Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells

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Androgens and the androgen receptor (AR) play important roles in male fertility, although the detailed mechanisms, particularly how androgen/AR influences spermatogenesis in particular cell types, remain unclear. Using a Cre-Lox conditional knockout strategy, we generated a tissue-specific knockout mouse with the AR gene deleted only in Sertoli cells (S-AR$^{-/y}$). Phenotype analyses show the S-AR$^{-/y}$ mice were indistinguishable from WT AR mice (B6 AR$^{+/y}$) with the exception of testes, which were significantly atrophied. S-AR$^{-/y}$ mice were infertile, with spermatogenic arrest predominately at the diplotene premeiotic stage and almost no sperm detected in the epididymides. S-AR$^{-/y}$ mice also have lower serum testosterone concentrations and higher serum leuteinizing hormone concentrations than B6 AR$^{+/y}$ mice. Further mechanistic studies demonstrated that S-AR$^{-/y}$ mice have defects in the expression of anti-Müllerian hormone, androgen-binding protein, cyclin A1, and sperm-1, which play important roles in the control of spermatogenesis and/or steroidogenesis. Together, our Sertoli cell-specific AR knockout mice provide *in vivo* evidence of the need for functional AR in Sertoli cells to maintain normal spermatogenesis and testosterone production, and ensure normal male fertility.

knockout mice | anti-Müllerian hormone | testosterone

Sertoli cells are the first cell type to become recognizably differentiated in the fetal gonad, an event which enables seminiferous cord formation, prevents germ cell entry into meiosis/differentiation, and enables the function of Leydig cells (1). Before puberty, in an environment with relatively low levels of androgen, Sertoli cells specifically secrete anti-Müllerian hormone (AMH), also named Müllerian inhibiting substance, which ensures regression of the Müllerian duct (1). At puberty, the Sertoli cell responds to androgen and takes on roles supporting spermatogenesis, and likely affects the function of the steroid-producing Leydig cells through complex cell–cell interactions.

Spermatogenesis is a multistep process, with mature spermatozoa developing from spermatogonia, the stem cells of the germ cell lineage (2). This process involves the complex interaction of germ cells and Sertoli cells within the seminiferous tubules (3, 4). Coculture of Sertoli cells with spermatogenic cells results in stimulation of germ cell RNA and DNA synthesis (5), induction of germ cell surface antigen presentation (6), and maintenance of glutathione synthesis in developing germ cells (7).

In addition to interacting with and providing essential cellular factors to germ cells, Sertoli cells are able to functionally communicate with Leydig cells. The location of Leydig cells in the interstitium between seminiferous tubules, and the presence of peritubular myoid cells at the basement membrane of the seminiferous tubules prevent direct physical contact between Sertoli and Leydig cells. However, damage to seminiferous tubule function, with cytotoxic agents or fetal irradiation, causes abnormal cytological features and function in adjacent Leydig cells (8), suggesting that a regulatory interaction exists between Sertoli cells and Leydig cells.

Androgen and the androgen receptor (AR) (9–12) have been shown to play critical roles in testis function (13). The local actions of androgen on testis function were initially demonstrated when testosterone alone, in the absence of the gonadotropins, leuteinizing hormone (LH), and follicle-stimulating hormone (FSH), could support spermatogenesis (14). AR has been detected in Sertoli, Leydig, peritubular myoid, and spermatid cells (round and elongated) (15, 16), and mice lacking functional AR develop testicular feminization syndrome (17). However, the consequences of AR loss in particular types of testicular cells remain largely unknown.

Given the central role of Sertoli cells in development of functional testes (1), we were interested in generating Sertoli cell-specific AR knockout (S-AR$^{-/y}$) mice, using the AMH-Cre/Lox system (17–20). By mating floxed AR mice (17) with a transgenic line possessing AMH promoter-driven expression of the Cre recombinase (20), we obtained male S-AR$^{-/y}$ mice with the AR gene deleted only in Sertoli cells.

Analysis of S-AR$^{-/y}$ mice revealed incomplete germ cell development and lowered serum testosterone levels, which resulted in azoospermia and infertility. Mechanistic studies further suggest that several key molecules, including AMH, androgen-binding protein (ABP), cyclin A1, and sperm-1, but not insulin-like growth factor 1 (IGF-1) or IGF-1 receptor (IGF-1R), may contribute to the defective spermatogenesis and testosterone production/secretion observed in S-AR$^{-/y}$ mice.

Methods

Generation of Sertoli Cell-Specific AR$^{-/y}$ Mice. Protocols for use of animals were in accordance with National Institutes of Health standards. Transgenic AMH-Cre (C57-B6/SJL) male mice expressing Cre recombinase, under the control of the AMH gene promoter (20), were mated with floxed AR (C57-B6/129 SEVE) female mice (Fig. 1). The generation of floxed AR gene-targeted mice has been described (17, 18). The expression of AMH promoter-driven Cre recombinase can efficiently and selectively delete the floxed AR gene in Sertoli cells. S-AR$^{-/y}$ mice express floxed AR and Cre alleles in tail genomic DNA. We genotyped 21-day-old pups from tail snips by PCR as described (17, 18).

Fertility Assessment. We investigated the reproductive capacities of S-AR$^{-/y}$ and WT mice by mating one male with two females for 2 wk. Female mice were checked for vaginal plugs each

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Abbreviations: ABP, androgen-binding protein; AMH, anti-Müllerian hormone; AR, androgen receptor; FSH, follicle-stimulating hormone; IGF-1, insulin-like growth factor 1; IGF-1R, IGF-1 receptor; LH, leuteinizing hormone; S-AR, Sertoli cell-specific AR.

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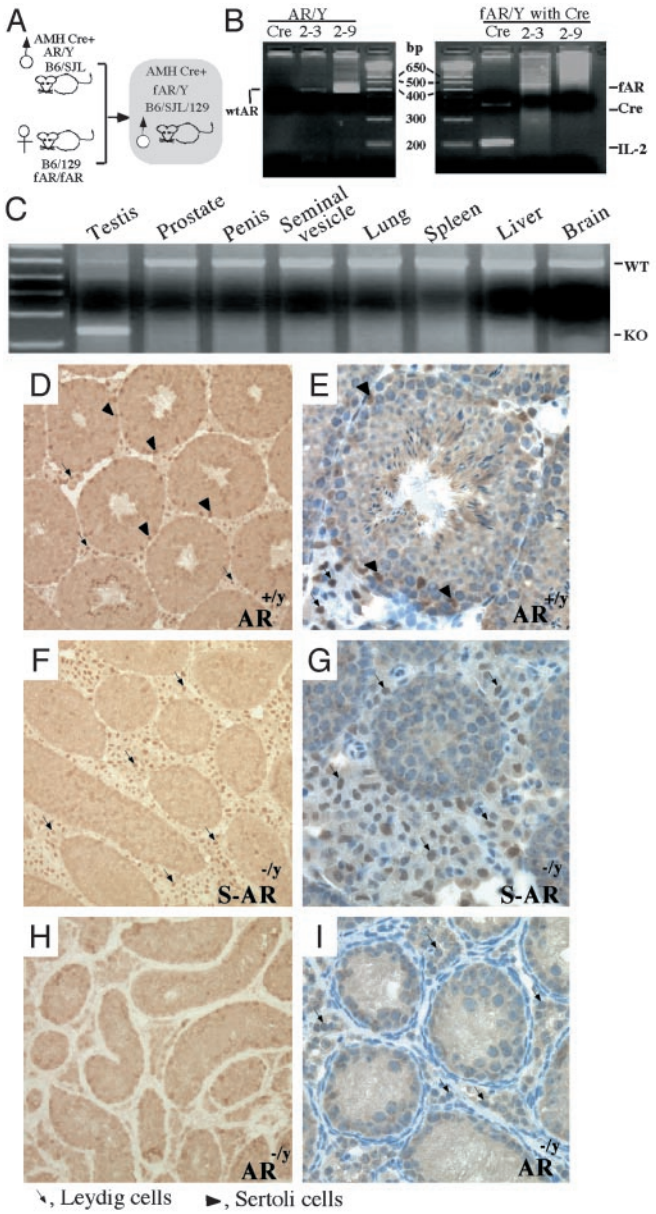

Fig. 1. Generation of mice with conditional knockout of AR in Sertoli cells (S-AR$^{-/y}$). (*A*) Mating strategy to generate S-AR$^{-/y}$ mice. (*B*) Identification and confirmation of S-AR$^{-/y}$ mice. Genomic DNA was isolated from tail snips and used as template for PCR with primers ''select and 2–3'' and ''select and 2–9.'' The detailed method and primer sequences have been described (17, 18). The expression of floxed AR, Cre, and internal control IL-2 in the tail genomic DNA of S-AR$^{-/y}$ mice was confirmed by PCR. (*C*) Genotyping of various tissues harvested from S-AR$^{-/y}$ mice. Only the genomic DNA from the testes of the S-AR$^{-/y}$ mice show the recombinant allele when primers ''select and 2–9'' are used. (*D–I*) Immunostaining of AR protein in testicular sections from AR$^{+/y}$, S-AR$^{-/y}$, and AR$^{-/y}$ mice. (*D* and *E*) In AR$^{+/y}$ testis, AR staining was found in Sertoli cells and Leydig cells. (*F* and *G*) The S-AR$^{-/y}$ testis shows no AR staining in Sertoli cells; however, Leydig cells retain positive staining (arrows). (*H* and *I*) There is no AR staining found in the testis of AR$^{-/y}$ mice. Arrows indicate the location and negative AR staining of Leydig cells. (*E*, *G*, and *I*) The tissue immunostained for AR is counterstained with hematoxylin to reveal the location of cell nuclei.

morning, and litter sizes were recorded on delivery, after three successive matings (21).

Evaluation of Epididymal Sperm. The epididymides were removed and minced in 1.5 ml of KSOM 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. Vitality and motility of at least 200 epididymal spermatozoa were assessed by means of light microscopy (22).

Propidium Iodide Staining and Flow Cytometry. Ethanol-fixed germ cells (1×10^6) 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 FACScan flow cytometer (BD Immunocytometry Systems) (23).

Immunohistochemistry, BrdUrd Incorporation, and Apoptosis Assay. For immunohistochemistry, mouse testes were fixed overnight in 4% paraformaldehyde at room temperature. The detailed procedure for immunohistochemistry has been described (18). The proliferative activity of testicular cells was detected by BrdUrd incorporation, using a BrdUrd labeling kit (Roche Applied Science). 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). The positive signals were counted and averaged from 30 seminiferous tubules from testicular sections (22).

Assessment of Serum Hormone Levels. Male S-AR$^{-/y}$, AR$^{-/y}$, and B6 AR$^{+/y}$ mice were killed 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 $\times$ *g*, the serum was collected and stored at −20°C before analysis. Total testosterone, LH, and FSH levels were measured by using ELISA kits (Assay Designs, Ann Arbor, MI, and Amersham Biosciences).

Results

Generation of Mice with Conditional Knockout of AR in Sertoli Cells. Using a Cre-Lox conditional knockout strategy, we mated C57-B6/129 SEVE female floxed AR mice with male AMH-Cre C57-B6/SJL mice to generate S-AR$^{-/y}$ (Fig. 1*A*). In tail genomic DNA from 21-day-old S-AR$^{-/y}$ mice, we detected Cre and the floxed AR DNA fragment (Fig. 1*B*). In 12-wk-old S-AR$^{-/y}$ mice, only the testes revealed a recombined 260-bp DNA fragment of the AR knockout allele (Fig. 1*C*), suggesting that selective disruption of AR occurred in the testes. Immunohistochemical staining with an anti-AR-ligand binding domain antibody was applied to confirm that the loss of AR was specific to Sertoli cells. As a control, AR-positive staining in Sertoli cells was found in AR$^{+/y}$ testes (Fig. 1 *D* and *E*), and no positive staining was found in Sertoli, Leydig, or germ cells in total knockout AR$^{-/y}$ testes (Fig. 1 *H* and *I*). As shown in Fig. 1 *F* and *G*, AR-positive staining was shown in Leydig cells of S-AR$^{-/y}$ mice, and in contrast, no AR staining was found in Sertoli cells within the seminiferous tubules of S-AR$^{-/y}$ mice, suggesting that AR expression was lost specifically in Sertoli cells. Together, results from genotyping of genomic DNA and immunohistochemical staining reveal that the AR gene was specifically knocked out in Sertoli cells of S-AR$^{-/y}$ mice.

Infertility with Azoospermia in S-AR$^{-/y}$ Mice. To evaluate fertility in S-AR$^{-/y}$ mice, three each of 8-wk-old AR$^{+/y}$ and S-AR$^{-/y}$ mice were mated with 8-wk-old C57BL/6 female mice. Although there were always vaginal plugs the morning after mating, S-AR$^{-/y}$ mice failed to impregnate their mates in three successive sets of 2-wk pairings, with six different female mice (see the fertility assessment in Table 1). As a control, these same female mice (after three sets of 2-wk-matings with S-AR$^{-/y}$) were always impregnated after mating with AR$^{+/y}$ mice (fertility assessment in Table 1; results from three female mice are shown). Furthermore, in analysis of epididymal sperm we observed that only debris was present in

Table 1. Fertility assessment and epididymal content analysis

Mice	Mate no. 1	2	3	Vaginal plug	Sperm count	Motility, %	Vitality, %
$AR^{+/y}$	8.5 ± 0.5	7.5 ± 0.5	7.0 ± 1.0	+	12.2×10^6 per ml	48	54
S-$AR^{-/y}$	0	0	0	+	0	0	0
$AR^{-/y}$	0	0	0	−	No epididymis		

S-$AR^{-/y}$ mice (data not shown). In contrast, we observed active sperm with normal motility and vitality in the epididymides of $AR^{+/y}$ mice (see the epididymal content analysis in Table 1). Together, results from Table 1 show that S-$AR^{-/y}$ mice are infertile and azoospermic.

Decreased Testis Size and Hypotestosteronemia in S-$AR^{-/y}$ Mice. Phenotype analyses show there was no difference in most of the genitourinary organs or in total body weight (30 ± 1.5 g for $AR^{+/y}$ vs. 29.5 ± 1.7 g for S-$AR^{-/y}$) between 12-wk-old B6 $AR^{+/y}$ and S-$AR^{-/y}$ mice. However, the size of the testis in S-$AR^{-/y}$ mice is only one-third that of the $AR^{+/y}$ mice (0.07 ± 0.02 vs. 0.24 ± 0.06 g for 12-wk-old S-$AR^{-/y}$ vs. $AR^{+/y}$ mice). In contrast, the total knockout $AR^{-/y}$ mice have even smaller testes and no seminal vesicles, epididymi, or prostate (Fig. 2*A*). Serum hormone assays show that S-$AR^{-/y}$ mice have lower serum testosterone levels (Fig. 2*B*), a compensatory increase in ABP expression (Fig. 2*C*), and higher serum LH levels but marginally increased (10%) FSH serum levels as compared to B6 $AR^{+/y}$ mice (Fig. 2 *D* and *E*).

Arrest of Spermatogenesis Predominately at the Premeiotic Diplotene Stage in S-$AR^{-/y}$ Mice. To determine why epididymal sperm are absent in S-$AR^{-/y}$ mice, we analyzed spermatogenesis by determining the relative distribution of germ cell populations in the testes of S-$AR^{-/y}$ and $AR^{+/y}$ mice. Using flow cytometric scanning of propidium iodide-labeled $AR^{+/y}$ germ cells, we detected three main histogram peaks of DNA content, which correspond to haploid (spermatids and spermatazoa), diploid (spermatogonia, preleptotene primary spermatocytes, and secondary spermatocytes), and tetraploid cells (spermatogonia, leptotene, zygotene, pachytene, and diplotene primary spermatocytes). S-$AR^{-/y}$ mice show an increase in tetraploid cells and a reduction in cells with haploid DNA content. The diploid to tetraploid ratio found in S-$AR^{-/y}$ mice suggests that spermatogenic arrest occurs in S-$AR^{-/y}$ mice before the first meiosis (Fig. 3*A*). This conclusion is further supported by dramatically decreased active-binding capping protein α3 mRNA in the S-$AR^{-/y}$ testis (Fig. 3*B*). Earlier studies demonstrated that capping protein α3 was expressed specifically in haploid germ cells (25), and functional expression of capping protein α3 has been linked to acrosome development during spermiogenesis (26). The lack of capping protein α3 expression supports our finding of a loss of mature germ cells that would have undergone acrosome development.

Finally, results from Fig. 3 *C–H* demonstrate very little evidence of meiosis and no spermatids or spermatozoa, either elongated or round, in the S-$AR^{-/y}$ mouse testis. In contrast to $AR^{+/y}$ mice (Fig. 3*C*), the seminiferous tubules of S-$AR^{-/y}$ mice display poor germ cell differentiation and reduced cellularity, with most germ cell maturation ceasing at the diplotene stage before the first meiotic division (Fig. 3 *F–H*). In comparison, we found that spermatogenesis was arrested at the pachytene stage in total knockout $AR^{-/y}$ testes (Fig. 3*E*), in agreement with previous findings (17).

Enhancement of Apoptosis and Disruption of Proliferation of Germ Cells in the S-$AR^{-/y}$ Testis. To further determine why S-$AR^{-/y}$ mice are infertile and azoospermatic, with spermatogenesis arrested at the premeiotic diplotene stage, we compared testis cell proliferation and apoptosis between $AR^{+/y}$ and S-$AR^{-/y}$ mice. Results from BrdUrd staining suggest that $AR^{+/y}$ testes have more active cellular proliferation per tubule and more proliferative tubules per testis. Positive BrdUrd signal was observed predominately in spermatogonia (Fig. 4*A*), the proliferative germ cell type, as expected. In contrast, S-$AR^{-/y}$ testes have fewer proliferating spermatogonia (Fig. 4*B*). We also found that S-$AR^{-/y}$ testes contain increases in apoptotic pachytene and metaphase spermatocytes compared to $AR^{+/y}$ testes (Fig. 4 *E* vs. *D*). Arrowheads indicate the positive TUNEL staining for apoptotic cells. This proliferative (Fig. 4*C*) and

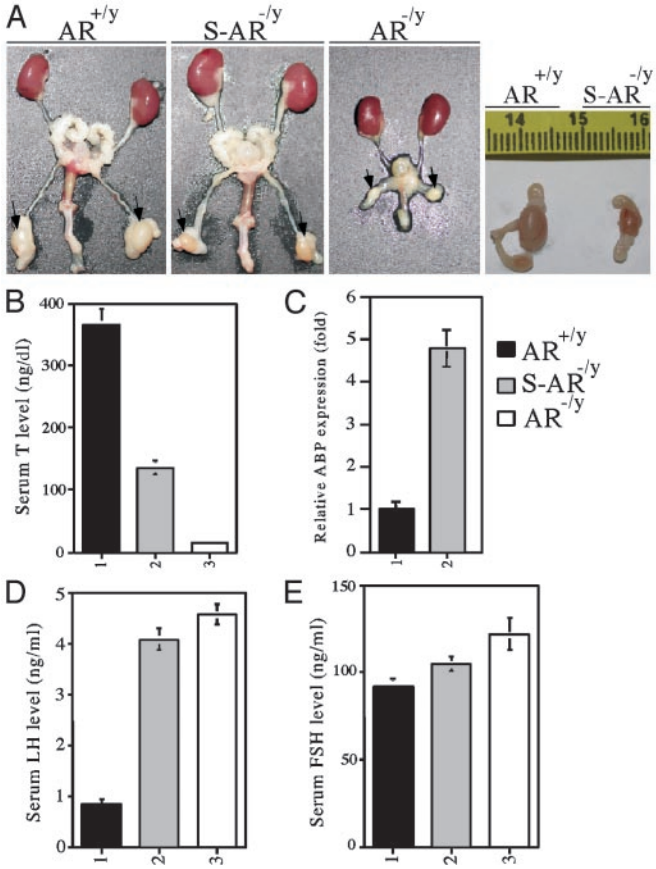

Fig. 2. Morphology of testes from 12-wk-old B6 $AR^{+/y}$, S-$AR^{-/y}$, and $AR^{-/y}$ mice. (*A*) Decreased testes size in S-$AR^{-/y}$ mice. S-$AR^{-/y}$ mice have genitourinary organs of similar size as $AR^{+/y}$ mice except for smaller testes, which were one-third the size of $AR^{+/y}$ testes. In contrast, there are small testes but no seminal vesicle, epididymi, or prostate in $AR^{-/y}$ mice. Arrows indicate testes. (*B*) Serum testosterone levels in 12-wk-old male B6 $AR^{+/y}$, S-$AR^{-/y}$, and $AR^{-/y}$ mice. S-$AR^{-/y}$ and $AR^{-/y}$ mice have lower serum testosterone levels than B6 $AR^{+/y}$ mice. (*C*) Increased expression of ABP is found in S-$AR^{-/y}$ mice. Total RNA was extracted from testes of 12-wk-old $AR^{+/y}$ or S-$AR^{-/y}$ mice and quantitated by real-time PCR (18, 24). The primers for ABP are 5′-CTGCTTCTGCTGTTGCTACTA-3′ and 5′-TTGGTGAGGTCAATGGTCATAAC-3′. Our results indicate increased expression of ABP mRNA in S-$AR^{-/y}$ testis. (*D*) Compensatory elevation of LH in S-$AR^{-/y}$ mice reveals Leydig cell dysfunction. (*E*) There was only a marginal change in FSH level in male S-$AR^{-/y}$ and $AR^{-/y}$ mice compared to the age-matched $AR^{+/y}$ mice.

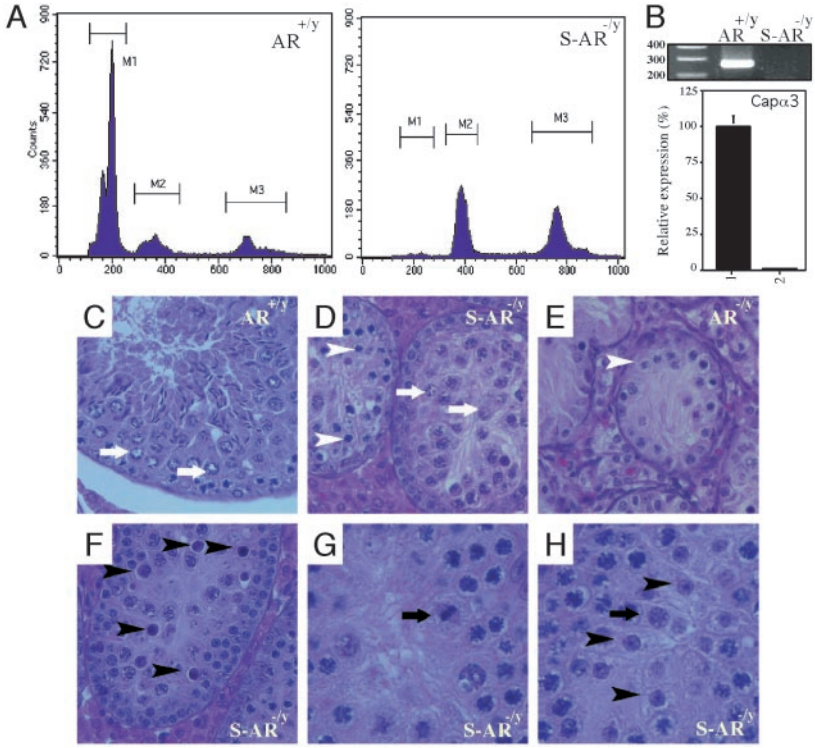

Fig. 3. (*A*) Analyses of germ cell DNA content of AR$^{+/y}$ and S-AR$^{-/y}$ mice by using flow cytometry. M1 represents haploid cells, M2 represents diploid cells, and M3 represents tetraploid cells. (*B*) Capα3 expression is absent in S-AR$^{-/y}$ testes. Total RNA from testes of 12-wk-old AR$^{+/y}$ and S-AR$^{-/y}$ mice and quantitated by real-time PCR with Capα3 forward primer 5′-TGGCTCTCAGTTACGCAAGG-3′ and reverse primer 5′-CCAACTCTATCCAAGCACTTACTC-3′. (*C–H*) The maturation of spermatocyte ceases in diplotene stage in S-AR$^{-/y}$ testes. (*C*) WT testis at tubule stage XI. White arrows indicate diplotene spermatocytes. (*D*) S-AR$^{-/y}$ testes. No lumen formation is observed in tubules. Some segments of tubule contain many pachytene spermatocytes (white arrowheads). Diplotene spermatocytes (white arrows) can be found in the central region of some tubules. (*E*) AR$^{-/y}$ testes. Only a small number of pachytene spermatocytes (white arrowheads) can be occasionally seen in the tubules. (*F*) S-AR$^{-/y}$ testes. Many apoptotic bodies (or degenerate germ cells; black arrowheads) were located in the stage with late pachytene or diplotene spermatocytes. (*G*) S-AR$^{-/y}$ testes. Meiosis with spindle formation (black arrow) can only rarely be found in the tubules. (*H*) In S-AR$^{-/y}$ testes, residual secondary spermatocyte (black arrow) and round spermatids (black arrowheads), which have escaped degeneration, are occasionally found in the central region of the tubule.

apoptotic (Fig. 4*F*) dysregulation of spermatogenesis likely contributes to the spermatogenic arrest that occurs at the diplotene stage before meiosis I.

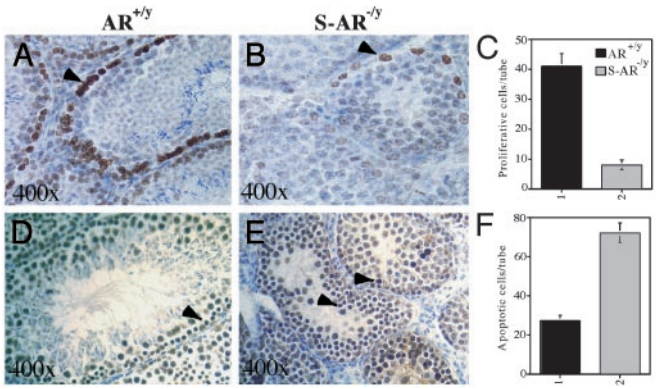

Fig. 4. Apoptosis and proliferative activity of AR$^{+/y}$ and S-AR$^{-/y}$ testes. (*A* and *B*) Proliferative activity of germ cells detected in 12-wk-old male AR$^{+/y}$ and S-AR$^{-/y}$ testes. (*C*) Quantitation of proliferative cells per seminiferous tubule based on BrdUrd incorporation. (*D* and *E*) Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay to detect the apoptotic cells in 12-wk-old male AR$^{+/y}$ and S-AR$^{-/y}$ testes. (*F*) Quantitation of apoptotic cells per seminiferous tubule in AR$^{+/y}$ and S-AR$^{-/y}$ testes.

Loss of AR Increases AMH Expression, Which May Lead to Hypotestosteronemia. To understand how the loss of AR in Sertoli cells results in hypotestosteronemia, we hypothesized that AR disruption causes increased secretion of AMH from Sertoli cells and, consequently, reduced Leydig cell production/secretion of testosterone. AMH is a paracrine factor secreted specifically from Sertoli cells. In male transgenic mice overexpressing AMH, the differentiation of Leydig cell precursors and the expression of mRNA of steroidogenic enzymes were decreased (27). Clinical studies have also demonstrated a reverse correlation between serum AMH and testosterone concentration at puberty (28). Real-time RT-PCR quantitation demonstrates that AMH mRNA expression is higher (3.5-fold) in S-AR$^{-/y}$ testes compared to expression in testis tissue from age-matched AR$^{+/y}$ mice (Fig. 5*A*). Results from immunohistochemical staining also indicate high AMH expression in S-AR$^{-/y}$ testes (Fig. 5*B*). The increased AMH expression in the testes may then suppress testosterone synthesis/secretion from Leydig cells.

Decreased Expression of Cyclin A1 and Sperm-1 May Contribute to Defects in Spermatogenesis in S-AR$^{-/y}$ Mice. Earlier studies have shown that cyclin A1 and sperm-1 expression begins at the end of the first meiotic prophase and suggested that these proteins play essential roles in the subsequent meiotic division (29, 30). To clarify why spermatogenesis ceases predominately at the diplotene spermatocyte stage in S-AR$^{-/y}$ testes, we assayed the expression of cyclin A1 and sperm-1. Our results show decreased expression of

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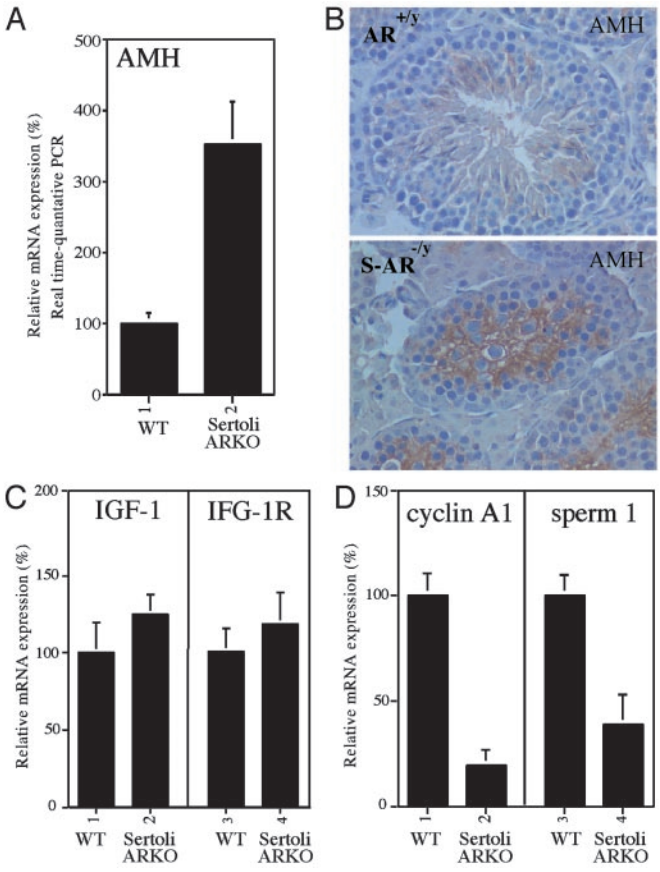

Fig. 5. Expression of AMH, cyclin A1, sperm-1, and IGF-1/IGF-1R in AR$^{+/y}$ and S-AR$^{-/y}$ mice. (*A*) Increased AMH expression in S-AR$^{-/y}$ testes by real-time PCR quantitation. (*B*) Increased AMH staining in S-AR$^{-/y}$ testis. (*C*) The expression of IGF-1 and IGF-1R show no significant change in the testes of S-AR$^{-/y}$ mice as compared to age-matched B6 AR$^{+/y}$ mice. (*D*) Reduced sperm-1 and cyclin A1 expression in S-AR$^{-/y}$ testis by real-time RT-PCR. The detailed real-time PCR method and primer sequences have been described (18). Total RNA and tissue sections were obtained from the testes of 12-wk-old AR$^{+/y}$ and S-AR$^{-/y}$ mice ($n = 5$ for each group).

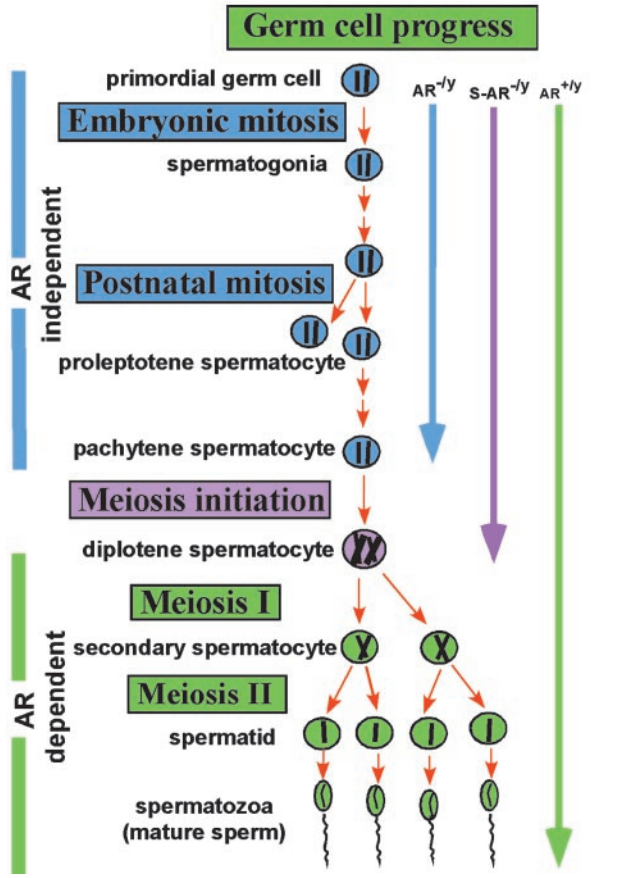

Fig. 6. Diagram of germ cell progression in AR$^{+/y}$, AR$^{-/y}$, and S-AR$^{-/y}$ testes. AR$^{+/y}$ testes can achieve full germ cell progression. However, spermatogenesis in the AR$^{-/y}$ and S-AR$^{-/y}$ testes ceases predominately in the pachytene and diplotene stages, respectively.

cyclin A1 and sperm-1, and are consistent with spermatogenic arrest before completion of the first meiotic division (Fig. 5*D*).

IGF-1/IGF-1R signaling has been reported to be part of a growth factor pathway important in several organs including the testis (31), and our finding that IGF-1/IGF-1R mRNA expression levels show no significant change in S-AR$^{-/y}$ mice (Fig. 5*C*) suggests that IGF-1 signals may not play a major role in the AR-mediated function of Sertoli cells. Together, the results from Fig. 5 suggest that increased AMH and reduced cyclin A1 and sperm-1 production contribute to the defects in germ cell maturation and resultant spermatogenic arrest before the first meiotic division.

Discussion

The testis contains four major cell types including Sertoli, Leydig, germ, and peritubular myoid cells. Early studies suggest that the testis has two major functions, synthesis of steroid hormones (steroidogenesis) and production of spermatozoa (spermatogenesis), which are achieved through coordination among various cell types within the testes (32). It is speculated that androgen/AR plays important roles in both of these processes (13, 33). Results from mice lacking AR showed that functional AR is critical for proper development and function of testes (16, 17). However, the effects of AR signaling within and between particular testicular cell types remain largely uncharacterized.

Sertoli cells are radially distributed at the basal aspect of the seminiferous tubules. The cytoarchitectural arrangements between Sertoli cells and developing germ cells provide one of the most complex examples of cell–cell interaction. Earlier studies demonstrated that an individual Sertoli cell is able to contact five adjacent Sertoli cells at the basal surface of each cell, as well as 47 adjacent germ cells at various stages of development (34). The columnar and convoluted structure of Sertoli cells provides physical support for spermatogonia undergoing mitosis, spermatocytes undergoing meiosis, and spermatids undergoing spermatogenesis to become spermatozoa (35). Sertoli cell-mediated environmental interactions are essential in the creation of the blood–testis barrier and maintenance of the unique microenvironment within the seminiferous tubule required for germ cell development (36). However, an earlier study showed that *in vitro* cultured Sertoli cells were unable to fully respond to some treatments, including androgen (37). Thus, the study of AR effects on Sertoli cell-mediated functions requires a plausible *in vivo* model. Indeed, results from our S-AR$^{-/y}$ mice provide the first *in vivo* evidence demonstrating a critical role for Sertoli cell-expressed AR in spermatogenesis (summary as Fig. 6).

Interestingly, a functional linkage between androgen/AR in Sertoli cells to the production/secretion of testosterone in Leydig cells was also identified in the S-AR$^{-/y}$ mice. Results from earlier studies indicated that LH acts on Leydig cells to stimulate the production of androgen, which subsequently acts on peritubular myoid cells to stimulate the production of the peritubular modification for Sertoli function (PmodS) protein, which in turn modulates Sertoli cell function and provides nutritional interactions

essential for germ cell development (38). This androgen-mediated regulatory interaction among Leydig and other testicular cells is postulated to be important for the maintenance of testicular functions, including spermatogenesis,** and to provide a potentially important, indirect mode of androgen action in the testis. The effects of PmodS on Sertoli cells, and the inability of purified Sertoli cells to respond to androgens *in vitro*, support the existence of indirect effects of androgen action that follows a signaling pathway from the Leydig cells, to the peritubular myoid cells, to the Sertoli cells, and then finally to germ cells.

Although *in vitro* cell culture studies have suggested that factors secreted by Sertoli cells affect Leydig cell function (39), mechanisms for androgen/AR signaling from Sertoli cells to Leydig cells remains speculative, because there has been no clear *in vivo* evidence. Our results from S-AR$^{-/y}$ mice provide *in vivo* evidence of AR-mediated Sertoli to Leydig cell signaling in the form of increased AMH expression and low testosterone levels compared to AR$^{+/y}$ mice.

Transgenic mice chronically overexpressing AMH show Leydig cell hypoplasia and impaired steroidogenesis (27), suggesting that AMH acts as a negative modulator for Leydig cell differentiation and testosterone secretion. Androgens are known to suppress Sertoli cell production of AMH, and AMH levels are elevated in humans with androgen insensitivity syndrome, a disorder caused by loss of AR function (40, 41). Results from the analysis of S-AR$^{-/y}$ mice provide *in vivo* evidence that loss of AR specific to Sertoli cells causes increased production of AMH, which affects Leydig cell production/secretion of testosterone. These *in vivo* data add to the evidence that a complicated functional network of cell–cell signaling is affected by loss of AR in Sertoli cells, likely leading to increased AMH that signals to the Leydig cells (reduced androgen level), to peritubular myoid cells (reduced PmodS protein), to Sertoli cells (defects in gene expression other that AR and AMH), and finally to germ cells (abnormal spermatogenesis).

ABP is a secretory protein produced by Sertoli cells that may regulate spermatogenesis and sperm maturation by maintaining high androgen levels in the testis and epididymis (42). It has also been proposed as a biochemical marker for Sertoli cell function and formation of the blood–testis barrier (43). Furthermore, ABP bound to androgen has been found to contribute to the regulation of fertility and spermatogenesis in various experimental models (44, 45). ABP transgenic mice display progressive structural and functional abnormalities in their testes, eventually leading to infertility (46). These data support our current findings showing that loss of AR in Sertoli cells, with resulting higher expression of ABP, may contribute to the defects in spermatogenesis and fertility observed in the S-AR$^{-/+}$ mice. It is also possible that the higher ABP could be a compensation for lower serum androgen levels.

Together, our results represent *in vivo* evidence showing that the knockout of AR specifically in Sertoli cells affects both the testosterone production/secretion function of Leydig cells, as well as spermatogenesis, with increased AMH and ABP expression and decreased cyclin A1 and sperm-1 expression, along with reduced serum testosterone levels in S-AR$^{-/y}$ mice. Further studies using microarray analysis to systematically compare Sertoli cell gene expression in AR$^{+/y}$ and S-AR$^{-/y}$ mice may allow us to dissect more completely the *in vivo* molecular mechanisms mediating the effects of androgen/AR signaling on spermatogenesis and/or steroidogenesis.

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