BIOLOGY OF REPRODUCTION **83,** 759–766 (2010)
Published online before print 21 July 2010.
DOI 10.1095/biolreprod.110.085472

Gene Expression Alterations by Conditional Knockout of Androgen Receptor in Adult Sertoli Cells of *Utp14b*$^{jsd/jsd}$ (*jsd*) Mice[1]

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ABSTRACT

Spermatogenesis is dependent primarily on testosterone action on the Sertoli cells, but the molecular mechanisms have not been identified. Attempts to identify testosterone-regulated target genes in Sertoli cells have used microarray analysis of gene expression in mice lacking the androgen receptor (AR) in Sertoli cells (SCARKO) and wild-type mice, but the analyses have been complicated both by alteration of germ cell composition of the testis when pubertal or adult mice were used and by differences in Sertoli-cell gene expression from the expression in adults when prepubertal mice were used. To overcome these limitations and identify AR-regulated genes in adult Sertoli cells, we compared gene expression in adult *jsd* (*Utp14b*$^{jsd/jsd}$, juvenile spermatogonial depletion) mouse testes and with that in SCARKO-*jsd* mouse testes, since their cellular compositions are essentially identical, consisting of only type A spermatogonia and somatic cells. Microarray analysis identified 157 genes as downregulated and 197 genes as upregulated in the SCARKO-*jsd* mice compared to *jsd* mice. Some of the AR-regulated genes identified in the previous studies, including *Rhox5*, *Drd4*, and *Fhod3*, were also AR regulated in the *jsd* testes, but others, such as proteases and components of junctional complexes, were not AR regulated in our model. Surprisingly, a set of germ cell–specific genes preferentially expressed in differentiated spermatogonia and meiotic cells, including *Meig1*, *Sycp3*, and *Ddx4*, were all upregulated about 2-fold in SCARKO-*jsd* testes. AR-regulated genes in Sertoli cells must therefore be involved in the regulation of spermatogonial differentiation, although there was no significant differentiation from spermatocytes in SCARKO-*jsd* mice. Further gene ontogeny analysis revealed sets of genes whose changes in expression may be involved in the dislocation of Sertoli cell nuclei in SCARKO-*jsd* testes.

androgen receptor, conditional knockout, meiosis, Sertoli cells, spermatogenesis, testis, testosterone

[1]Supported by National Institutes of Health (NIH) Research grants HD-40397 (to M.L.M.) and NIH Cancer Center Support grant CA 16672 to M.D. Anderson Cancer Center, HD-10808 (to M.D.G.) and HD-16229 (to Joanne Richards, Baylor College of Medicine, Houston, Texas).
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Received: 19 April 2010.
First decision: 1 June 2010.
Accepted: 6 July 2010.
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eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

INTRODUCTION

Androgens are essential for normal spermatogenesis and fertility. Androgen regulation of spermatogenesis must occur through the Sertoli cell because selective knockout of the androgen receptor (AR) in these cells, using a floxed *Ar*-gene and a Sertoli cell–specific *cre*, impedes spermatogenesis [1–3]. Further studies indicated that the genomic activity of AR in Sertoli cells plays the essential role in spermatogenesis [4], but specific genes involved are not known. Studies have attempted to identify androgen-regulated genes affecting spermatogenesis using microarray analysis [reviewed in 5] on 10-day-old SCARKO (Sertoli-cell androgen receptor knockout) mice [6], 20-day-old testicular feminized (*Tfm*) mice [7], hypogonadal (*hpg*) mice briefly treated with testosterone [8], and AR elimination in Sertoli cells of adult mice that were already hypomorphic for AR function [9]. Whereas these studies have provided valuable information, they all have some limitations. Conclusions from studies employing immature animals may be limited to the effects of androgen on immature Sertoli cells, which differ from the effects in adults. Conclusions from studies in adult animals are complicated by alterations in germ cell compositions resulting from elimination of AR. Studies that were limited to the acute changes induced by the hormones may have missed some important functional changes that result from chronic treatment, such as indirect regulation of targets.

We used a recently constructed SCARKO model, based on mice homozygous for the juvenile spermatogonial depletion (*jsd*) mutation [10] in the *Utp14b* gene [11, 12] (referred to as *jsd* mice) to circumvent these problems. The *Utp14b* gene is primarily expressed in germ cells and only very weakly in Sertoli cells [13], and Sertoli cells in testis of *jsd* mice can support differentiation of transplanted normal spermatogonia [14]. The *jsd* mice become sterile after the first few waves of spermatogenesis [15] and the only germ cells remaining in most seminiferous tubules of adult *jsd* mice are undifferentiated type A spermatogonia [16]. It has been shown that suppression of testosterone by gonadotropin-releasing hormone analogues can reverse the block of spermatogonial differentiation [17–19]. However, it appeared that Sertoli cell AR was not responsible for the spermatogonial block in the *jsd* mice, as SCARKO-*jsd* mice showed no histological evidence of recovery of spermatogenesis, although their testes did support spermatogonial differentiation when systemic levels of testosterone were suppressed [20]. Nevertheless, the absence of AR action on Sertoli cells of *jsd* mice resulted in significant alteration of the localization of their nuclei.

Because germ cell compositions of adult *jsd* and SCARKO-*jsd* mice are the same, they make an excellent model with which to identify the genes regulated by chronic deficiency of testosterone action in adult Sertoli cells by microarray analysis, without the complication introduced by changes in cellular

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components. In this report we identify changes in gene expression resulting from selective and prolonged elimination of AR in adult Sertoli cells of *jsd* mice.

MATERIALS AND METHODS

Animals

Transgenic mice with floxed-*Ar* (Ar^{flox}, official symbol $Ar^{tm1Verh}$), on a 129/Sv genetic background, were obtained from the Catholic University of Leuven, Belgium [1]. Both males and females were crossed with mice carrying the *jsd* mutation on a C57BL/6 background, and the offspring were intercrossed for several generations to generate Ar^{flox} and *jsd* homozygous double mutant females ($Ar^{flox/flox}$, $Utp14b^{jsd/jsd}$). Mice expressing cre recombinase under the control of the anti-Mullerian hormone (*Amh*) promoter (*Amh-cre mice*,$cre^{+/-}$, official symbol Tg(Amh-cre)8815Reb, on a 129/Sv background were obtained from the University of Washington, Seattle [3]. Both males and females ($cre^{+/-}$) were crossed with mice carrying the *jsd* mutation on a C3H-B6–129 (HB129) mixed background [21] for one or more generations to generate double-mutant males that were heterozygous for both *Amh-cre* and *jsd* ($cre^{+/-}$, $Utp14b^{+/jsd}$). The $Ar^{flox/flox}$, $Utp14b^{jsd/jsd}$ females were then mated with $cre^{+/-}$, $Utp14b^{jsd/+}$ males to produce the triple-mutant test model males ($Ar^{flox/Y}$, $cre^{+/-}$, $Utp14b^{jsd/jsd}$, SCARKO-*jsd*). The *jsd* mice used as controls carried Ar^{flox}. Genotyping of SCARKO-*jsd* and control *jsd* mice was described earlier [20]. Note that the same breeding protocol was used as in the earlier report [20], although some of the background strains were misidentified in that report. All animal studies were approved by the M.D. Anderson Cancer Center Institutional Animal Care and Use Committee.

Testicular Histology

After the mice were euthanized, testes were removed and fixed in Bouin's solution and embedded in methacrylate. The blocks were sectioned and stained with hematoxylin and periodic acid–Schiff reagent.

Stereology Analysis

Stereological analysis using the optical dissector approach [22] was performed on 25-μm thick sections with Stereo Investigator version 8.0 software (MicroBrightField, Inc., Williston, VT) under a stage-controlled microscope (Leica DMLB 100S, Leica Microsystems, Germany) using a 100× oil immersion lens. A 60 μm × 60 μm counting frame was positioned at multiple sampling sites across the section by the systematic random sampling method. The total sampling volume (Vs) for a given section was calculated as the product of counting frame area, number of sampling sites, and dissector height (15 μm). The total numbers of germ, Sertoli, interstitial, vascular smooth muscle, and peritubular myoid cells per testis (Nt) were then calculated as follows: Nt = Ns × (Vt/Vs), where Ns is the number of nuclei of the specific cells counted in the total sampling volume and Vt is the testis volume, which was determined by dividing the testis weight by the testicular tissue density of 1.05 g/cm^3 [23].

RNA Preparation and Microarray Hybridization

Testis tissue samples were removed from three 12-wk-old control *jsd* and three 12-wk-old SCARKO-*jsd* mice and immediately placed in RNAlater (Qiagen, Valencia, CA) and stored at −20°C until total RNA was extracted using the RNeasy Mini kit (Qiagen). To normalize both microarray and qRT-PCR data on a per testis basis, we added 4 μl of a 1:10 000 dilution of *B. subtilis* RNA containing the *dap* gene with an artificial poly A tail (GeneChip Poly-A RNA Control Kit, Affymetrix, Santa Clara, CA) to each testis sample. We then extracted total RNA using the RNeasy Mini kit. RNA quality was determined by both electrophoresis and by 230-, 260-, and 280-nm absorption readings (NanoDrop, Wilmington, DE).

Ten micrograms of total RNA from each sample was labeled using a GeneChip One-Cycle Target Labeling Kit (Affymetrix) to produce labeled cRNA, which was then hybridized to the Affymextrix GeneChip Mouse Genome M430 2.0 array (Affymetrix). Production of cDNA and cRNA, hybridization to arrays, and evaluation of hybridization quality were completed by the Laboratory for Biotechnology and Bioanalysis I at Washington State University, Pullman, WA.

Microarray Analysis

Expression analysis was performed using the Robust Multi-array Averaging function [24] from the Affy package (v1.5.8) through the BioConductor software (http://www.bioconductor.org/ [25]). Then, genes differentially expressed between *jsd* and SCARKO-*jsd* mice were identified with Significance Analysis of Microarrays software [26]. The data were deposited into the National Center for Biotechnology Information Gene Expression Omnibus, with accession number GSE20918. Analysis of the levels of *dap* RNA in the normalized microarray data showed that there were equivalent levels of *dap* RNA in the *jsd* and the SCARKO-*jsd* samples (Supplemental Table S1, all Supplemental Data are available online at www.biolreprod.org). This result demonstrated that the relative values of gene expression obtained in the normalized microarray data were indeed equivalent to those that would be calculated on a per testis basis [27].

Real-time RT-PCR

Testis samples were collected from 12-wk-old *jsd* and SCARKO-*jsd* mice (n = 5 for *jsd* and n = 4 for SCARKO-*jsd*, which includes the samples used in the microarray), and total RNA was extracted using RNeasy Mini kit (Qiagen), with DNase I treatment to digest genomic DNA. Total RNA (3 μg) was used to generate the cDNA with a Transcriptor First Strand cDNA synthesis kit (Roche Applied Sciences, Indianapolis, IN), which was then used as the template for real-time RT-PCR. Quantitative real-time PCR was performed using the Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia) and SYBR Green (JumpStart Taq ready mix, Sigma, St. Louis, MO). The housekeeping gene, beta-actin, was used to normalize concentration values for each sample. The ratios of beta-actin to *dap* RNA were identical in the samples from the *jsd* and the SCARKO-*jsd* testes (Supplemental Table S1), demonstrating that values presented relative to beta-actin were equivalent to those on a per testis basis. The PCR reaction volume of 10 μl contained 5 μl of SYBR Green JumpStart Taq Mix, 4.8 μl of first-strand cDNA product (1:100 dilution), and 0.1 μl each of specific forward and reverse primers, with a final concentration of 0.1 μM (Sigma Genosys, Houston, TX). Cycling conditions were as follows: 2 min 95°C hold, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C, and 1 min at 72°C. Fluorescence was measured and acquired at 72°C. The primer sequences are listed in Supplemental Table S2. All samples were run in triplicate. Relative levels of gene expression were calculated using Rotor-Gene 6.0 software.

Gene Ontology Analysis

Genes that were differentially regulated in SCARKO-*jsd* mouse testis were analyzed by DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov).

RESULTS

Stereological Analysis of Cellular Components

Both morphological and stereological analysis demonstrated that the cellular compositions of the SCARKO-*jsd* testes were essentially identical to those of *jsd* testes (Fig. 1). About 42% of the cells in the testes of these mice were Sertoli cells and only 6% were germ cells. Of the germ cells, 96% were premeiotic (spermatogonia or preleptotene spermatocytes) and there were only a few spermatocytes in meiotic prophase. This suggests that microarray analysis of differential gene expression between adult testes with and without active AR in the Sertoli cells would largely reflect changes in Sertoli cell gene expression, with minimal possibility of false positives due to changes in cellular composition.

Microarray Analysis

The RNA samples from SCARKO-*jsd* and *jsd* mouse testes were analyzed by microarray. The results for all probes are presented in a searchable Excel file (Supplemental Table S3), which can be used for further analyses. By using ±1.8-fold as a cutoff and a false discovery rate of less than 10%, 157 genes (probes with unique UniGene identifiers) in the SCARKO-*jsd* mice were shown to be downregulated and 197 genes were upregulated compared to *jsd* mice (Supplemental Table S3). The 12 most strongly downregulated and 10 most strongly upregulated genes identified in SCARKO-*jsd* testis are listed in Table 1.

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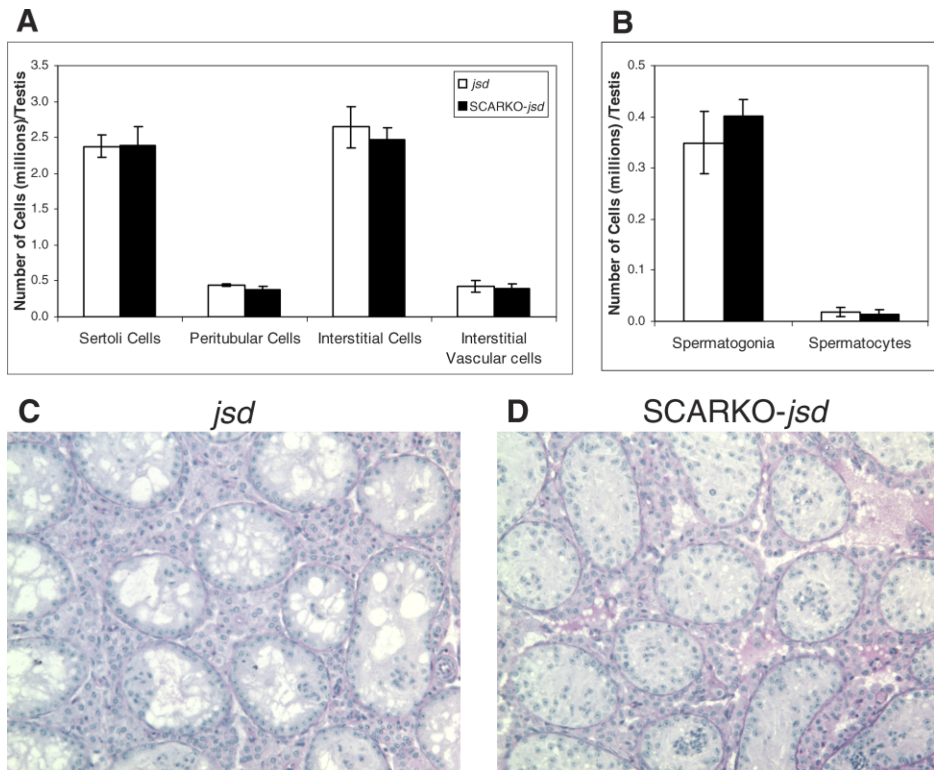

FIG. 1. Cellular composition and histology of the SCARKO-*jsd* mouse testis compared to that of *jsd* mouse testis. Numbers of somatic (**A**) and germ (**B**) cells of SCARKO-*jsd* and *jsd* mouse testis analyzed by stereology. Error bars represent mean ± SEM. We could not reliably distinguish preleptotene spermatocytes from B spermatogonia and so counted them with the spermatogonia. **C**) Cross-section of *jsd* mouse testis showing Sertoli cells and a few spermatogonia at the periphery of seminiferous tubules. **D**) Cross-section of SCARKO-*jsd* mouse testis showing disorganization of Sertoli cells within the tubules. Original magnification (**C**, **D**) ×200.

Confirmation of Microarray Data

Real-time RT-PCR confirmed the gene expression changes for 21 of the 22 genes that appeared to be strongly regulated (Table 1). The only exception was probe set 1431417_at, which was one of five probes representing junction adhesion molecule 2 (*Jam2*). Since the other four microarray probe sets for *Jam2* exhibited minimal changes between *jsd* and SCARKO-*jsd* testes (Supplemental Table S3), we conclude that the downregulation of *Jam2* probe set 1431417_at in SCARKO-*jsd* testis was a false-positive result and should be discarded. This result also demonstrates that caution must be taken when multiple probe sets representing the same gene exhibit different expression patterns.

TABLE 1. Genes most strongly downregulated and upregulated in SCARKO-*jsd* mouse testes vs. *jsd* testes.

Genes	SCARKO-*jsd* vs. *jsd* (fold changes in microarray)	SCARKO-*jsd* vs. *jsd* (fold changes in real-time PCR)
Downregulated in SCARKO-*jsd*		
Reproductive homeobox 5 (*Rhox5*)	−20.4	−43.7 (±13.5)
Corin (*Corin*)	−8.3	−11.9 (±2.3)
Myozenin 2 (*Myoz2*)	−4.9	−7.4 (±1.3)
Cysteine dioxygenase 1, cytosolic (*Cdo1*)	−4.9	−5.9 (±1.0)
Dopamine receptor 4 (*Drd4*)	−4.8	−5.7 (±1.1)
Low density lipoprotein receptor-related protein 8 (*Lrp8*)	−4.7	−4.2 (±0.2)
TRAF2 and NCK interacting kinase (*Tnik*)	−4.6	−2.4 (±0.2)
DIX domain containing 1 (*Dixdc1*)	−3.9	−5.6 (±1.7)
Aquaporin 8 (*Aqp8*)	−3.9	−15.0 (±2.2)
Carbonic anhydrase 7 (*Car7*)	−3.8	−11.9 (±2.8)
Cortexin 1 (*Ctxn1*)	−3.6	−5.5 (±0.8)
Upregulated in SCARKO-*jsd*		
Mitogen activated protein kinase 13 (*Mapk13*)	+8.5	+44.4 (±14.6)
Formin homology 2 domain containing 3 (*Fhod3*)	+8.1	+14.6 (±4.9)
Calcium channel, voltage-dependent, gamma subunit 5 (*Cacng5*)	+8.0	+14.4 (±0.5)
Histocompatibility 2, Q region locus 10 (*H2-Q10*)	+7.4	+12.7 (±4.0)
Dopachrome tautomerase (*Dct*)	+6.7	+18.7 (±4.4)
Secreted phosphoprotein 1 (*Spp1*)	+5.8	+7.5 (±2.1)
Alpha-2-HS-glycoprotein (*Ahsg*)	+5.1	+9.6 (±1.6)
Heparan sulfate 6-O-sulfotransferase 2 (*Hs6st2*)	+4.6	+4.8 (±0.5)
Aldo-keto reductase family 1, member C12 (*Akr1c12*)	+4.6	+6.2 (±1.9)
Tenomodulin (*Tnmd*)	+4.5	+15.0 (±3.8)
Variable Result with different probes		
Junction adhesion molecule 2 (*Jam2*, probe set ID 1431417_at)	−3.9	−1.1 (±0.2)[a]

[a] Not verified by real-time PCR; further investigation revealed that only one weak probe out of five probes for *Jam2* showed downregulation, the other microarray four probes showed minimal changes in expression.

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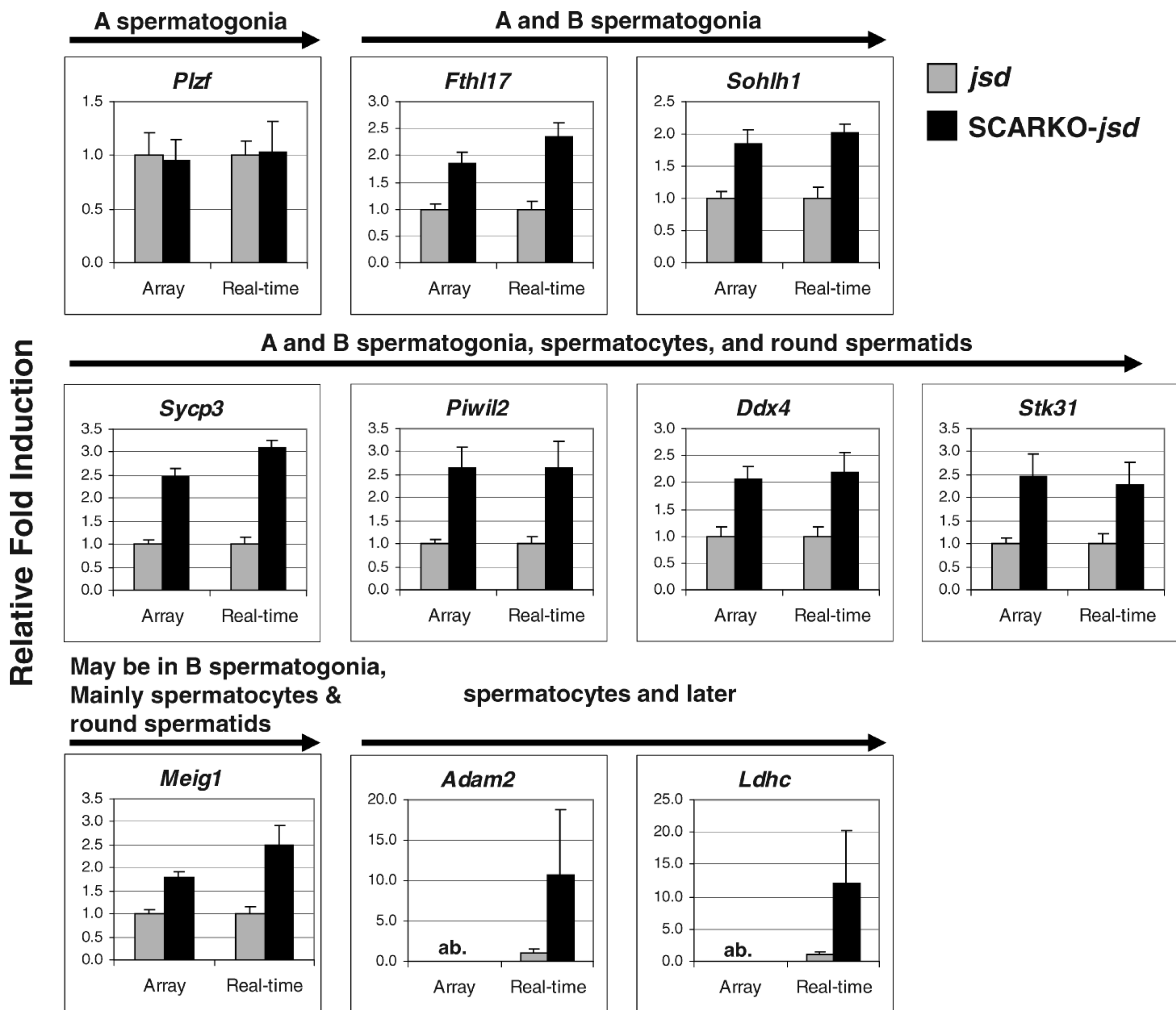

FIG. 2. Expression of germ cell–specific genes in *jsd* and SCARKO-*jsd* mouse testis. Cell types expressing the mRNA of the genes are indicated above arrows. Data are presented as fold induction compared to *jsd* testis. Error bars represent mean ± SEM. ab., scored as absent in microarray data.

Ontology Analysis

Gene ontology studies revealed that, among the significantly regulated genes resulting from the absence of AR in Sertoli cells, there was an overrepresentation of genes involved in bone mineralization (*Ank*, *Spp1*, *P2rx7*, *Ahsg*, and *Cd276*) ($P = 5 \times 10^{-5}$) and neuron differentiation (*Ank3*, *Gas7*, *Tiam1*, *Olig1*, *Mtap1b*, *Ptprz1*, *Id4*, *Nrcam*, *Alcam*, *Bex1*, *Ret*, *Pigt*, and *Mcf2*) ($P = 0.002$). Most surprisingly, the most significantly overrepresented category was meiosis, which included the genes *Syce1*, *Meig1*, *Dazl*, *Sycp3*, *Stra8*, and *Piwil2* ($P = 3 \times 10^{-5}$). Further examination of the microarray data and real-time RT-PCR (Fig. 2) revealed that *Zbtb16* (formerly known as *Plzf*), a gene present only in undifferentiated A spermatogonia, was unchanged in SCARKO-*jsd* testes. Genes expressed in only A and B spermatogonia (*Fthl17*, *Sohlh1*), as well as those whose expression continued in later stages (*Sycp3*, *Piwil2*, *Ddx4*, and *Stk31*), were all upregulated 2- to 3-fold in SCARKO-*jsd* testis. Whereas *Meig1*, whose expression appeared to begin in B spermatogonia, was also upregulated in SCARKO-*jsd* testes, genes that were first expressed in spermatocytes in meiotic prophase, such as *Ldhc* and *Adam2*, were essentially absent in *jsd* testes and very weakly and variably expressed in SCARKO-*jsd* testes, and no statistically significant upregulation could be demonstrated. These observations indicate that the absence of AR in the Sertoli cells induced significant expression of genes associated with differentiated spermatogonia in *jsd* mouse testis.

DISCUSSION

In this study, we used the selective elimination of AR from Sertoli cells in adult *Utp14b* mutant (*Utp14b*$^{jsd/jsd}$) mice as a unique model system for identifying the androgen-regulated genes in adult Sertoli cells that may be involved in the

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TABLE 2. Comparisons of models used for microarray analysis of androgen regulated genes in Sertoli cells.

Genetic deficient model	Control	Age	Duration of differential androgen action	Germ cell populations in deficient	Comparable germ cell populations (model vs. control)
SCARKO-*jsd* (current study)	*Jsd*-(*Ar-flox*)	Adult	~8 wk	A gonia	Yes
SCARKO [6]	*Ar-flox*	10 day	~2–7 days[e]	Up to preleptotene	Yes
hpg [8][a]	*hpg* + testosterone	35–66 day	4–24 h	Up to pachytene (spermatid with T pretreatment) [f]	Yes
Tfm [7][b]	Wild-type	20 day	~12–17 days[e]	Up to early spermatocytes	No
Sertoli knockout of AR hypomorph [9][c]	AR-hypomorph[d]	Adult	~8 wk	Up to round spermatids	No

[a] Includes androgen regulated genes in all somatic cells of the testis.
[b] Genes primarily expressed in germ interstitial cells were eliminated to obtain genes that are androgen regulated primarily in Sertoli cells.
[c] Mice with a floxed (exon 1-neo) of the androgen receptor, which becomes inverted in Sertoli cells and forms a null allele in the presence of *Amh-Cre*.
[d] Mice carrying mutant floxed (exon 1-neo) allele of the androgen receptor.
[e] Based on the reported appearance of androgen receptor in Sertoli cells either on Days 3–5 [6] or Day 8 of development.
[f] Some data were generated by short-term testosterone treatment of *hpg* mice which only have pachytene spermatocytes; and some data were generated by pretreatment with testosterone (T) followed by 2 wk without treatment, and then short term testosterone treatment.

testosterone-dependent functions of these cells, such as the progression of germ cells through meiosis.

Whereas no system is perfect, our current system fulfills many criteria for an ideal system to uncover important hormonally regulated Sertoli cell genes (Table 2). First, it detects gene expression changes due to loss of androgen action, specifically on the Sertoli cells, whereas other models, such as *hpg* [8] and *Tfm* [7], involve reduced androgen action on multiple testicular cells. In the study using the *Tfm* model, this problem was partially overcome by employing methods to eliminate genes primarily expressed in germ or interstitial cells. Second, it involves the use of adult animals, which is preferable since Sertoli cells from immature mice may not express the genes necessary for the maturation of germ cells, as exemplified by the elimination of Sertoli-cell AR having no effect on germ cell populations in 10-day-old mice [6]. Third, it involves prolonged differential levels of androgen action between the test model and the control, since the SCARKO mice are deficient in AR in Sertoli cells from birth. Fourth, our model showed minimal differences in composition of germ cells from the control, thus eliminating the secondary effects of changing germ cell populations on the expression of genes by the Sertoli cells. Furthermore, the almost total absence of germ cells, which make up the bulk of the cells in a normal testis, increases the efficiency of detection of Sertoli cell genes in the *jsd* mice. However, the absence of germ cells may be a shortcoming of the model that could result in differences from the normal testis, since germ cells affect gene expression in Sertoli cells and induce stage-specific cyclical changes. Therefore, the Sertoli cells in *jsd* or SCARKO-*jsd* mice do experience some different physiological conditions from those in mice with a full complement of germ cells. Also, the current model and others previously reported [1–6] eliminate AR from Sertoli cells in the embryonic testis, which may alter Sertoli

TABLE 3. Comparison of regulation of genes strongly regulated by Sertoli-cell AR in adult *jsd* mice with the AR regulation of these genes in immature SCARKO and *Tfm* mice.

Gene symbol	SCARKO-*jsd* vs. *jsd* (adult; current study)	SCARKO vs. wild-type (10 day; [6])[a,b]		*Tfm* vs. wild-type (20 day [7])[a,c]	
Rhox5	−20	−18	A	−7	A
Corin	−8	−4	A	−2.8	A
Myoz2	−5	−1.7	A	NR	
Cdo1	−5	−2.3	A	NR	
Drd4	−5	−6	A	−5	A
Lrp8	−5	(−1.1)	D	−1.9	A
Tnik	−5	Nf		NR	
Dixdc1	−4	Nf		NR	
Aqp8	−4	Ab		−2.2	A
Car7	−4	Nf		−1.3	Q
Ctxn1	−4	Nf		NR	
Mapk13	+9	1.5	Q	NR	
Fhod3	+8	2.3	A	4.1	A
Cacng5	+8	−1.4	D	3.5	A
H2-Q10	+7	−1.4	D	2.3	A
Dct	+7	1.3	Q	2.5	A
Spp1	+6	(−1.1)	D	NR	
Ahsg	+5	(1.1)	D	2.0	A
Hs6st2	+5	−1.2	D	4.4	A
Akr1c12	+5	Ab		NR	
Tnmd	+5	Ab		1.6	Q

[a] A, in agreement with the current study; Q, in qualitative agreement with current study; D, in disagreement with the current study.
[b] Ab, probe was scored as absent on microarray; nf, probe not found in data set available.
[c] NR, not reported on list of significantly regulated genes; therefore, likely in qualitative disagreement with the current study.

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cell development during neonatal development and cause some gene expression changes in adult testes, which differ from those that would be observed by elimination of AR specifically in the adult testis. This can be improved in the future by using a conditional knockout model that will knockout AR in Sertoli cells when the animals have entered adulthood.

As reviewed above (Table 2), the various model systems used to identify androgen-regulated genes in Sertoli cells employ different conditions. We propose that the genes that are AR-regulated under multiple conditions are more likely to be directly regulated by AR and be the ones that are regulated by AR in the normal adult testis. Conversely, the ones that are regulated by AR only in one specific model system may be false positives, indirect effects, or in populations other than Sertoli cells, and they may not be regulated by AR in the Sertoli cells of the normal adult testis. In Table 3, we have compared the changes in expression of the highly regulated genes in SCARKO-*jsd* (Table 1) with the regulation of these genes in two other studies of androgen-regulated Sertoli-cell gene expression [6, 7]. Of the 21 genes, only three downregulated genes, *Rhox5*, *Corin*, and *Drd4*, and one upregulated gene, *Fhod3*, are qualitatively regulated in the same direction in all three systems. The androgen regulation of *Rhox5* has been well studied, occurs in rat as well as mouse [28], and is a direct target of AR [29]. Using the PATSER tool (http://rsat.ulb.ac.be/rsat/) [30] with a cutoff of Weight Score 7 ($P < 10^{-4}$), we have identified at least one potential ARE binding site for all of the genes in Table 1 (from −5000 bp upstream of the transcription start site to the end of transcription) except for *Akr1c12* (data not shown). An attempt to determine whether these genes were regulated similarly in the rat using the irradiated rat testis model [31] was not informative, as these genes either could not be found on the rat 230 2.0 expression array or their expression was too low to be detected on that array.

The present results can also be used to test the robustness of reports of effects of AR elimination on changes in expression of functional groups of genes, such as junctional complex proteins, proteases and protease inhibitors, proteins involved in vitamin A metabolism, and solute carriers [6, 7, 9, 32] in Sertoli cells (Supplemental Table S4). Some major proteins involved in the tight junctions between Sertoli cells [33], such as occludin (*Ocln*), gelsolin (*Gsn*), and claudin 11 (*Cldn11*), are not AR-regulated in the *jsd* model. Only alpha actinin 3 (*Actn3*) and thrombospondin 1 (*Thsb1*) showed consistent downregulation in several Sertoli AR knockout models. In addition, none of the proteases or protease inhibitors or genes regulating the vitamin A levels showed the same regulation by elimination of AR in the *jsd* model as in other models; only eppin (*Spinlw1*), which was downregulated 8-fold in 10-day-old SCARKO mice, and alcohol dehydrogenase (*Adh1*), which was upregulated 9-fold in 20-day-old *Tfm* mice, showed the same direction of regulation, but these changes were only 1.4- and 1.7-fold, respectively. Finally, none of the solute transport proteins, except the cationic amino acid transporter *Slc7a4*, were regulated by elimination of Sertoli-cell AR in the *jsd* model. These results suggest that caution should be exercised in making broad generalizations as to groups of genes regulated by AR in Sertoli cells, when only one model of a specific age and germ cell composition is used.

Since *Rhox5* was strongly downregulated in the absence of AR in SCARKO-*jsd* testes, we investigated whether genes reported to be regulated by *Rhox5* in other models would also be regulated by AR in adult *jsd* testes. However, of the 13 genes reported to be regulated by *Rhox5* [34, 35], only *Tmem176a* showed a change in SCARKO-*jsd* testis in the same direction as predicted, but the change was very small (only −1.3-fold downregulation), and one gene, *Ins2*, which had been reported to be upregulated by *Rhox5* [34], was actually upregulated 2.5-fold in SCARKO-*jsd* Sertoli cells, cells whose *Rhox5* was dramatically reduced (Supplemental Table S3). Thus, these results suggest that the regulation of these genes by *Rhox5* is highly dependent on the developmental stage and environment of the Sertoli cells and/or they are also regulated by AR-dependent mechanisms independently of *Rhox5*, which overcome the effects of the *Rhox5* regulation. In either case, it does not appear that changes in expression of *Rhox5*-regulated genes are responsible for the dislocation of Sertoli cell nuclei in SCARKO-*jsd* mice.

To investigate whether the Sertoli cell AR-regulated genes identified in multiple model systems are important for spermatogenesis, we reviewed the effects of targeted disruption. Only homozygous mice with targeted disruption of *Lrp8* [36] and *Rhox5* [37], but not those with null mutation for *Corin*, *Myoz2*, *Drd4*, and *Thbs1*, have been reported to exhibit male infertility. However, in the case of *Lrp8* null mice, spermatogenesis proceeds normally and the sperm defects develop in the epididymis; in *Rhox5* null mice, germ cells pass through meiosis and the mice still retain some fertility. Since there is no evidence that any specific androgen-regulated gene is critical for the completion of spermatogenesis, it is likely that when AR is eliminated from Sertoli cells, spermatogenesis is disrupted as a result of multiple gene alterations.

The biological significance of the dramatic overrepresentation of a group of genes involved in bone mineralization among the Sertoli cell AR–regulated genes in *jsd* testes is not clear. It is tempting to speculate that since the Sertoli cells and bone cells are both involved in tissue remodeling processes, there is some overlap in gene expression. Three of the genes identified (*Spp1*, *Ank*, and *Cd276*) are preferentially expressed in the Sertoli cells, and hence alterations of their expression in testis could be directly affected by changes in the Sertoli cells. Also, since bone mineralization is stimulated by androgens, it would be expected that bone mineralization genes would be preferentially found among an androgen-regulated set. However, only one of the genes identified in the group (*Spp1*, also known as osteopontin) is known to be downregulated by androgen [38]. Alternatively, it is quite possible that, although these genes all share the ''bone mineralization'' functional category, it is their other functions, including cell adhesion, signal transduction, and cell differentiation and/or proliferation, that make them stand out in this analysis, and these functions explain the disrupted Sertoli cell nuclear localization and detachment from the basement membrane in the SCARKO-*jsd* testes [20].

The neuron differentiation genes may have been identified by ontology analysis due to the same principle. Their functions include cell adhesion (*Alcam*, *Nrcam*, *Ret*), signal transduction (*Alcam*, *Ank3*, *Mcf2*, *Tiam1*), cell differentiation and/or proliferation (*Bex1*, *Gas7*, *Id4*), and cytoskeleton formation (*Mtap1b*, *Nrcam*), which may have been responsible for the alteration of Sertoli cell nuclear localization in SCARKO-*jsd* mouse testis. It is also possible that the cell elongation and induction of asymmetry, which occur during both neuron differentiation and Sertoli cell maturation (Fig. 1C), involve the same cellular processes, which appear to be lost in the SCARKO-*jsd* testes.

Previous studies have shown that the selective elimination of AR from Sertoli cells disrupted the localization of Sertoli cell nuclei both in adult SCARKO mouse testes [32] and in adult SCARKO-*jsd* testes [20]. With only a few type A spermatogonia left in the seminiferous tubules, Sertoli cell

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nuclear localization was more drastically altered in SCARKO-*jsd* testis [20] than in the SCARKO testis. When we examined genes that were identified as significantly altered in expression in postnatal day 10.5 SCARKO mouse testis and were considered to play roles in Sertoli cell nucleus dislocation [32], including *Vim*, *Lama5*, *Cldn11*, *Ocln*, *Gsn*, *Cacna1a*, and *Plat*, we found them in SCARKO-*jsd* testis to be unchanged compared to *jsd* testis or not expressed at all (Supplemental Table S3). The lack of changes in expression of these genes in SCARKO-*jsd* mice indicates that they are not the main causes of Sertoli cell nuclear dislocation and their changes in the day-10.5 SCARKO mice could result from a difference between prepubertal and adult animals. Therefore, changes in expression of the bone mineralization (*Ank*, *Spp1*, *P2rx7*, *Ahsg*, and *Cd276*) and neuron differentiation (*Ank3*, *Gas7*, *Tiam1*, *Olig1*, *Mtap1b*, *Ptprz1*, *Id4*, *Nrcam*, *Alcam*, *Bex1*, *Ret*, *Pigt*, and *Mcf2*) genes identified in our array data provide reasonable explanations for the alteration in Sertoli cell nucleus localization. These genes make good candidates with which to further explore the detailed molecular mechanisms of AR regulation of Sertoli cell structure.

The upregulation of a group of genes that are expressed in differentiated germ cells in the SCARKO-*jsd* mice was surprising, since no morphological changes in germ cell populations were detected (Fig. 1) [20]. Microarray and real-time RT-PCR results demonstrated that the genes known to be expressed in differentiated A spermatogonia and B spermatogonia were increased 2–3 fold when the AR was eliminated from Sertoli cells, whereas an undifferentiated spermatogonial marker remained unchanged and spermatocyte makers were not significantly detectable. These results demonstrate that the presence of AR in Sertoli cells did contribute, to a small extent, to the inhibition of spermatogonial differentiation in *jsd* mice. One of the histological endpoints that showed no significant difference between SCARKO-*jsd* and *jsd* testes [20], the tubule differentiation index, which measures the percentage of tubules with cells at the B spermatogonial stage or later [39], had a large variance in that study so that a 2-fold change may not have been detected. Although there is no 2-fold increase in spermatogonial numbers in SCARKO-*jsd* testes (Fig. 1), we cannot rule out the possibility of a relative increase in the more differentiated spermatogonial stages in the SCARKO-*jsd* mice since all spermatogonia were counted together. In any case, the degree of spermatogonial differentiation induced by elimination of AR from the Sertoli cells of *jsd* mice is minor compared to that caused by total elimination of AR [19] or addition of AR antagonists [18]. This further demonstrates that testosterone acts on the AR in other somatic cell types to produce the factors that inhibit *jsd* spermatogonial differentiation.

In summary, this study, using a unique model of specific elimination of AR in adult Sertoli cells in a testis essentially lacking germ cells, has identified a group of genes that are consistently AR-regulated in Sertoli cells of different ages and environments and has demonstrated that other previously reported genes are AR-regulated in Sertoli cells only under specific conditions. We have also identified groups of genes common to other biological processes that are regulated by AR in adult Sertoli cells, which may provide insight into the cause of the nuclear dislocation of the Sertoli cells in SCARKO-*jsd* testis. Finally, the results demonstrate that AR action in Sertoli cells contributes to a small but significant degree to the inhibition of spermatogonial differentiation in *jsd* mutant mice.

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