Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids

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Accepted 20 October 2003

Development 131, 459-467
Published by The Company of Biologists 2004
doi:10.1242/dev.00957

Summary

Androgen receptor function is required for male embryonic sexual differentiation, pubertal development and the regulation of spermatogenesis in mammals. During spermatogenesis, this requirement is thought to be mediated by Sertoli cells and its genetic and pharmacological disruption is manifested in spermatocytes as meiotic arrest. Through studies of a hypomorphic and conditional allele of the androgen receptor (*Ar*) gene, we have uncovered a dual post-meiotic requirement for androgen receptor activity during male germ cell differentiation. Observations in *Ar* hypomorphic animals demonstrate that terminal differentiation of spermatids and their release from the seminiferous epithelium is AR dependent and maximally sensitive to AR depletion within the testis. Cell-specific disruption of *Ar* in Sertoli cells of hypomorphic animals further shows that progression of late-round spermatids to elongating steps is sensitive to loss of Sertoli cell AR function, but that progression through meiosis and early-round spermatid differentiation are surprisingly unaffected.

Supplemental data available online

Key words: Androgen receptor, Sertoli, Spermatogenesis, Mouse

Introduction

Androgen receptor (AR) function is essential in males for proper sexual differentiation and for the maintenance of normal spermatogenesis. AR activity is regulated by the steroid ligand testosterone (T) and its derivative dihydrotestosterone (DHT), the binding of which initiates nuclear translocation and the transcriptional regulatory function of AR (Lindzey et al., 1994). Testosterone production is regulated by the gonadotrophin luteinizing hormone (LH), whose release from the pituitary stimulates the steroidogenic Leydig cells in the testis (Mendis-Handagama, 1997). Not surprisingly, AR itself also plays an important role in the feedback regulation of T levels. This regulation occurs through autocrine feedback on the Leydig cells, via effects on hypothalamic gonadotrophin releasing hormone (GnRH) production, and through inhibition of LH secretion by the pituitary (Amory and Bremner, 2001).

In both humans and mice, XY individuals carrying a hemizygous null mutation in the X chromosome-linked *Ar* gene exhibit complete Androgen insensitivity syndrome (cAIS), which is characterized by pseudohermaphroditism and sterility (Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov: 80/entrez/dispomim.cgi?id=300068). Although the phenotype of these individuals clearly demonstrates the crucial requirement for AR in development, the suitability of AIS as a model for studying the spermatogenic function of *Ar* is poor. Testicular descent fails in mice with cAIS (Ar^{tfm}), and the spermatogenic phenotype mimics that of cryptorchidism in an otherwise normal male, namely early meiotic arrest (Lyon and Hawkes, 1970). Thus, it is impossible to distinguish the contribution to the phenotype of loss of AR function from that resulting from the abdominal positioning of the testes. Mice homozygous for a mutation in the gonadotrophin releasing hormone gene (*Gnrh*), which have dramatically lowered serum testosterone levels (Singh et al., 1995), present a testicular phenotype similar to cAIS (Cattanach et al., 1977). Spermatogenesis in these animals can be qualitatively rescued by androgen replacement therapy (Singh et al., 1995). This occurs in the absence of appreciable levels of the gonadotrophins LH and follicle stimulating hormone (FSH). Further, FSH alone fails to significantly rescue spermatogenesis beyond the meiotic stages (Haywood et al., 2003; Singh and Handelsman, 1996), suggesting that T and/or DHT is the major hormonal regulator of spermatogenesis.

This observation is further supported by classic androgen withdrawal experiments in rats. Removal of androgens from adult rats by hypophysectomy (Hx) leads to an acute, stage-specific regression of the seminiferous epithelium (Ghosh et al., 1991; Russell and Clermont, 1977). After long-term Hx and elimination of residual testosterone by flutamide, spermatogenesis rarely proceeds beyond meiosis, with very few round spermatids observed and elongated spermatids nearly nonexistent (Franca et al., 1998). As with *Gnrh*-null mice, androgen or LH replacement leads to qualitative recovery of spermatogenesis in hypophysectomized rats while FSH has little direct stimulatory effect on spermatogenesis (El Shennawy et al., 1998; Elkington and Blackshaw, 1974; Russell and Clermont, 1977). Similar results are seen in response to suppression of *Gnrh* activity (Szende et al., 1990), and destruction of Leydig cells with the Leydig-specific cytotoxin EDS (Kerr et al., 1993; Sharpe et al., 1990).

In the mouse testis, AR protein is expressed in the somatic Leydig, myoid and Sertoli cells (Zhou et al., 2002). Although expression in Leydig and myoid cells is continuous, Sertoli cell expression of AR occurs in a stage-dependent fashion. Interestingly, the stages during which AR expression in Sertoli cells is highest correspond directly with those most acutely affected by androgen withdrawal. Sertoli cells are also the only somatic cell type in direct contact with differentiating germ cells. They provide both physical and nutritional support for spermatogenesis, which occurs in the intercellular spaces between Sertoli cells (McGuiness and Griswold, 1994). Taken together, these observations have led to the general belief that Sertoli cells are the primary mediators of AR regulation of spermatogenesis. Loss of AR activity from Sertoli cells would thus be responsible for the spermatogenic phenotypes outlined above, primarily the failure to efficiently complete meiosis.

To crucially examine this hypothesis, we have created a conditional null allele of the *Ar* gene in mice which allows the selective removal of AR function from Sertoli cells. This conditional *Ar* allele also proved to be a general hypomorph because of an inhibitory effect of the inserted *Pgk-neo* cassette on *Ar* mRNA processing.

Materials and methods

Generation of transgenic animals

A genomic DNA fragment containing the first exon of *Ar* was isolated from a mouse genomic BAC library (Research Genetics). From this clone a 12.5 kb *Eco*RV fragment centered around the first exon was subcloned into pBluescript. A 34 base-pair (bp) loxP sequence was inserted into a *Stu*I site 2.5 kb 5′ of exon 1, while a 2 kb neomycin resistance cassette was cloned into a *Bam*HI site 150 bp 3′ of the same exon. The Pgk:neo cassette was flanked by frt sequences to allow FLP mediated excision of the cassette following selection of homologous recombinants (Meyers et al., 1998). In addition, this cassette contains a loxP sequence at its 3′ end, such that it would be retained in the genome following recombination by FLP. This loxP site was inverted in its sequence relative to the other loxP site in the construct. Finally, a 1.5 kb diphtheria toxin cassette was inserted on the 3′ flank of the genomic fragment for negative selection of non-homologous recombinants.

The targeting construct was linearized at the 5′ end with *Not*I and electroporated into the FWB2 mouse embryonic stem cell line, derived in our laboratory from the 129/SvJaeSor strain of mice. Two correctly targeted clones were identified. One of these clones, Ar206, was then injected into C57Bl/6J derived blastocysts to produce chimeric offspring. Males were identified that transmitted the Ar206 *Ar* allele through the germline. The line of mice derived from these chimeras has been designated Ar^{tm1Reb}, referred to throughout the text as $Ar^{flox(ex1\text{-}neo)}$.

To allow Sertoli cell-specific ablation of AR activity, a *Cre* recombinase transgene driven by the anti-Mullerian hormone (*Amh*) promoter was generated. A 1.5 kb *Bam*HI-*Kpn*I *Amh* genomic fragment containing the entire promoter region (Beau et al., 2001) as well as the complete first exon and intron 1 was digested with *Nhe*I to linearize the template downstream of the *Amh* translational initiation codon. The *Amh* AUG and the remainder of exon 1 were replaced with a Myc-tagged *Cre* cDNA by homologous recombination in yeast. Intron 1 of the *Amh* gene was left intact, fused to the 3′ end of *Cre*. This *Amh-cre* construct was isolated from the vector by *Kpn*I-*Not*I digestion and introduced into FVB/N zygotes by pronuclear injection. Founder animals were identified by transgene specific PCR and functionally screened for CRE activity by mating to the *R26R* tester strain (Soriano, 1999) and their progeny assayed for testis specific β-galactosidase activity. Two independent lines expressing *Amh-cre* specifically in Sertoli cells were identified and one (line 8815) backcrossed into the 129/SvJaeSor strain for at least three generations prior to their use in these experiments.

Southern blots

Genomic DNA (5-10 μg) was digested overnight with EcoRV and separated on 1% agarose gels by electrophoresis for approximately 19 hours at 40 volts (V). DNAs were transferred to Genescreen nylon membrane (New England Nuclear) by capillary transfer in 20×SSC. Blots were UV crosslinked and pre-hybridized 60 minutes at 65°C in [2×SCP, 1% sarkosyl, 0.01% BSA]. A random primed ^{32}P-labelled DNA probe was generated from a 400 bp *Ar* exon 1 PCR fragment and hybridized to the membrane overnight at 65°C in [2×SCP, 1% sarkosyl, 0.01% BSA, 1×Denhardt's solution, 0.1 mg/ml salmon sperm DNA]. The blots were washed 20 minutes at 65°C in [2×SSC, 1% SDS], 20 minutes at 42 degrees in [0.1×SSC, 0.1% SDS], and 20 minutes at room temperature in [0.1×SSC, 0.1% SDS], followed by overnight exposure to X-ray film.

Phenotypic analysis of $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males

Eight-week-old males of the desired genotypes and wild-type littermate controls were euthanized by CO_2 asphyxiation and body weight measurements taken. Blood was collected by aortic puncture with a 23-gauge needle coated with heparin to prevent clotting. Red blood cells were pelleted by centrifugation and the overlying serum recovered for testosterone, LH, FSH and estradiol assays. All hormone assays were performed by the University of Virginia Ligand Core Laboratory.

Testes, epididymides and seminal vesicle were collected from each animal and testis and seminal vesicle weights recorded. A single testis and epididymis from each animal was fixed overnight at 4°C in either Bouin's fixative or neutral-buffered formalin (NBF), rinsed with 70% ethanol, embedded in paraffin wax, cut into 5 μm sections, and mounted on glass slides for histological and immunocytochemical examination. The second epididymis was minced in 1 ml sperm motility buffer (135 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 30 mM HEPES pH 7.4, 10 mM sodium lactate, 1 mM sodium pyruvate, 20 mg/ml BSA, 25 mM $NaHCO_3$) and sperm allowed to swim out overnight at room temperature. Numbers of epididymal sperm were determined by hemacytometer counts. The remaining testis was subdivided for protein, DNA and RNA isolation. For protein, one-half testis was homogenized in 500 μl buffer A [10 mM HEPES (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT plus PMSF, leupeptin, and pepstatin A as protease inhibitors] followed by addition of 50 μl buffer B [0.3 M HEPES (pH 7.6), 1.4 M KCl, 30 mM $MgCl_2$]. Cellular debris was pelleted by centrifugation and soluble protein extracts stored at –70°C. One-quarter testis was homogenized in 1 ml Trizol reagent (Invitrogen) and total RNA isolated following the manufacturer's instructions. Finally, one-quarter testis was used for DNA purification using the Qiagen DNEasy Tissue kit following the manufacturer's protocol.

Immunocytochemistry and β-galactosidase detection

Sections from testis and epididymis (5 μm) were deparaffinized and rehydrated [2×10 minutes Xylenes, 2×5 minutes 100% ethanol, 1×5 minutes 95% ethanol, 1×5 minutes 70% ethanol, 1×5 minutes 50% ethanol, 1×5 minutes phosphate buffered saline (PBS)], followed by quenching of endogenous peroxidase activity (1×15 minutes 0.5% H_2O_2). AR immunostaining was performed on NBF fixed tissues as previously described (Zhou et al., 2002). MSY4 and PRM2 immunostaining was performed on Bouin's fixed tissues as previously described (Giorgini et al., 2002). Immunostained sections were counterstained with Hematoxylin and mounted in GVA mounting solution (Zymed).

For assessment of *Amh-cre* function, fetal gonads and mesonephroi

were dissected from embryonic day 14.5 animals. The dissected tissues, as well as the remainder of the fetus, were fixed 30 minutes in 4% paraformaldehyde at room temperature. All tissues were then rinsed 2×30 minutes in *lacZ* rinse buffer (200 mM $Na_2HPO_4 \cdot 7H_2O$ pH 7.3, 2 mM $MgCl_2$, 0.02% Nonidet P-40, 0.01% sodium desoxychoate). The rinsed tissues were incubated overnight at 37°C in *lacZ* stain [*lacZ* rinse buffer containing 0.02 M $K_3Fe(CN)_6$, 0.02 M $K_4Fe(CN)_6$, and 1 mg/ml X-gal] to detect β-galactosidase activity. After staining, tissues were rinsed in PBS prior to imaging.

Western and northern blots

To determine AR protein levels in testes of control and experimental mice, 20 μg total testis protein was separated on a 10% SDS-PAGE minigel (BioRad). Proteins were transferred to Protran nitrocellulose membrane (Schleicher & Schuell) 30 minutes at 20 V on a Trans-Blot SD apparatus (BioRad). Membranes were blocked 30 minutes in PBS + 5% dry milk prior to addition of primary antibody. The membrane was cut in half horizontally through the 84 kilodalton (kDa) molecular weight marker. Proteins greater than 84 kDa were probed with rabbit anti-AR antibody N-20 (sc-816; Santa Cruz Biotechnology) diluted 1:500. As a loading control, proteins smaller than 84 kDa were probed with mouse anti-α-tubulin monoclonal antibody (Zymed Laboratories) diluted 1:3000. Primary antibody incubations were overnight at 4°C in PBS + 5% dry milk. Membranes were washed 2×15 minutes in PBS + 0.1% Tween-20. Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad), diluted 1:5000 in PBS + 5% dry milk, for 2-3 hours at room temperature. The membranes were washed as before followed by detection of peroxidase activity by ECL kit (Amersham) and exposure to X-ray film.

Pem and *Prm1* mRNA levels were determined by northern blot. Total testis RNA (10 μg) was run 2.5 hours at 100 V on a 1.5% formaldehyde-agarose gel. RNA was then transferred overnight to GeneScreen nylon membrane (New England Nuclear) by capillary transfer in 20×SSC. Random primed ^{32}P-labelled probe DNA was generated from cDNAs corresponding to the *Pem*, *Prm1* and β-actin genes. The RNA was UV crosslinked to the membrane followed by hybridization overnight at 42°C in hybridization buffer (50% formamide, 5×SSC, 50 mM $NaPO_4$ pH 6.5, 250 μg/ml salmon sperm DNA, 1×Denhardt's solution, 0.5% SDS, 6.25% dextran sulfate). The membrane was washed 30 minutes at 65°C in [2×SSC, 1% SDS, 0.1% sodium pyrophosphate] followed by 30 minutes at 65°C in [0.5×SET, 0.1% sodium pyrophosphate]. The membranes were then exposed to a storage phosphor screen and imaged on a Storm 820 phosphorimager (Amersham). Bands corresponding to the RNA of interest were then quantified with ImageQuant software (Amersham). RNA levels for *Pem* and *Prm1* were normalized to the β-actin loading control to allow between sample comparisons. Membranes were probed sequentially and stripped in boiling 1% SDS between probes.

Statistical analyses

Between group comparisons for all variables were performed by one-way ANOVA followed by Tukey's post hoc analysis using the Statistical Package for Social Sciences (SPSS) version 10.0.

Results

Construction and analysis of *Ar* conditional allele

To determine the requirement for Sertoli cell AR function during spermatogenesis, we constructed a conditional *Ar* allele whose expression can be specifically ablated in Sertoli cells (Fig. 1A,B). After generation of chimeric animals from the Ar206 ES cell line, two males were identified who transmitted only the $Ar^{flox(ex1-neo)}$ allele to their progeny. In litters sired by these males, all male offspring were wild-type for *Ar* while all females were heterozygous ($Ar^{flox(ex1-neo)/+}$), further confirming correct targeting to the X-linked *Ar* locus (Fig. 1C).

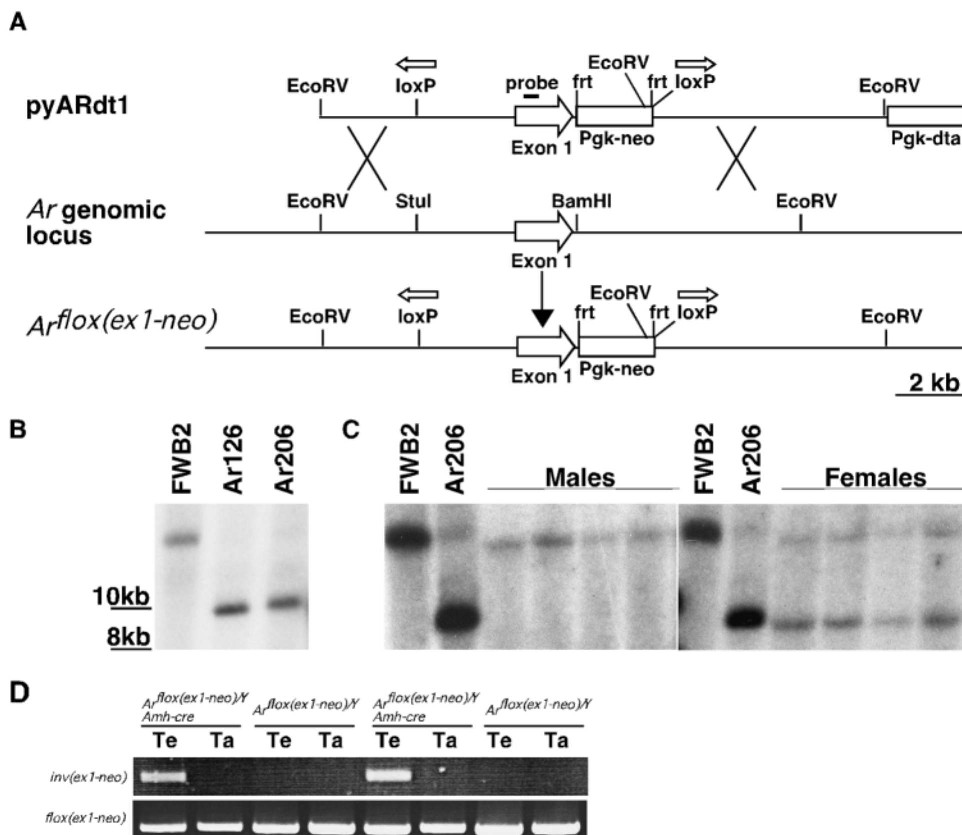

Fig. 1. Construction and characterization of *Ar* conditional allele. (A) Schematic of targeting construct (pyARdt1), wild-type and conditional hypomorphic ($Ar^{flox(ex1-neo)}$) alleles of *Ar*. Arrows over loxP sites indicate orientation of sites relative to one another. Probe used for Southern blots is indicated above exon 1. (B) *Eco*RV digest and Southern blot of ES cell DNAs. Probe detects presence of 12.5kb *Ar* fragment in wild-type FWB2 ES cells. Correctly targeted ES cell clones Ar126 and Ar206 contain only the 9 kb fragment representative of the correctly targeted allele. (C) Chimeric males transmit the X-linked $Ar^{flox(ex1-neo)}$ allele exclusively to female offspring. (D) PCR genotyping detects the *Ar* inversion allele, *inv(ex1-neo)*, specifically in the testes of $Ar^{flox(ex1-neo)/Y}$; *Amh-cre* animals. No product is observed in tails from the same animals or in testes or tails from $Ar^{flox(ex1-neo)/Y}$ males. The non-recombinant hypomorphic allele, *flox(ex1-neo)*, is detected in all samples. Te, testis; Ta, tail.

Table 1. Reproductive organ weights and sperm counts are reduced in *Ar* mutant males

Genotype	Body weight (g)*	Testis weight (mg)*	Seminal vesicle weight (mg)*	Number of sperm ($\times 10^4$/epi.)*
$Ar^{+/Y}$	25.2±2.8 (*n*=23)	107.0±12.8^{a,b} (*n*=23)	157.0±26.5^{d,e} (*n*=23)	1493.9±637.4^{f,g} (*n*=23)
$Ar^{flox(ex1\text{-}neo)/Y}$	26.4±4.4 (*n*=23)	87.7±8.9^{a,c} (*n*=23)	125.1±30.1^{d} (*n*=19)	58.6±83.9^{f} (*n*=22)
$Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre*	26.6±3.6 (*n*=8)	64.1±7.5^{b,c} (*n*=8)	127.8±26.5^{e} (*n*=8)	12.8±21.6^{g} (*n*=8)

*Values are mean±s.d.
Values with the same superscript are significantly different (a,b,c, $P<0.001$; d, $P<0.005$; e, $P<0.05$; f,g, $P<0.001$).

To verify that CRE-mediated recombination of $Ar^{flox(ex1\text{-}neo)}$ generates a null allele, $Ar^{flox(ex1\text{-}neo)/+}$ females were mated to both *Sycp1-cre* and *eIIa-cre* males, which have been previously shown to express CRE in two- to four-cell and one-cell embryos, respectively (Lakso et al., 1996; Vidal et al., 1998). Previous studies of *Ar* mutant alleles have established the *Ar* null phenotype as XY pseudohermaphroditism (Lyon and Hawkes, 1970; Yeh et al., 2002). Therefore, we genotyped female progeny of these matings by PCR for the Y-linked *Sry* gene. We identified multiple phenotypic females which were found to be Sry^{+}. These animals all proved to be hemizygous for the CRE-induced inversion allele, $Ar^{inv(ex1\text{-}neo)}$ by PCR (data not shown). Internal examination revealed the presence of small, abdominal testes (see Fig. S1A at http://dev.biologists.org/supplemental) whose germ cells were arrested at the pachytene stage of meiosis, thus establishing $Ar^{inv(ex1\text{-}neo)}$, as a null. RT-PCR demonstrated the absence of wild-type *Ar* transcripts from the undescended testes of these individuals (see Fig. S1B at http://dev.biologists.org/supplemental). We were also able to confirm the presence of an *Ar* exon1-neomycin fusion RNA within these same testes. Presumably, this fusion RNA is generated by transcription of the inverted exon 1 from inverted *Ar* promoter elements. Fusion of exon 1 to the neomycin transcript provides transcriptional termination and polyadenylation signals, thus producing a stable transcript. Sequencing of this fusion RNA has shown that the *Ar* and neomycin sequences are out of frame, thus any peptide produced would truncate shortly after entering the neomycin coding sequence (data not shown).

In order to specifically remove AR function from Sertoli cells, we created a transgene in which *Cre* cDNA expression is regulated by the Sertoli cell specific anti-Mullerian hormone promoter (*Amh-cre,* see Fig. S2 at http://dev.biologists.org/supplemental). To confirm an interaction between *Amh-cre* and $Ar^{flox(ex1\text{-}neo)/Y}$, PCR was performed to detect the expected inversion allele generated by recombination between the loxP sites (Fig. 1D). As anticipated, an inversion specific product is observed in DNA from the testes of $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males, but not in the tails of these animals. No product is amplified from tail or testis DNA of $Ar^{flox(ex1\text{-}neo)/Y}$ males, though an $Ar^{flox(ex1\text{-}neo)}$ specific PCR product is detected in all samples, confirming the integrity of the DNA.

Morphological and behavioral analysis of *Ar* mutant phenotypes

Previous work has shown that inclusion of the neomycin phosphotransferase cassette used in our targeting construct is likely to create a hypomorphic allele of the gene into which it is inserted (Meyers et al., 1998). Therefore we decided to examine the phenotype of $Ar^{flox(ex1\text{-}neo)/Y}$ males as potential hypomorphs, along with the Sertoli cell mutant $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* animals. Unlike an Ar^{tfm} null male, $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males were indistinguishable from wild-type male littermates by external examination. In addition, $Ar^{flox(ex1\text{-}neo)/Y}$ males produced copulatory plugs when housed with superovulated females, indicating male sexual behavior is properly imprinted. However, as shown in Table 1, further characterization uncovered significant differences not only between mutant animals and wild type, but also between the mutants themselves.

Animals of the three genotypes examined did not differ in their overall body weights. However, testis weights were reduced 19% in the hypomorphic $Ar^{flox(ex1\text{-}neo)/Y}$ animals. $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males exhibited a further 27% drop in testis weight, down 40% relative to $Ar^{+/Y}$ controls. Reductions in epididymal sperm numbers were even more pronounced. Sperm counts were 3.9% and 0.9% in $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre*, respectively, relative to $Ar^{+/Y}$ males. Though not statistically significant, counts were reduced ~78% in $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males compared with $Ar^{flox(ex1\text{-}neo)/Y}$ alone. Finally, seminal vesicle (SV) weights were measured as an androgen-sensitive endpoint. In both $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males, SV weights were reduced 20% when compared with wild type. There was no difference in SV weights between the $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* animals.

Systemic and molecular aspects of reduced *Ar* function

To further characterize the phenotypes of the mutant animals, measurements were made of serum gonadotrophin (LH and FSH), testosterone (T) and estradiol levels. Although no differences were observed between the two mutant classes, serum LH and T levels were drastically and significantly increased in both $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre*

Table 2. Serum hormone levels are significantly elevated in *Ar* mutant males

Genotype	T (ng/dl)*	LH (ng/ml)*	FSH (ng/ml)*	Estradiol (pg/ml)*
$Ar^{+/Y}$	38.8±33.7^{a,b} (*n*=21)	0.21±0.40^{c,d} (*n*=23)	22.1±6.4^{e,f} (*n*=20)	25.5±14.0 (*n*=17)
$Ar^{flox(ex1\text{-}neo)/Y}$	1542.5±975.0^{a} (*n*=22)	5.10±3.70^{c} (*n*=22)	50.3±18.9^{e} (*n*=22)	33.6±24.8 (*n*=16)
$Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre*	1633.7±1075.1^{b} (*n*=8)	4.65±3.37^{d} (*n*=8)	57.3±12.4^{f} (*n*=7)	37.3±9.8 (*n*=6)

*Values are mean±s.d.
Values with the same superscript are significantly different (a,b,c,e,f, $P<0.001$; d, $P<0.005$).

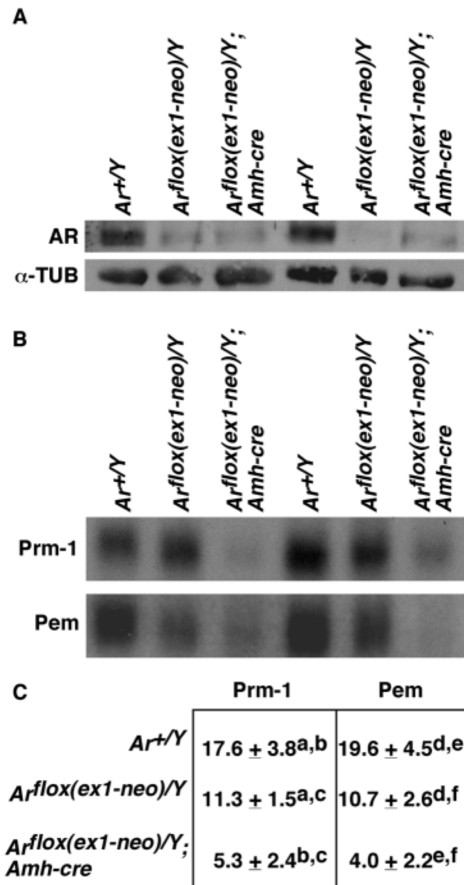

Fig. 2. Analysis of protein and RNA levels in adult testes. (A) Western blot confirming that AR protein levels are reduced in testes of $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males. (B) Northern blot showing decrease in *Prm1* and *Pem* mRNA expression in testes of $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males. (C) Quantification of mRNA levels from B. Values with the same superscript are significantly different from one another (a,c,d,f, $P<0.05$; b,e, $P<0.001$). $n=4$ for all groups.

males in comparison with $Ar^{+/Y}$ animals (Table 2). LH levels were increased 23-fold with a concomitant 40-fold increase in T levels in the serum. FSH levels were also significantly elevated in mutant animals, though only approx. twofold relative to wild type. Interestingly, estradiol levels were unaffected in *Ar* mutant males.

Western blot analysis of total testis protein confirmed the expected reduction in AR protein levels in $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* mice (Fig. 2A). Comparison to the α-tubulin loading control shows that total testis AR is depleted in mutant males relative to wild-type littermates. Testis AR levels in $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males are approximately equal, suggesting that the majority of AR protein detected in $Ar^{flox(ex1\text{-}neo)/Y}$ testes is due to expression by peritubular myoid and Leydig cells.

Northern blots of *Prm1* and *Pem* mRNAs also show significant differences attributable to loss of AR function (Fig. 2B). *Prm1* was used as a marker for round and elongating spermatid numbers (Mali et al., 1989), while *Pem* is a Sertoli cell specific transcript whose expression is directly regulated by AR (Rao et al., 2003). Phosphorimaging and normalization to β-actin levels show that *Prm1* mRNA is reduced 36% in $Ar^{flox(ex1\text{-}neo)/Y}$ and 70% in $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* testes, whereas *Pem* mRNA is reduced 45% and 80%, respectively. Pairwise comparisons show that these reductions from wild type are significant, as are the differences between $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* animals for both transcripts (Fig. 2C).

Testicular and epididymal abnormalities associated with *Ar* mutations

Histological analysis of testes and epididymides from *Ar* mutant and wild-type males confirmed and extended our previous findings. Overall size and organization of the epididymis was similar between genotypes. However, numbers of mature sperm were reduced in the epididymides of $Ar^{flox(ex1\text{-}neo)/Y}$ males whereas spermatozoa were histologically undetectable in $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* epididymides (Fig. 3A-C). Epididymal smooth muscle hyperplasia was also apparent in males of both mutant genotypes. Additionally, cellular debris was commonly observed in the epididymal lumen. In particular, large numbers of what appear to be highly vacuolated round spermatids are observed in the epididymides of $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males.

Within the testes of $Ar^{flox(ex1\text{-}neo)/Y}$ males, seminiferous tubules of all spermatogenic stages are observed (Fig. 3E,H,K). However, abnormal tubules are also seen, including those with apparent reductions in expected classes of spermatids. In addition, elongated spermatids are frequently seen degenerating between Sertoli cells (Fig. 3K, arrowhead), a condition rarely observed in wild-type testes. Examination of testes from $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males presented a more severe histological phenotype. These testes are virtually devoid of elongated spermatids and normal tubules of stages I-VIII are nonexistent (Fig. 3F,I,L). Occasionally, tubules at stages IX-XII are observed with all cell types present, though these tubules appear to have greatly reduced numbers of elongating spermatids that are lost entirely by stage I-II (data not shown).

Protein marker analysis by immunocytochemistry provided further insight into the phenotypes of $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* animals. MSY4, the expression of which marks mid-stage pachytene spermatocytes through round spermatids (Davies et al., 2000), is largely unaffected in the *Ar* mutants (Fig. 4A-F). These images also demonstrate the reduction in numbers of elongating spermatids at stage X-XI in our *Ar* mutant animals, with the loss being most severe in $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males. PRM2, which is expressed in elongated spermatids (Stanker et al., 1987), is present in $Ar^{flox(ex1\text{-}neo)/Y}$ testes, although the numbers of positive spermatids appears to be reduced (Fig. 4H). PRM2 positive spermatids are further reduced in $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* testes (Fig. 4I), consistent with previous observations from sperm counts and epididymal and testicular histology. As expected, AR protein was detected in the myoid, Leydig and Sertoli cells of $Ar^{flox(ex1\text{-}neo)/Y}$ testes, even in the most severely affected tubules (Fig. 4K). Within the testes of $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males, AR is clearly expressed in myoid and Leydig cells, while Sertoli cells are AR negative (Fig. 4L). Occasionally, faint AR immunoreactivity is detected within Sertoli cells (see Fig. S3A at http://dev.biologists.org/supplemental); however, this signal is due to expression of a

non-functional, N-terminal peptide generated from an inverted exon 1-neomycin fusion RNA, as described above. No full-length AR protein is detected within the Sertoli cells of *Ar*$^{flox(ex1-neo)/Y}$; *Amh-cre* males (see Fig. S3C at http://dev.biologists.org/supplemental).

Discussion

We have created a conditional, hypomorphic allele of the *Ar* gene to study the role of AR function in the regulation of spermatogenesis. As proof-of-principal, we demonstrated that inversion of *Ar* exon 1 in early embryos by CRE recombinase phenocopies the previously established *Ar*-null phenotype observed in several laboratories (Lovell, 1979; Lyon and Hawkes, 1970; Tanaka et al., 1994; Yeh et al., 2002). To confirm that the phenotype of *Ar*$^{flox(ex1-neo)/Y}$ males is due to hypomorphism at the *Ar* locus, heterozygous *Ar*$^{flox(ex1-neo)/+}$ females were also mated to *R26*$^{fki/fki}$ males, which express FLP recombinase under the direction of the *ROSA26* promoter (Awatramani et al., 2001). We found that excision of *Pgk-neo* by FLP in male progeny of this cross restores epididymal sperm numbers and serum hormone concentrations to wild-type levels (data not shown). Therefore we conclude that the *Ar*$^{flox(ex1-neo)}$ allele manifests a hypomorphic phenotype because of insertion of *Pgk-neo* into exon 1 of the *Ar* gene.

Characterization of *Ar* hypomorphic males has provided unexpected insight into the function of AR in prenatal development and spermatogenesis. Our results clearly demonstrate sensitivity to AR function during sexual differentiation distinct from the requirement for normal spermatogenesis. *Ar*$^{flox(ex1-neo)/Y}$ males are morphologically normal in spite of reduced levels of AR protein, as evidenced by western blot analysis of testis protein extracts. The dysregulation of serum gonadotrophin levels also implies decreased AR function in the hypothalamus and pituitary. In addition, AR activity appears to be reduced based on the decrease in Sertoli cell transcript levels from the AR-regulated *Pem* gene. These results together indicate an overall depression of AR activity in *Ar*$^{flox(ex1-neo)/Y}$ animals. Not surprisingly, evaluation of epididymal sperm numbers and testicular histology reveals a severe disruption of spermatogenesis. Thus, it seems that quantitatively normal spermatogenesis requires a higher level of AR function than does male sexual differentiation. This result mimics that found for the mildest forms of androgen-insensitivity syndrome (AIS) in humans, but to our knowledge this is the first example of partial AIS (pAIS) in an animal model (Giwercman et al., 2001; Hiort et al., 2000). It is also worth noting that the *Ar*$^{flox(ex1-neo)/Y}$ phenotype results from a reduction in wild-type AR protein levels, rather than a reduction in the activity of individual AR molecules as is nearly always the case in human pAIS (Androgen Receptor Gene Mutations Database, http://ww2.mcgill.ca/androgendb/).

As mentioned above, *Ar*$^{flox(ex1-neo)/Y}$ males also exhibit dysregulation of serum gonadotrophin and testosterone (T)

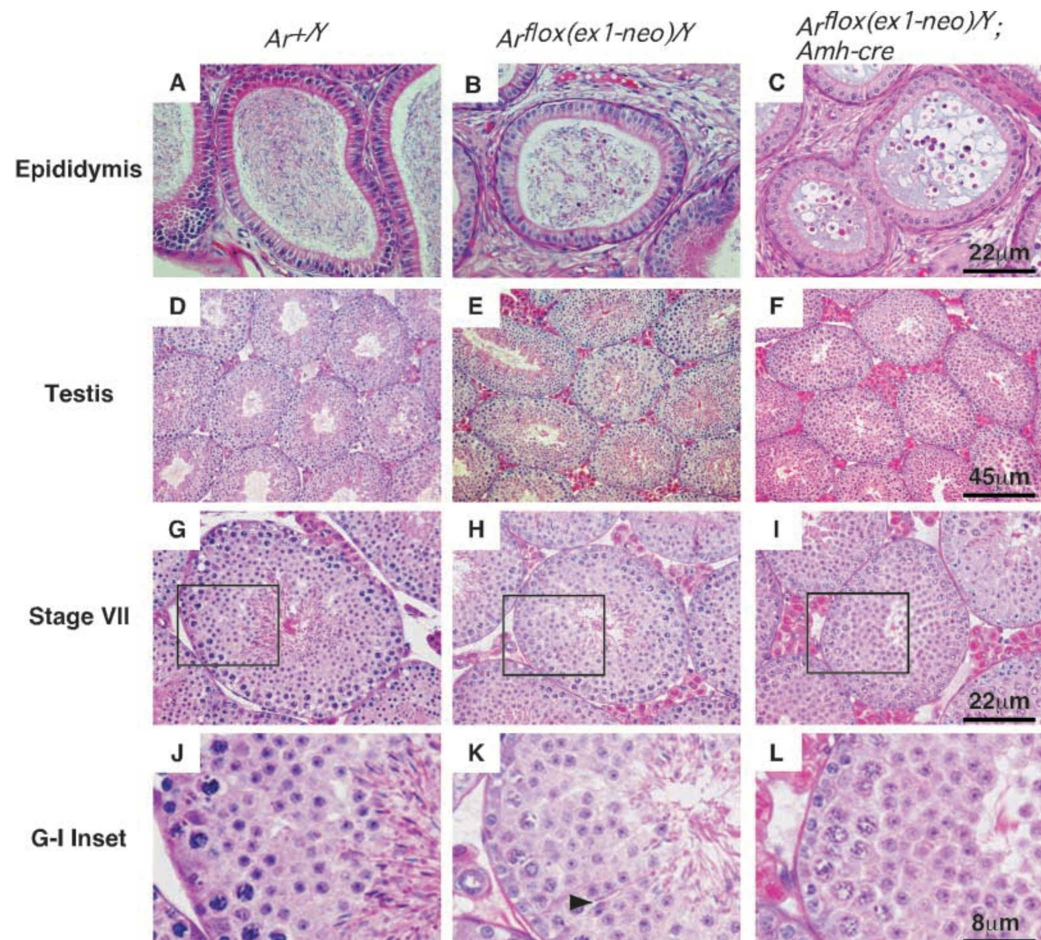

Fig. 3. Histological examination of epididymis and testis. (A-C) Transverse section through the epididymis. Spermatazoa are infrequent in *Ar*$^{flox(ex1-neo)/Y}$ and absent from *Ar*$^{flox(ex1-neo)/Y}$; *Amh-cre* males. Pyknotic and vacuolated round spermatids populate the *Ar*$^{flox(ex1-neo)/Y}$; *Amh-cre* epididymis. Smooth muscle hyperplasia is apparent in *Ar* mutants. (D-F) Low magnification view of testis transverse section. Elongated spermatids are depleted from *Ar*$^{flox(ex1-neo)/Y}$; *Amh-cre* testes. (G-I) Stage VII tubules show decrease in (*Ar*$^{flox(ex1-neo)/Y}$) or complete absence of (*Ar*$^{flox(ex1-neo)/Y}$; *Amh-cre*) elongated spermatids. (J-L) Higher magnification of G-I. Black arrowhead indicates presence of degenerating sperm in *Ar*$^{flox(ex1-neo)/Y}$ males.

levels. Interestingly, given their interrelated modulation by GnRH, LH concentration is more dramatically affected than is that of FSH. This differential effect on LH and T versus FSH can be explained in the context of known mechanisms of gonadotrophin regulation. In studies of human gonadotrophin production, decreased T levels have been shown to increase the frequency and magnitude of LH and FSH secretion, whereas increases in T levels decrease the frequency of the GnRH pulse from the hypothalamus, thus suppressing release of LH and FSH from the pituitary (Matsumoto and Bremner, 1984). In addition, the frequency of the GnRH pulse has been shown to differentially affect LH and FSH secretion, with high frequency pulses favoring secretion of LH while less frequent pulses favor FSH (Wildt et al., 1981). Paradoxically, our model is expected to mimic the effect of testosterone withdrawal in spite of the elevated levels of serum T. Owing to the decrease in functional AR, the system is effectively experiencing androgen depletion. Thus, we would expect the frequency of the GnRH pulse to be increased, with the outcome being preferential secretion of LH over FSH.

FSH secretion is also regulated by factors that do not overlap with the regulation of LH. Inhibin B, which is produced by Sertoli cells in response to FSH stimulation, feeds back directly on the pituitary to limit FSH secretion (Anawalt et al., 1996). Experiments in *Ar*-null mice have also shown that T and estradiol act to suppress serum FSH concentration in the absence of functional androgen receptor, implying estrogen receptor mediated negative feedback on FSH levels (Schleicher et al., 1989). These mechanisms of FSH regulation, which are expected to be wholly intact in our model, are thus likely to account for the differential effect on LH and FSH levels in the $Ar^{flox(ex1\text{-}neo)/Y}$ hypomorphic male. By contrast, our results provide strong evidence for a direct and primary role for AR in the feedback regulation of LH and T levels. This presumably occurs through direct effects on the hypothalamus and pituitary as well as through autocrine regulation of T production by the Leydig cells.

The role of AR function during spermatogenesis has been the subject of intense interest for many years. It has been assumed, reasonably so, that the major cellular mediator of this regulatory function of AR is the Sertoli cell, based on its intimate contact with germ cells and stage-specific AR expression (McGuiness and Griswold, 1994; Zhou et al., 2002). Abundant prior work on the role of testosterone and AR in spermatogenesis has led to the expectation that Sertoli cell AR function is required for the completion of meiosis and the transition of spermatocytes to haploid round spermatids. As previously cited, multiple studies of androgen withdrawal and disruption of AR activity, either by surgical, chemical or genetic means, have produced similar results: in the absence of

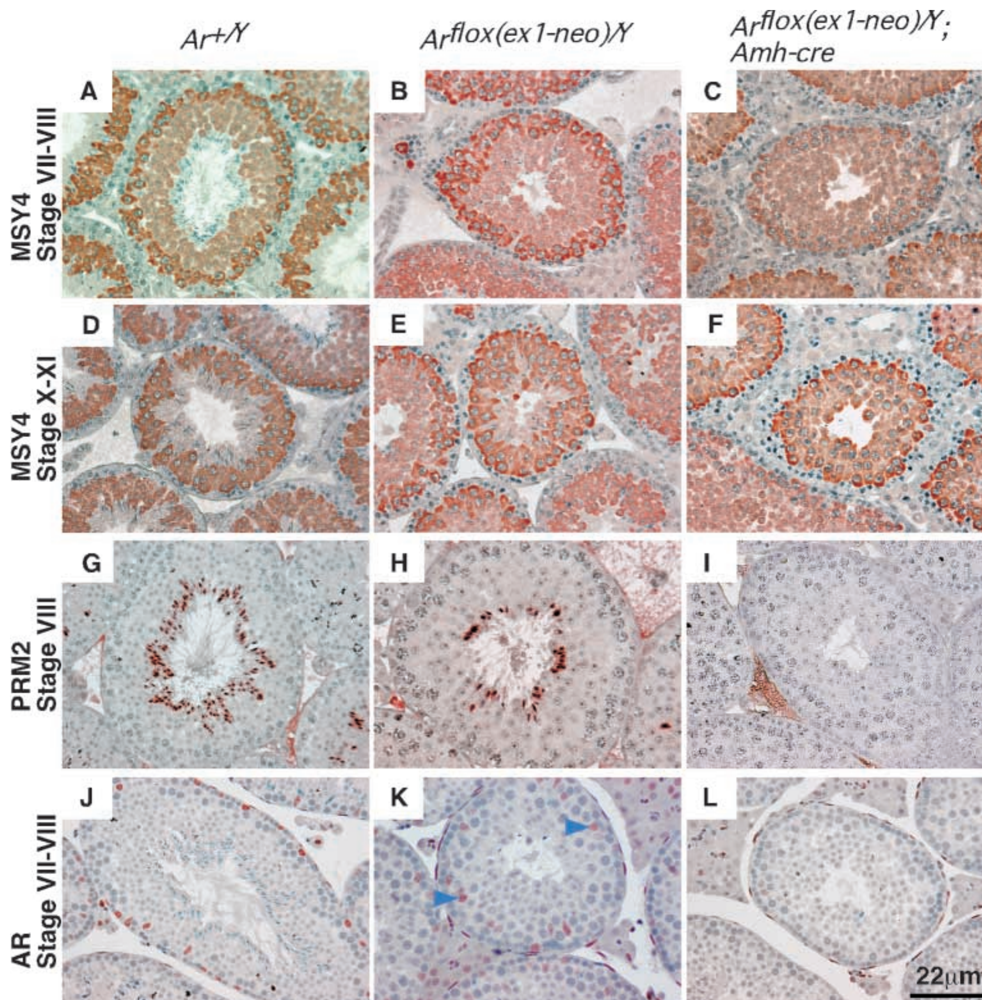

Fig. 4. Evaluation of somatic and germ cell protein expression. (A-F) MSY4 expression in pachytene spermatocytes and round spermatids is unaffected in *Ar* mutant males. Elongated spermatids are reduced in (B,E) and absent from (C,F) the tubules of $Ar^{flox(ex1\text{-}neo)/Y}$ and $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre*, respectively. (G-I) PRM2-positive elongated spermatids are decreased in $Ar^{flox(ex1\text{-}neo)/Y}$ males (H) and absent from $Ar^{flox(ex1\text{-}neo)/Y}$; *Amh-cre* males (I) at time of spermiation. (J-L) AR protein is expressed in somatic cells of the testis. AR is present in Sertoli cells of $Ar^{flox(ex1\text{-}neo)/Y}$ males (K, blue arrowheads).

androgens and AR function, spermatogenesis rarely proceeds beyond meiosis. In all of these model systems, very few round and even fewer elongated spermatids are observed. This point was made clearly in a recent study (Haywood et al., 2003). In the presence of physiological levels of transgenic FSH, significant stimulation of Sertoli cell and spermatogonial proliferation was observed in the testes of *Gnrh*-null animals. However, only limited numbers of haploid spermatids were detected whose development was arrested during elongation. Efficient completion of meiosis and development of mature spermatozoa required the presence of androgens.

In the current study we have provided evidence for the presence of two AR-sensitive steps during spermatogenesis. The first, which is most acutely sensitive to a reduction in AR function, occurs in the late stages of spermatid differentiation near the time of spermiation. Unexpectedly, the second step, which is sensitive to loss of Sertoli cell AR activity, occurs during the transition from the round to elongating stages of spermiogenesis, and may involve a loss of adhesion of round spermatids to the seminiferous epithelium. Based on our observations, it is possible that a primary role of AR function in Sertoli cells is to regulate spermatid adhesion to the seminiferous epithelium. AR would thus be required for maintenance of adhesion of round spermatids during their differentiation to elongated spermatozoa. Conversely, AR function is then required for the execution of spermiation and release of mature sperm into the tubule lumen. We are currently testing this hypothesis through examination of the cell-to-cell contacts between Sertoli and germ cells.

Sertoli cell *Ar* expression does not appear to be required for the completion of meiosis or the differentiation of round spermatids prior to elongation. However, androgens are clearly required at some level for normal meiotic progression. It has been recently shown that progestins, which like the androgens act through a nuclear hormone receptor, are capable of exerting a non-genomic effect on cell proliferation in the absence of functional progesterone receptor (Sager et al., 2003). Thus, we suggest that androgens may still be required in Sertoli cells for execution of meiosis, but that these hormones are also capable of acting non-genomically, in the absence of AR function, to promote spermatogenesis. Alternatively, our results indicate that the search for localization of the meiotic AR requirement be refocused outside of the seminiferous tubules. One possible candidate is the peritubular myoid cell, which expresses AR at all stages of spermatogenesis and is in close proximity to the spermatogonial stem cells.

We thank Jake Bernstein, Steve Eacker, Kathy Kafer, Dr Flaviano Giorgini, Dr Bill Buaas and Dr Charles Connolly for technical assistance and thoughtful discussions over the course of the current work. We also thank Dr Richard Behringer, Dr Phil Soriano, Dr Gail Martin and Dr Rex Hess for the generous provision of critical reagents. Finally, we thank Dr Laura Holdcraft for her expert assistance with statistical analysis of the data. This research was supported by NICHD/NIH through cooperative agreements (HD12629) and (HD28934 'Center for Cellular and Molecular Studies in Reproduction'), as part of the Specialized Cooperative Centers Program in Reproduction Research.

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