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ORIGINAL PAPER

Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells

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Abstracts Androgen and the androgen receptor (AR) have been shown to play critical roles in male fertility. Our previous data demonstrated that mice lacking AR ($AR^{-/y}$) revealed incomplete germ cell development and lowered serum testosterone levels, which resulted in azoospermia and infertility. However, the consequences of AR loss in Leydig cells remain largely unknown. Using a Cre-LoxP conditional knockout strategy, we generated a tissue-specific knockout mouse (L-$AR^{-/y}$) with the AR gene deleted by the anti-Müllerian hormone receptor-2 (Amhr2) promoter driven Cre expressed in Leydig cells. Phenotype analyses show that the outside appearance of L-$AR^{-/y}$ mice was indistinguishable from wild type mice ($AR^{+/y}$), but with atrophied testes and epididymis. L-$AR^{-/y}$ mice were infertile, with spermatogenic arrest predominately at the round spermatid stage and no sperm could be detected in the epididymis. L-$AR^{-/y}$ mice also have lower serum testosterone concentrations and higher serum leuteinizing hormone and follicle-stimulating hormone concentrations than $AR^{+/y}$ mice. Further mechanistic studies demonstrated that hypotestosteronemia in L-$AR^{-/y}$ mice is not caused by reducing numbers of Leydig cells, but instead by the alterations of several key steroidogenic enzymes, including 17β-HSD3, 3β-HSD6, and P450c17. Together, L-$AR^{-/y}$ mice provide in vivo evidence that functional AR in Leydig cells is essential to maintain normal spermatogenesis, testosterone production, and required for normal male fertility.

Keywords Androgen receptor · Leydig cell · Steroidogenesis · Spermatogenesis

Introduction

The primary function of the Leydig cell is the production of testosterone (T). Fertility in the sexually mature male is dependent on the postnatal development of adult Leydig cells, which is achieved by morphological differentiation from spindle-shaped progenitor cells. The progenitor cells first transform into round immature Leydig cells, further increase in size, mature into adult Leydig cells, and then acquire the capacity for T production [1]. The amount of T secreted by Leydig cells is determined by a balance between T biosynthetic and metabolizing enzyme expressions and activities. In general, the mRNA levels for T biosynthetic enzymes increase and androgen-metabolizing enzymes decline postnatally as adult Leydig cells mature [2–6].

The local actions of androgen on testicular function were initially demonstrated, when T alone, in the absence

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of the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH), could support spermatogenesis [7]. Androgen receptor (AR) [8–10] has been detected in Leydig, Sertoli, peritubular myoid, and spermatid cells (round and elongated) [11, 12], and mice lacking functional AR develop testicular feminization syndrome. Together, these observations indicated that androgen and AR play critical roles in male fertility [13]. We demonstrated that Sertoli specific AR knockout mice (S-AR$^{-/y}$) have incomplete germ cell development and low serum T levels, which result in azoospermia and infertility [14]. Given the important role of Leydig cells in the production of T, we were interested in generating Leydig cell-specific AR knockout (L-AR$^{-/y}$) mice. By mating floxed AR mice [15] with a transgenic line possessing anti-Müllerian hormone receptor-2 (Amhr2) promoter-driven expression of the Cre recombinase [16], we obtained male L-AR$^{-/y}$ mice.

In the present studies, analysis of L-AR$^{-/y}$ mice revealed an incomplete germ cell development and low serum T levels [17], which resulted in azoospermia and infertility. Mechanistic studies suggest that several key steroidogenic enzymes, including type 3 17-beta-hydroxysteroid dehydrogenase (17β-HSD3), 3β-HSD6, and P450c17 were affected, and might contribute to the defects in spermatogenesis and T production/secretion observed in L-AR$^{-/y}$ mice.

Materials and methods

Generation of L-AR$^{-/y}$ mice

Protocols for use of animals were in accordance with National Institutes of Health standards, and follow protocols approved by University of Rochester Department of Laboratorial Animal Medicine. Transgenic Amhr2-Cre (129 S/SvEv × C57BL/6) male mice expressing Cre recombinase, under the control of the Amhr2 gene promoter [16], were mated with floxed AR (129 S/SvEv × C57BL/6) female mice (Fig. 1). The generation of floxed AR gene-targeted mice has been described [15]. The expression of Amhr2 promoter-driven Cre recombinase can efficiently and selectively delete the floxed AR gene in Leydig cells [17]. L-AR$^{-/y}$ mice express floxed AR and Cre alleles in tail genomic DNA. Genomic DNA was isolated from tail snips and used as template for PCR with primers "select" and "2–3." The detailed methods and primer sequences have been described previously [18].

Fertility assessment

We investigated the reproductive capacities of L-AR$^{-/y}$ and wild-type (AR$^{+/y}$) mice by mating one male with two females for 2 wk in multiple trials. Female mice were checked for vaginal plugs each morning, and litter sizes were recorded on delivery, from three successive matings.

Evaluation of epididymal sperm

The epididymides were removed and minced in 1.5 ml of potassium-modified simplex optimized medium and 3% BSA for 30 min at 37°C, to release sperm into the medium. Spermatozoa were extracted from the whole epididymis, and the total sperm count was assessed in the final suspension by using a hemacytometer. Motility of at least 200 epididymal spermatozoa was assessed by means of light microscopy.

Propidium iodide staining and flow cytometry

Ethanol-fixed germ cells (1×10^6) from testes were washed twice in PBS and incubated in 500 µl of 0.2% pepsin for 10 min at 37°C. After centrifugation, the cells were stained with a solution containing 25 µg/ml propidium iodide, 40 µg/ml RNase, and 0.3% Tween 20 in PBS at room temperature for 20 min. The stained cells were analyzed by using a FACS Calibur flow cytometer (BD Immunocytometry Systems, San Jose, CA).

Immunohistochemistry, BrdU incorporation, and apoptosis assay

For immunohistochemistry, mouse testes were fixed overnight in 4% paraformaldehyde at room temperature. The tissue immunostained for AR was counterstained with hematoxylin to reveal the location of cell nuclei. The detailed procedure for immunohistochemistry has been described previously [14]. The proliferative activity of testicular cells was detected by Bromodeoxyuridine (BrdU) incorporation, using a BrdU labeling kit (Roche Applied Science, Indianapolis, IN). Apoptosis was evaluated by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay for in situ visualization of DNA fragmentation with commercial reagents (Roche Applied Science, Indianapolis, IN).

Assessment of serum hormone levels

Male L-AR$^{-/y}$, AR$^{-/y}$, and AR$^{+/y}$ mice were sacrificed at 12 wk. A mid-line sternotomy was performed and 1 ml of blood was drawn by cardiocenthesis. After 15 min of centrifugation at 3,000 × g, the serum was collected and

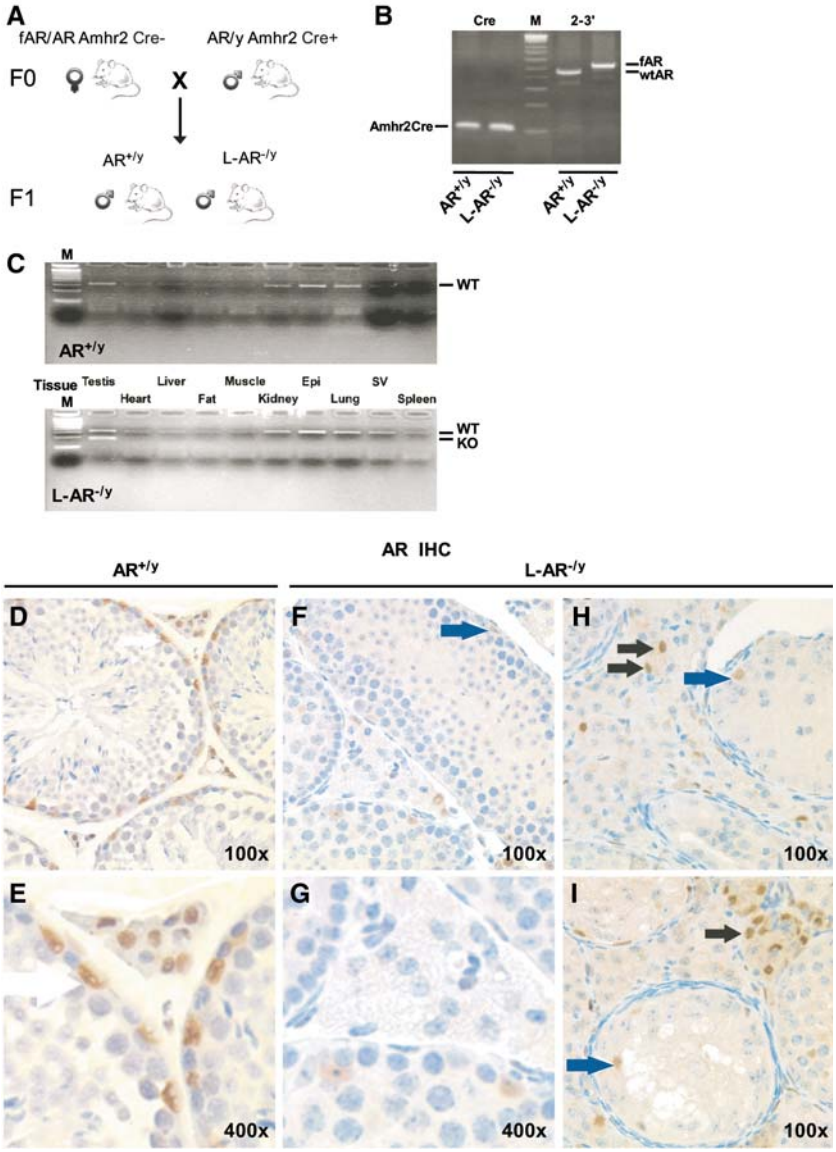

Fig. 1 Generation of mice with conditional knockout of AR in Leydig cells (L-AR$^{-/y}$). (**a**) Mating strategy to generate L-AR$^{-/y}$ mice. (**b**) Identification and confirmation of L-AR$^{-/y}$ mice. The expression of floxed AR and Cre in the tail genomic DNA of L-AR$^{-/y}$ mice was confirmed by PCR; (**c**) RT-PCR of various tissues harvested from L-AR$^{-/y}$ and AR$^{+/y}$ mice. Only the mRNA from the testes of the L-AR$^{-/y}$ mice show the truncated KO allele when specific primers are used; (**d**–**i**), Immunostaining of AR protein in testicular sections from AR$^{+/y}$ and L-AR$^{-/y}$, mice. Data are representative images from the two experimental groups (n = 5); (**d** and **e**), In AR$^{+/y}$ testis, AR staining was found in Sertoli cells and Leydig cells; (**f**–**i**) The L-AR$^{-/y}$ testis shows no or few AR staining (black arrows) in Leydig cells; however, Sertoli cells retain weak positive staining (dark blue arrow)

stored at –20°C until analysis. Total T, LH, and FSH levels were measured using ELISA kits (Assay Designs, Ann Arbor, MI, and Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions.

Cell culture

TM3 cells were derived from immature mouse Leydig cells (d14) [19] and were grown in a 1:1 mixture of F-12 medium and Dulbecco's modified Eagle medium (DMEM), supplemented with 4 mM glutamine and adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS). The cells were kept at 37°C in a humidified atmosphere containing air and carbon dioxide (95%/5% vol/vol). Before drug treatment, all transfected Leydig cells were steroid starved in F-12/DMEM (supplemented with 5% charcoal-stripped FBS) for 24 h. After steroid-starvation, the cells were treated with 1 nM DHT, or androgen-free F-12/DMEM media for 24 h.

Determination of leydig cell volume per testis

Sections of Bouin's fixed, paraffin-embedded testes were immunostained for 3β-HSD, and counterstained with hematoxylin. We determined the relative volume per testis for seminiferous tubule and interstitium using SigmaScan Pro software (ver.5, Systat, San Jose, CA). Volume of interstitium per testis was determined by multiplying the percentage value by fixed testis weight (volume), as shrinkage is minimal by using Bouin's fixation. The nuclei numbers of Leydig cells (3β-HSD positive) of equal fields

were counted and then numbers of Leydig cells are normalized by total volume of interstitium per testis. Three tissue sections (thickness, 5 μm) that were 10 μm apart and 200 cells per field were counted.

Real-time quantitative RT-PCR

Mouse testes from $AR^{+/y}$ and L-$AR^{-/y}$ mice were dissected, homogenized with an external luciferase control, and total RNA isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). cDNA synthesis and PCR were performed using SuperscriptTM RNase H-free Reverse Transcriptase and a cDNA cycle kit (Invitrogen Corp., Carlsbad, CA) in a 20 μl volume according to the manufacturer's instructions. Real-time PCR was performed using an icycler real-time PCR amplifier (Bio-Rad Laboratories, Hercules, CA). Each PCR reaction contained 1 μl cDNA, 50 μM each primer, and 12.5 μl iQTM SYBR green supermix reagent (Bio-Rad Laboratories, Hercules, CA) and was performed in triplicate.

Statistics

All data were expressed as mean ± SEM. Analysis was performed by two-tailed, unpaired Student's *t* test or by two-way ANOVA by using SigmaStat (Systat, San Jose, CA) as indicated in the figure legends. A P value ≤0.05 was considered significant.

Results

Generation of mice with conditional knockout of AR in leydig cells

Using a Cre-LoxP conditional knockout strategy, we mated (129 S/SvEv × C57BL/6) female floxed AR mice with male anti-Müllerian hormone receptor-2 (Amhr2)-Cre mice [16] to generate L-$AR^{-/y}$ mice and wild type ($AR^{+/y}$) littermates (Fig. 1a). In tail genomic DNA from 21-day-old L-$AR^{-/y}$ mice, we detected the Cre and floxed AR DNA fragments (Fig. 1b). In 12-wk-old L-$AR^{-/y}$ mice, we found that only the testes had a 260-bp DNA fragment of the AR knockout allele (Fig. 1c), suggesting that selective disruption of AR occurred in the testes.

We performed immunohistochemical staining to verify the deficient expression of AR using an anti-AR-ligand binding domain antibody (N-20). As a control, AR-positive staining in Leydig cells was found in testes of $AR^{+/y}$ mice (Fig. 1d and e). Some AR staining was found in the Leydig cells of L-$AR^{-/y}$ mice indicating that AR deficiency was not fully established and AR-positive staining was shown in the Sertoli cells within the seminiferous tubules of L-$AR^{-/y}$ mice (Fig. 1f and g). In L-$AR^{-/y}$ mice testes two patterns were observed including Sertoli cells only (SCO) syndrome and maturation arrest. In testes with maturation arrest pattern, we found significant deficiency of AR in both Leydig and Sertoli cells (Fig. 1f and g). Some Sertoli cells were AR-negative; however, some interstitium contained a few AR-immunoactive Leydig cells. In the testis showing SCO, most of the Leydig cells were negative and Sertoli cells were relatively weak in AR immunoactivity (Fig. 1h and i, dark blue arrows). A few Leydig cells had AR-positive nuclei (Fig. 1h, black arrows). Some AR-positive Leydig cells formed a small nest in the subcapsular region (Fig. 1i, black arrows). These findings suggested the knockout efficiency specific for Leydig cells by Amhr2 driven Cre strategy [16] was not fully established and Sertoli cells were influenced through unclear mechanisms.

Decreased testis size, epididymis size, and hypotestosteronemia in L-$AR^{-/y}$ mice

In order to assess the testicular development, body, testis, and epididymis weights were measured. There was no significant difference in the body weights (30.1 ± 1.5 g for $AR^{+/y}$ vs. 28.7 ± 0.9 g for L-$AR^{-/y}$) between 12-wk-old $AR^{+/y}$ and L-$AR^{-/y}$ mice. However, there was a significant reduction in testis (Fig. 2a) and epididymis (Fig. 2b) weights in L-$AR^{-/y}$ mice by 12-wks-old, L-$AR^{-/y}$ mice testes weigh only one-third that of the $AR^{+/y}$ mice, $n = 5$ per group (Fig. 2c). Weights of seminal vesicles and prostates were not significantly different in L-$AR^{-/y}$ mice compared to those in $AR^{+/y}$ mice (data not shown). In contrast, total knockout $AR^{-/y}$ mice have even smaller testes and no seminal vesicles, epididymides, or prostate [15]. In order to determine if endocrine abnormalities might underlie or contribute to the L-$AR^{-/y}$ mice phenotype, serum FSH, LH, and T were measured. Serum hormone assays show that L-$AR^{-/y}$ mice have lower serum testosterone levels (Fig. 2d), and higher serum LH and FSH levels, similar to $AR^{-/y}$ mice, as compared to $AR^{+/y}$ mice (Fig. 2e). In L-$AR^{-/y}$ mice, relative interstitial volume per testis was significantly increased when compared with $AR^{+/y}$ littermates (Fig. 2f), which could be due, in part, to increased ratios of Leydig cell contents per testis. L-$AR^{-/y}$ mice eventually develop comparable numbers of Leydig cells per testis (Fig. 2g and h).

Infertility with azoospermia in L-$AR^{-/y}$ mice

The first obvious functional defect of testes observed in L-$AR^{-/y}$ mice was azoospermia. In order to investigate the underlying cause for loss of fertility, 8-wk-old $AR^{+/y}$ and

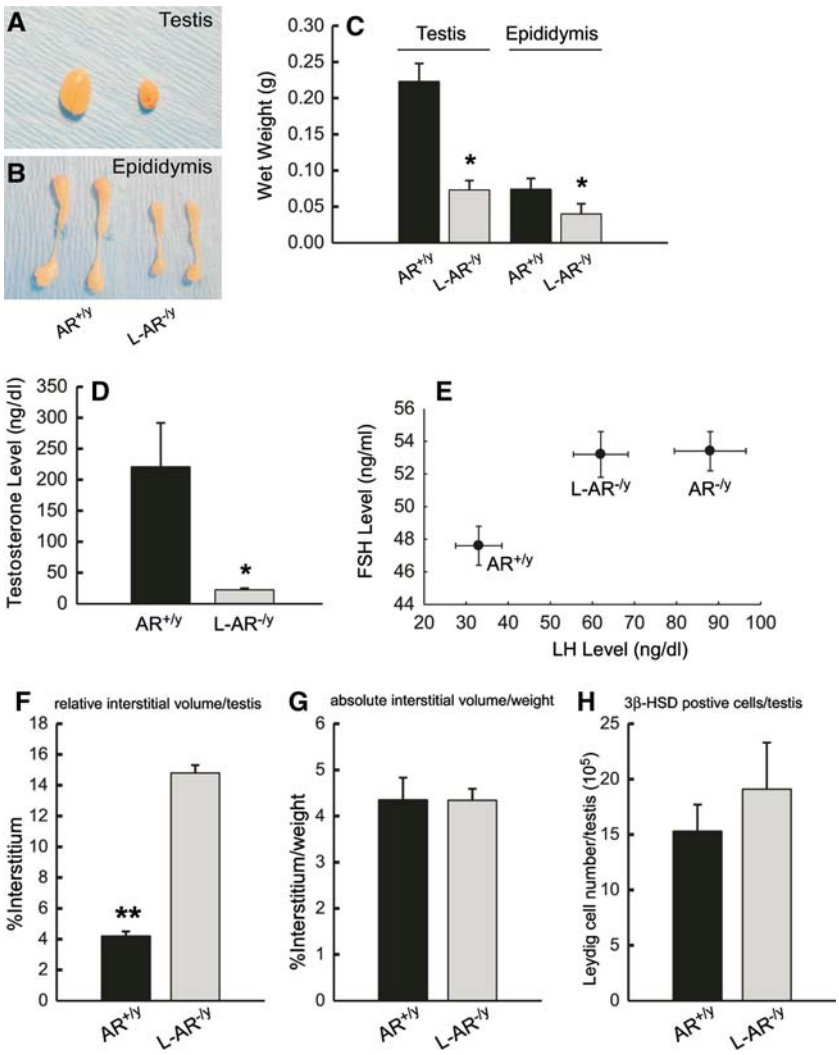

Fig. 2 Morphology of testes from 12-wk-old $AR^{+/y}$ and L-$AR^{-/y}$ mice. (**a** and **b**), Decreased testes and epididymides weight in L-$AR^{-/y}$ mice, Data are representative images from the two experimental groups ($n = 5$); (**c**) L-$AR^{-/y}$ mice have smaller testes and epididymides, which were one-third the size of that of $AR^{+/y}$; (**d**) Reduced serum T levels in L-$AR^{-/y}$ mice; (**e**) Compensatory elevation of LH in L-$AR^{-/y}$ mice reveals Leydig cell dysfunction. There was only a marginal change in FSH level in male L-$AR^{-/y}$ and $AR^{-/y}$ mice compared to the age-matched $AR^{+/y}$ mice ($n = 4$–5); (**f**), Relative interstitial volume (%) per testis was quantified from representative (**h** & **e**) sections of $AR^{+/y}$ and L-$AR^{-/y}$ testes; (**g**) Absolute volume (%) per interstitial weight was quantified using testes weight to normalize; (**h**) Absolute number per testis was quantified by number of 3β-HSD positive cells. $^*P < 0.05$, L-$AR^{-/y}$ versus $AR^{+/y}$; $^{**}P < 0.01$, L-$AR^{-/y}$ versus $AR^{+/y}$ (2-tailed, unpaired, t-test)

L-$AR^{-/y}$ mice were placed with fertile 8-wk-old C57BL/6 female mice. Although there were always vaginal plugs on the following morning after mating, which may suggest the libido is normal, L-$AR^{-/y}$ mice failed to impregnate their mates in three successive sets of 2-wk pairings (Table 1). As controls, the same female mice (after three sets of 2-wk-matings with L-$AR^{-/y}$ mice) were always impregnated after mating with $AR^{+/y}$ mice (Table 1; results from three female mice are shown). Furthermore, we observed the absence of sperm in the epididymides of L-$AR^{-/y}$ mice (data not shown). In contrast, we observed sperm with normal motility in the epididymides of $AR^{+/y}$ mice (Table 1). Therefore, the infertility of L-$AR^{-/y}$ mice is due to azoospermia.

Arrest of spermatogenesis predominately at the round spermatid stage in L-$AR^{-/y}$ mice

In order to determine why epididymal sperm are absent and if changes in mature germ cell number occur and underlie the defects in L-$AR^{-/y}$ mice testes, we analyzed spermatogenesis by determining the relative distribution of germ cell populations in the testes of L-$AR^{-/y}$ and $AR^{+/y}$ mice. Using flow cytometric scanning of propidium iodide-labeled germ cells, we detected three main histogram peaks of DNA content, which correspond to haploid (1N; spermatids and spermatozoa), diploid (2N; spermatogonia, preleptotene primary spermatocytes, and secondary spermatocytes), and tetraploid cells (4N; spermatogonia, leptotene, zygotene, pachytene, and diplotene primary spermatocytes) in $AR^{+/y}$ cells. Consistent with previous results [17], L-$AR^{-/y}$ mice germ cells show an increase in tetraploid cells and a reduction in cells with haploid DNA content, which included spermatids and spermatozoa (Fig. 3a). Testes from adult L-$AR^{-/y}$ male mice have several different histopathological patterns. The majority of testes showed maturation arrest at second spermatocyte or early round spermatid step. Focal accumulation of Sertoli cells was found in some seminiferous tubules. Leydig cells were relatively hypertrophic (Fig. 3e–i) as compared those

Table 1 Fertility test and epididymal content analysis

Genotype	Mate number			Vaginal plug	Sperm count	Motility %
	1	2	3			
$AR^{+/y}$	6.5 ± 1.0	7.5 ± 0.5	8.0 ± 0.5	+	2.56×10^7/ml	72
L-$AR^{-/y}$	0	0	0	+	0	0
T-$AR^{-/y}$	0	0	0	–	No epididymis	–

$AR^{-/y}$, general AR knockout mice

in testis of $AR^{+/y}$ mice (Fig. 3b–d). One extreme condition was the SCO syndrome. Almost no germ cell was found in the seminiferous tubule and Sertoli cells showed fibrillary degeneration (S in Fig. 3h). Interstitium increased and Leydig cells were hypertrophic and contained abundant foamy cytoplasm (L in Fig. 3h). Another atypical pattern was hypospermatogenesis. Elongated spermatids in the seminifeous epithelium display poor differentiation and reduced cellularity (Fig. 3g, arrows). Sertoli cells were relatively normal in morphology. Each of these patterns can also be found in the testicular pathology from infertile men with oligozoospermia or azoospermia [20]. In comparison, we found that spermatogenesis was arrested at the much earlier pachytene stage in total knockout $AR^{-/y}$ testes (data not shown), consistent with previous results [14].

Dysregulation of apoptosis and disruption of proliferation of germ cells in the L-$AR^{-/y}$ testis

In order to determine why L-$AR^{-/y}$ mice are infertile and azoospermic with spermatogenesis arrested predominately at the round spermatid stage, we compared testis cell proliferation and apoptosis in $AR^{+/y}$ and L-$AR^{-/y}$ mice. Results from BrdU incorporation studies show that positive BrdU staining was observed predominately in proliferating spermatogonia of $AR^{+/y}$ testes (Fig. 4a). In contrast, L-$AR^{-/y}$ testes have less proliferating spermatogonia (Fig. 4b). Overall, $AR^{+/y}$ testes have a greater number of proliferating cells per tubule and more tubules per testis. We also found that L-$AR^{-/y}$ testes contain fewer apoptotic pachytene and metaphase spermatocytes compared to $AR^{+/y}$ testes (Fig. 4e vs. d). Arrows indicate the positive TUNEL staining for apoptotic cells (Fig. 4e). This proliferative and apoptotic dysregulation of spermatogenesis likely contributes to the spermatogenic arrest that occurs mainly at the round spermatid stage after secondary meiosis in L-$AR^{-/y}$ mice.

Loss of AR leads to hypotestosteronemia that might be via reduction of specific steroidogenic genes expression

In order to understand how the loss of AR in Leydig cells results in hypotestosteronemia, we hypothesized that Leydig specific AR disruption causes reduced transcription of specific steroidogenic enzyme genes in Leydig cells and, consequently, reduced Leydig cell production/secretion of T. Real-time RT-PCR quantification demonstrated that testicular 17β-HSD3, 3β-HSD6, P450c17, and Relaxin-Like Factor (RLF) mRNA levels are lower, whereas Thrombospondin 2 (TSP2) and LH receptors (LHR) mRNA levels are higher in L-$AR^{-/y}$ mice compared to those from age-matched $AR^{+/y}$ mice (Fig. 5a; steroidogenic pathway illustrated). In addition, no significant changes in testicular mRNA levels of cytochrome P450 side-chain-cleavage (P450scc) and steroidogenic acute regulatory protein (StAR) were observed in L-$AR^{-/y}$ mice compared to those from age-matched $AR^{+/y}$ mice (Fig. 5a). The decreased mRNA expression of specific steroidogenic enzyme genes may suppress testosterone synthesis/secretion from Leydig cells. QPCR quantification in TM3 murine Leydig cells and TM3 murine Leydig cells with transient transfected functional AR demonstrated that testicular 17β-HSD3 and 3β-HSD6 mRNA levels are upregulated in TM3 cells with transfected functional AR upon DHT treatment (Fig. 5b). These results, both in vivo and in vitro, suggest that functional AR is essential in Leydig cells for key enzymes expression of steroidogenesis.

Discussion

The primary aim of the present study was to evaluate the effect of Leydig cell selective knockout of AR on the testicular function and development. AR deficiency in Leydig cells has a major effect on the phenotype of affected males, causing hypotestosteronemia and infertility. Spermatogenesis is arrested mainly at round spermatids and disrupted with a loss of normal germ cell development, due to hypotestosteronemia. These defects are consistent with abnormal adult Leydig cell development and impaired maturation in the absence of functional AR [21].

Leydig AR is essential for production of testosterone

T is the principal steroid produced and secreted by the testis, although numerous other C18, C19, and C21 steroids

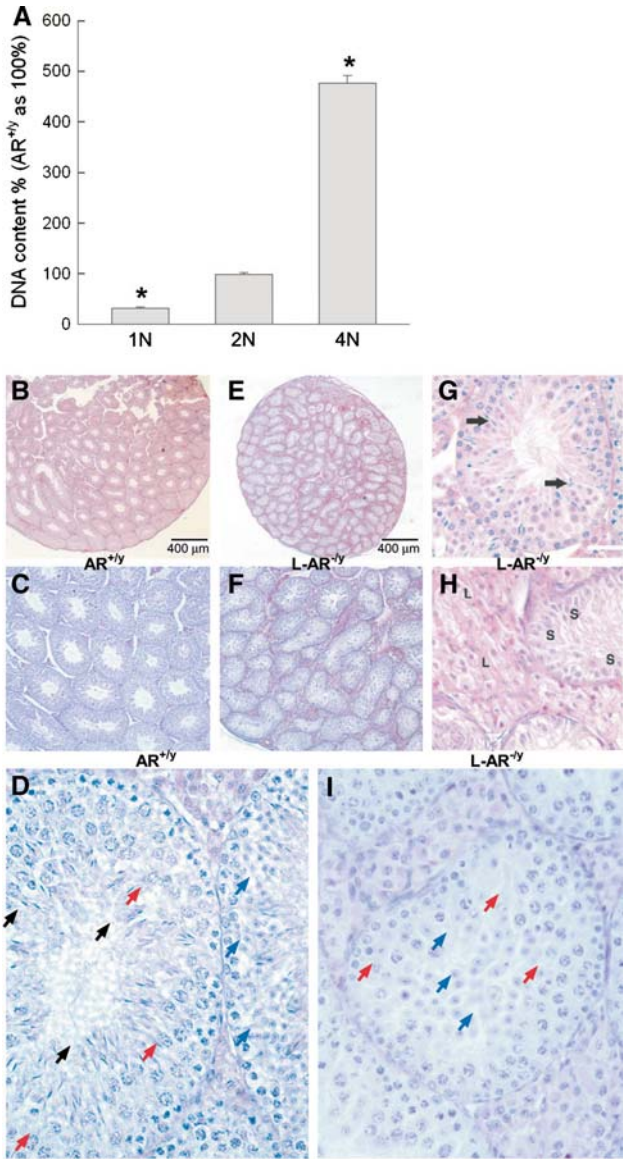

Fig. 3 Impaired spermatogenesis in L-AR$^{-/y}$ mice. (**a**) Analyses of germ cell DNA content of AR$^{+/y}$ and L-AR$^{-/y}$ mice using flow cytometry. 1N, haploid cells; 2N, diploid cells; and 4N, tetraploid cells ($n = 3$), $*P < 0.05$, L-AR$^{-/y}$ versus AR$^{+/y}$ (2-tailed, unpaired, *t*-test); (**b–d**) AR$^{+/y}$ testis at tubule stage XI; red arrows, diplotene spermatocytes; blue arrows, round spermatids; black arrows, spermatozoon; (**e–i**) The maturation of spermatocytes ceases in the round spermatid stage in L-AR$^{-/y}$ testes. No lumen formation is observed in tubules. Data are representative images from the two experimental groups ($n = 5$); (**g**) Elongated spermatids in the seminifeous epithelium display poor germ cell differentiation and reduced cellularity (arrows); (**h**) No germ cells were found in the seminiferous tubule and Sertoli cells (S) showed fibrillary degeneration. Interstitium increased and Leydig cells (L) were hypertrophic and contained abundant foamy cytoplasm; (**i**) Some segments of tubule contain many pachytene spermatocytes and diplotene spermatocytes (red arrows) can be found in the central region of some tubules. Apoptotic bodies (or degenerated germ cells were located in the stage with late diplotene or secondary spermatocytes. Round spermatids (blue arrows), which have escaped degeneration, are occasionally found in the central region of the tubule

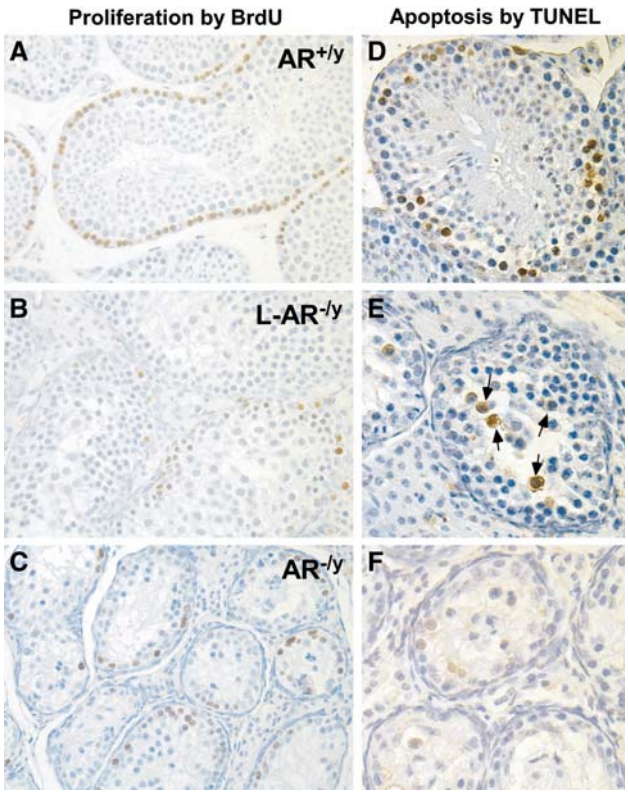

Fig. 4 Apoptosis and proliferative activity of AR$^{+/y}$, L-AR$^{-/y}$, and AR$^{-/y}$ testes. (**a**, **b**, and **c**) Proliferative activity of germ cells detected in 12-wk-old male AR$^{+/y}$, L-AR$^{-/y}$, and AR$^{-/y}$ testes, respectively; (**d**, **e**, and **f**) Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay in 12-wk-old male AR$^{+/y}$, L-AR$^{-/y}$, and AR$^{-/y}$ testes to detect the apoptotic cells (arrows), respectively. Data are representative images from the two experimental groups ($n = 4$)

are also produced [22–24]. In L-AR$^{-/y}$ mice the higher LH serum levels and LHR mRNA levels, subsequently induce expression of P450scc and StAR. However, other enzymes related to steroidogenesis including 3β-HSD, 17-βHSD, and P450c17 are decreased in L-AR$^{-/y}$ mice, suggesting the failure of adult Leydig cells maturation. This results in decreasing T production and illustrates the AR role in steroidogenic function of Leydig cells. The LH and FSH serum levels in L-AR$^{-/y}$ mice, like AR$^{-/y}$ mice, are both higher than AR$^{+/y}$ mice, suggesting lack of negative feedback due to hypotestosteronemia. The FSH level did not dramatically change in S-AR$^{-/y}$ mice, but the T level was decreased by three-fold. These data also demonstrate Leydig cell function is affected by the Sertoli cell, but the mechanism is unclear [14, 25].

Leydig AR is essential for normal spermatogenesis

The present studies show that spermatogenesis in L-AR$^{-/y}$ is arrested predominately at the round spermatid stage. In

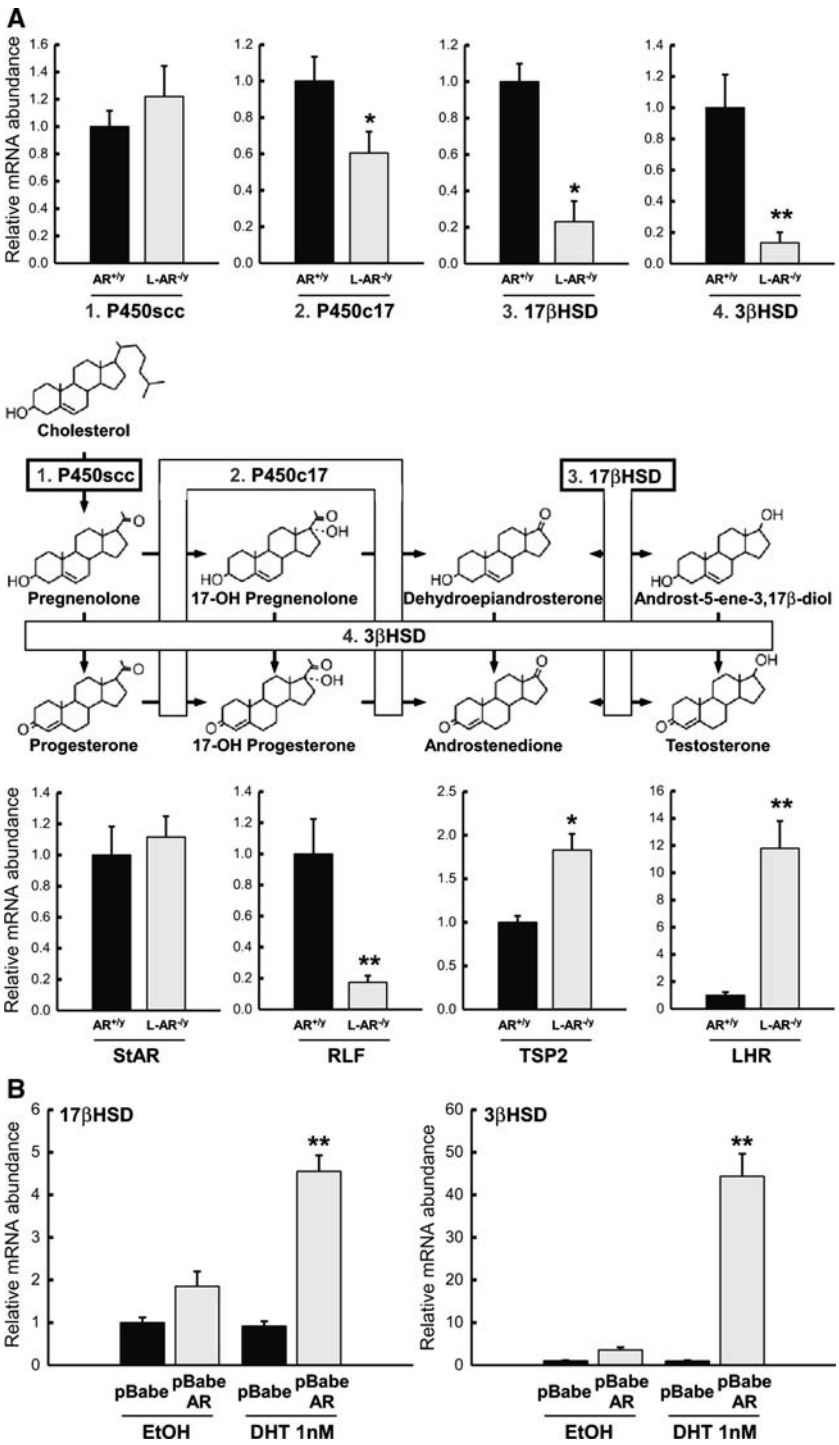

Fig. 5 Expression analysis of testicular genes involved in steridogenesis and Leydig cells maturation. (**a**) Quantitative Real-time RT-PCR was performed using RNA from 12-wk-old $AR^{+/y}$ and L-$AR^{-/y}$ testes using specific primer pairs ($n = 4$). $*P < 0.05$, L-$AR^{-/y}$ versus $AR^{+/y}$; $**P < 0.01$, L-$AR^{-/y}$ versus $AR^{+/y}$ (2-tailed, unpaired, *t*-test); (**b**) Quantitative Real-time RT-PCR was performed using RNA from TM3 murine Leydig cells and TM3 murine Leydig cells with transfected functional AR using specific primer pairs ($n = 3$). $**P < 0.01$, pBabeAR w/ DHT versus pBabe vector w/ DHT (ANOVA)

LHR knockout mice, spermatogenesis is also arrested at the round spermatid stage, adult-type Leydig cells are absent, T production is dramatically decreased, the animals are cryptorchid, and their accessory sex organs are atrophic [26]. In contrast, in L-$AR^{-/y}$ mice, significant increased serum levels of LH and testicular LHR mRNA levels, and similar arrested stage of spermatogenesis were observed. LH plays a crucial role in mediating the activity of Leydig cells in the testis. The LHR is expressed in the testis mainly in Leydig cells and stimulates steroidogenesis. In addition, LH is the primary factor required to maintain Leydig cell structure and fully differentiated function. Hypophysectomy or inhibition of LH secretion causes an atrophy of Leydig cells, a loss in their ability to secrete T, and a decrease of the number of LHR per Leydig cell [27]. Moreover, it has been suggested that LH or hCG is required

for Leydig cell proliferation and differentiation during fetal life and at puberty [28]. Although L-AR$^{-/y}$ mice have both higher LH serum levels and LHR expressions, maturation of adult Leydig cells are impaired indicating functional AR plays an essential role in establishment of a normal cohort of adult Leydig cells.

Comparison of spermatogenesis and testicular structure of AR$^{-/y}$, G-AR$^{-/y}$, S-AR$^{-/y}$, P-AR$^{-/y}$, and L-AR$^{-/y}$ mice

In the testis, AR is expressed in the somatic Leydig, Sertoli, and peritubular myoid cells. Some groups have reported the presence of AR within mouse fetal and postnatal germ cells, and rat spermatids [12, 29]. In order to examine the consequences of loss of AR function specifically in Leydig, Sertoli, peritubular myoid, and germ cells, we have created conditional alleles of the *Ar* gene, and used them to generate Leydig, Sertoli [14, 30], peritubular myoid [31], and germ [17] cell-specific ablation of *Ar*. The results of theses studies suggest there are multiple AR-dependent steps during spermatogenesis. Taken together, the observations from these studies reveal a differential requirement for AR activity in different cell types for at least three steps of spermatogenesis. AR is first required for progression through meiosis I, again during the transition from the round to elongated stages of spermatogenesis, and finally during the terminal stages of spermiogenesis. However, whether AR function in Sertoli cells also affects steroidogenesis through paracrinic crosstalking between different types of cells remains unclear. Interestingly, we found that knockout effectiveness in Leydig cells couldn't be fully established using Amhr2 Cre strategy [16] and Sertoli cells were influenced through unclear mechanisms. However, the spermatogenesis arrest later in L-AR$^{-/y}$ mice than that in S-AR$^{-/y}$ mice indicating function of Sertoli cells retains a certain degree and reduced T levels may account for the arrest of spermatogenesis at the stage of round spermatids [32].

Implication in male fertility and testicular biology

There are two growth phases of Leydig cells during testicular development in mammals [33]. In the first phase, fetal Leydig cells, which originate prenatally after testis differentiation in utero, produce androgen for fetal masculinization [34]. The second generation of Leydig cells appears postnatally along with puberty [21, 35]. The adult Leydig cells originate from undifferentiated fibroblast-like or mesenchymal progenitors in the testicular interstitium [36]. During normal testis development in rodents, adult Leydig cells first appear shortly before postnatal day 10, and there is a marked, LH-dependent, increase in adult Leydig cell number between postnatal days 10 and 20 [5, 37]. This is followed by a further increase between postnatal day 20 and adulthood, which establishes the normal adult cohort of cells. By using a Cre-Lox conditional knockout strategy, the AR gene disruption was effectively established after activation of the Amhr2 driven Cre expression in Leydig cells of the embryonic testes by embryonic day 11.5 [16]. In L-AR$^{-/y}$ mice, Leydig cell number was established as those in AR$^{+/y}$ mice; showing that establishment of the Leydig cell number is not androgen dependent. Hardy et al showed that AR and androgen are required postnatally for adult Leydig cell proliferation and maturation along with LH [38, 39]. RLF [5] is expressed in the rodent adult Leydig cell population, but not in the fetal population, and TSP2 [5] is expressed only in the fetal Leydig cell population. In L-AR$^{-/y}$ mice, testicular mRNA expression of RLF was reduced and TSP2 was increased indicating that the pre-pubertal rise in Leydig cell number was normal, but later adult Leydig cell differentiation was significantly attenuated. After postnatal day 20, Leydig cell number increased in both AR$^{+/y}$ and L-AR$^{-/y}$ mice, indicating that only the later part of the postnatal developmental process, which establishes differentiated adult Leydig cells, is androgen dependent [40]. Results from this study underline the importance of measuring Leydig cell number directly rather than inferring changes from apparent interstitial hyperplasia in L-AR$^{-/y}$ mice, largely because of reduced germ cell volume per testis.

In summary, our results provide in vivo evidence showing that the knockout of AR in Leydig cells affects both spermatogenesis and steroidogenesis, as well as expressions of several key steroidogenic enzymes, including 17β-HSD3, 3β-HSD6, and P450c17, which might contribute to the defective phenotype observed in L-AR$^{-/y}$ mice.

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