DNA ladder; lanes 1 and 2, wild-type males (*AR^{WT}*); lane 3, floxed *AR* male (*AR^{lox}*); lanes 4–6, tissue-specific *AR* knockout (ARKO) males (*AR^{lox}*; α -actin-cre). PCR shows no rearrangements in the targeted region and demonstrates that the floxed allele is functional, with deletion of exon 3 and the *neo* cassette occurring in *AR^{lox}*; α -actin-cre males, with the deleted allele (*AR^Δ*) detectable.

genic lines: MCK-cre (2) and α -actin-cre (13). There was no difference in body mass between the wild-type and *AR^{lox}* males (Fig. 2A). However, there was a significant increase in the mass of androgen-dependent tissues in the *AR^{lox}* mice, including the kidney (15.3% increase, $P < 0.001$; Fig. 2B), the seminal vesicle (18.6% increase, $P < 0.001$; Fig. 2C) and the highly androgen-dependent skeletal muscle levator ani (8.8% increase, $P = 0.001$; Fig. 2D). Prostate mass was not examined. There was also an increase in cardiac mass in the *AR^{lox}* mice (6.3% increase, $P = 0.05$; Fig. 2E), which can be considered cardiac hypertrophy because heart mass-to-body mass ratio was also significantly increased (5.69 ± 0.10 mg/g wild type vs. 6.13 ± 0.14 mg/g *AR^{lox}*, means \pm SE; $P = 0.01$). However, testis mass did not differ between wild-type and *AR^{lox}* mice (Fig. 2F). Because these changes are the reverse of the phenotype of androgen insensitivity occurring in the global ARKO mice, this suggests the converse phenotype in *AR^{lox}* males, which we term hyperandrogenization.

Alterations in androgen signaling in the testis can affect Sertoli cell development and cause arrest of spermatogenesis (23); therefore, we examined testis histology in 12-wk-old wild-type and *AR^{lox}* male mice. Although formal stereological analysis was not performed, testis sections from both wild-type and *AR^{lox}* males showed no pathological features, with normal spermatogenesis (Fig. 3) consistent with the observation that testis mass was normal in the *AR^{lox}* mice (Fig. 2F). This finding is not unexpected, because although testis mass and fertility are androgen dependent, it is predominantly lower or suprphysiological levels of androgens (1), or decreased AR function (24), that significantly alters mass, spermatogenesis, and fertility. In contrast, mild hyperandrogenization would be predicted to have little effect on these parameters. *AR^{lox}* males

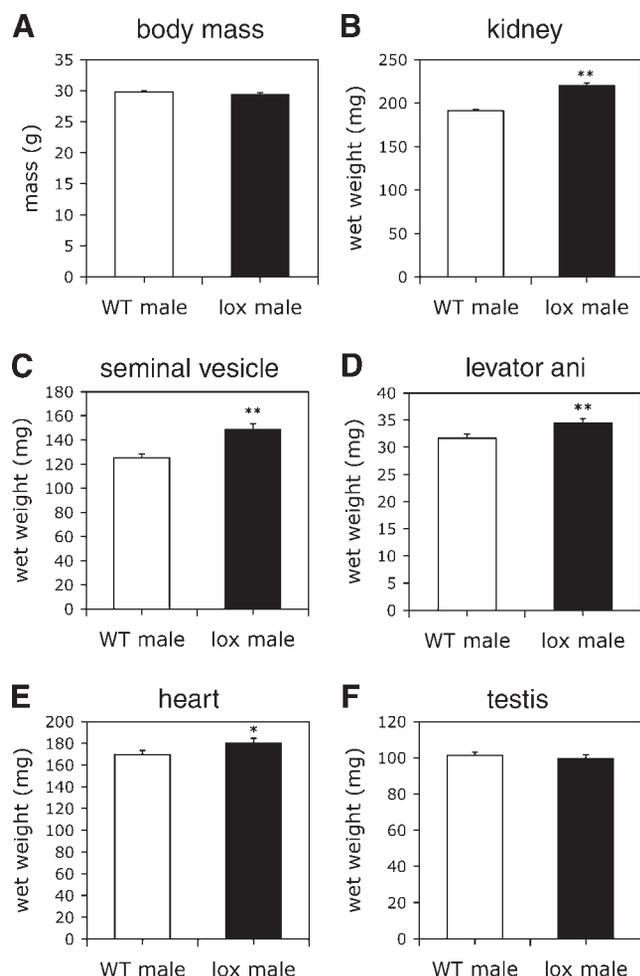


Fig. 2. Body and organ mass in 12-wk-old WT and AR^{lox} (lox) males. *A*: total body mass. *B*: kidney mass. *C*: seminal vesicle mass. *D*: levator ani mass. *E*: heart mass. *F*: testis mass. Data are means \pm SE; $n = 20$ –30/group. * $P < 0.05$, ** $P \leq 0.001$ vs. WT male (Student's *t*-test).

are fertile, with normal litter sizes produced (data not shown). AR^{lox} heterozygote females are also fertile (data not shown), but we have not generated AR^{lox} homozygous females.

Serum hormone levels not significantly altered. One explanation for the hyperandrogenization of AR^{lox} male mice is that they have elevated serum testosterone due to a tissue-specific loss of AR function. This would occur as a result of loss of the normal negative feedback on gonadotropin secretion by testosterone in the hypothalamic-pituitary-gonadal axis (3) due to tissue-specific loss of AR signal transduction in the brain. In this model, decreased signal transduction would arise because of a tissue-specific decrease in *AR* gene transcription in the AR^{lox} mice, caused by the presence of the *neo* cassette in intron 3 of the gene. Loss of feedback inhibition occurs in patients with complete androgen insensitivity syndrome (CAIS), which is caused by loss-of-function mutations in the *AR* gene. In CAIS patients, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone levels are significantly elevated (20), and LH is also elevated eightfold in a global ARKO mouse model (7). We showed previously (18) that serum testosterone levels at 9 and 30 wk of age were not different between wild-type and AR^{lox} male mice, although wide variations in testosterone values were observed within the groups.

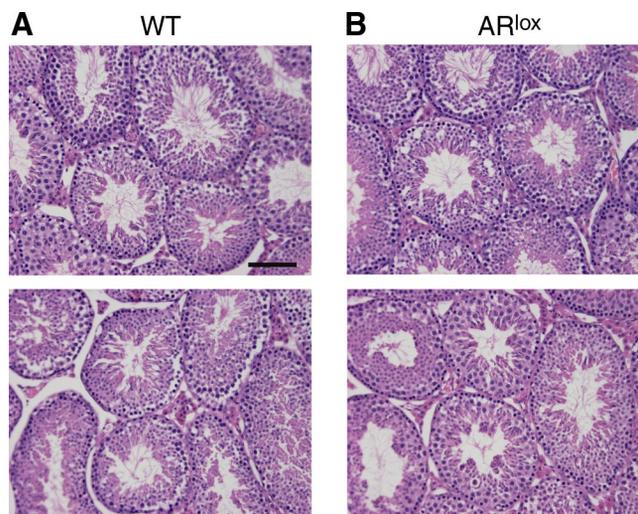


Fig. 3. Histological analysis of testis sections from 12-wk-old male mice. *A*: WT males. *B*: AR^{lox} males. Representative images from 2 mice/genotype are shown; sections were stained with hematoxylin and eosin. Bar, 50 μ m.

We extended these studies by measuring serum testosterone levels at 12 wk of age in an additional group of 23 wild-type and 24 AR^{lox} male mice. However, these data also showed no significant difference in serum testosterone between wild-type and AR^{lox} male mice (21.6 ± 6.5 nM wild type vs. 24.3 ± 3.8 nM AR^{lox} , means \pm SE). Our previous study (18) also showed no differences in serum LH or FSH levels between wild-type and AR^{lox} males, although the large intragroup variations mean that these assays will only detect large differences between groups. We have shown (1) that treatment of wild-type male mice with testosterone implants for 10 wk increases serum testosterone to an average of 54.9 nM and seminal vesicle mass is increased 250% compared with control treatment. Therefore, we cannot rule out the possibility that the milder 18.6% increase in seminal vesicle mass in the AR^{lox} mice is caused by a much more subtle elevation in testosterone levels that cannot be detected with serum assays. In addition, local production of 5α -dihydrotestosterone or weaker androgens could also potentially be increased, as occurs in some androgen-independent prostate cancers (22). However, it may be unlikely that these changes occur in the relatively normal cellular milieu of the

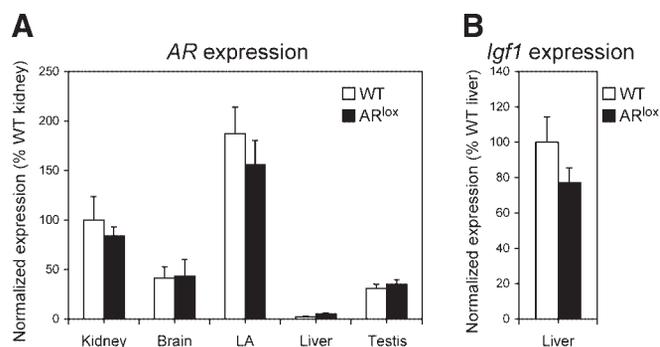


Fig. 4. Gene expression in 12-wk-old WT and AR^{lox} male mice quantitated with real-time PCR. *A*: *AR* mRNA levels in different tissues, normalized to expression in WT kidney. *B*: *Igf1* mRNA levels in liver, normalized to expression in WT liver. Data are means \pm SE; $n = 4$ –7/group.

AR^{lox} mice, as opposed to the adaptation to androgen deprivation occurring in prostate cancer cells.

No change in AR mRNA levels. As discussed above, the presence of the *neo* cassette in intron 3 could potentially disrupt AR mRNA transcription, splicing, or stability. This is observed in a number of cases where hypomorphic alleles are generated in floxed mice (13, 21, 26). Although perhaps less likely, the converse is also potentially true, that the *neo* cassette stabilizes the AR mRNA, because intronic sequences have previously demonstrated this capability (19). Therefore, an alternate hypothesis is that the phenotype of hyperandrogenization in the AR^{lox} male mice is caused by increased levels of AR mRNA leading to increased levels of AR protein in the presence of normal circulating levels of testosterone. To further investigate these alternate hypotheses we used quantitative real-time PCR to compare the levels of expression in 12-wk-old wild-type and AR^{lox} males. AR mRNA levels were quantitated in brain, kidney, liver, testis, and levator ani muscle and normalized to levels in wild-type kidney. Kidney and levator ani were examined because they represented two of the androgen-dependent tissues showing increased mass; brain and testis were examined because altered expression in these tissues could impact on testosterone production; and liver was examined because altered expression could impact on IGF-I production (11). In all these tissues, there was no significant difference between levels of AR mRNA in the wild-type and AR^{lox} male tissues (Fig. 4A). Thus the *neo* cassette in the floxed AR locus does not significantly alter the levels of AR mRNA in the tissues examined. This finding, combined with the fact that our global ARKO mice have normal serum IGF-I levels (data not shown) and the observation that *Igfl* gene expression was normal in the AR^{lox} liver (Fig. 4B), makes it unlikely that the AR^{lox} phenotype is caused by elevated IGF-I levels. It is also possible that the *neo* cassette and/or *loxP* sites could affect the AR gene or protein without altering mRNA levels, although the mechanism of these putative effects is not obvious.

Model of hyperandrogenization. To our knowledge, a phenotype of hyperandrogenization has not been described in men, despite the fact that over 600 unique mutations have been described in the human AR gene (10). However, hyperandrogenization, or androgen hypersensitivity, is observed in some hormone-independent prostate cancers (9). The potential effects of differences in testosterone levels within the normal male range are still controversial, with some studies suggesting differences including effects on fat mass, insulin sensitivity, muscle strength, bone density, lipid profile, or risk of prostate cancer (14, 16, 25, 28). Therefore, this hyperandrogenization model may be useful to further investigate the effect of a mild increase in androgen action in these androgen-responsive systems.

Our data demonstrate that male mice with a conditional targeted allele of the AR gene (AR^{lox} males) have an unexpected phenotype of hyperandrogenization. These differences occur in the absence of demonstrable changes in AR gene expression or serum hormone levels in AR^{lox} males compared with wild-type littermate controls. This lack of detectable difference in circulating testosterone and LH levels may be primarily due to the wide variation of both testosterone and LH levels among individual mice within the same genotype. Therefore, we believe that the most likely explanation of the AR^{lox} phenotype is that these mice have a mild increase in

serum testosterone levels due to a relative brain androgen insensitivity caused by a tissue-specific decrease in AR gene expression in the hypothalamus and pituitary. Because the hyperandrogenization phenotype of the AR^{lox} mice is the opposite of that resulting from loss of AR function, this does not invalidate this use of this AR^{lox} model in generating tissue-specific AR deletion with *cre/lox*. In fact, careful comparison of peripheral tissues from tissue-specific ARKO and AR^{lox} males may increase the probability of identifying modest effects of androgens that may be less apparent when compared with normal wild-type control males. This study reaffirms the need to investigate the potential phenotypic effects of floxed genes in the absence of *cre* in tissue-specific knockout studies. In addition, this hyperandrogenization model may be useful to further investigate the effects of subtle perturbations of androgen action in a range of androgen-responsive systems in the male.

ACKNOWLEDGMENTS

We thank David J. Handelsman for expert advice and helpful discussion. Present address of A. J. Notini: Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, VIC, 3052, Australia.

GRANTS

This research was supported by National Health and Medical Research Council of Australia (NHMRC) Project Grants 350334 and 350346 and an Eva and Les Erdi Major Research Grant. H. E. MacLean was supported by NHMRC Career Development Award 359226.

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