BioOne COMPLETE

Organotypic Cultures of Prepubertal Mouse Testes: A Method to Study Androgen Action in Sertoli Cells while Preserving their Natural Environment

Authors: De Gendt, Karel, McKinnell, Chris, Willems, Ariane, Saunders, Philippa T.K., Sharpe, Richard M., et al.

Source: Biology of Reproduction, 81(6) : 1083-1092

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.109.078360

BioOne Complete (complete.BioOne.org) is a full-text database of 200 subscribed and open-access titles in the biological, ecological, and environmental sciences published by nonprofit societies, associations, museums, institutions, and presses.

Your use of this PDF, the BioOne Complete website, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Complete content is strictly limited to personal, educational, and non - commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

BIOLOGY OF REPRODUCTION **81,** 1083–1092 (2009)
Published online before print 8 July 2009.
DOI 10.1095/biolreprod.109.078360

Organotypic Cultures of Prepubertal Mouse Testes: A Method to Study Androgen Action in Sertoli Cells while Preserving their Natural Environment

Karel De Gendt,[2] Chris McKinnell,[3] Ariane Willems,[2] Philippa T.K. Saunders,[3] Richard M. Sharpe,[3] Nina Atanassova,[4] Johannes V. Swinnen,[2] and Guido Verhoeven[1,2]

Laboratory for Experimental Medicine and Endocrinology,[2] Katholieke Universiteit Leuven, Leuven, Belgium
Medical Research Council Human Reproductive Sciences Unit,[3] Centre for Reproductive Biology, Edinburgh, Scotland, United Kingdom
Institute of Experimental Morphology and Anthropology Museum,[4] Bulgarian Academy of Science, Sofia, Bulgaria

ABSTRACT

Cluster analysis at Postnatal Day 8–20 of putative androgen-regulated genes in mice with Sertoli cell-selective knockout of the androgen receptor (SCARKO) has pinpointed three genes (*Spinlw1*, *Gpd1*, *Drd4*) with an expression pattern strongly resembling that of *Rhox5*, the definitive Sertoli cell (SC) androgen-regulated gene. We used organotypic testis cultures from Day 8 mice to study control of these genes by (anti)androgens and follicle-stimulating hormone (FSH). Testis morphology and androgen induction of the studied genes were preserved for 48 h. Preincubation with ketoconazole for 24 h to block endogenous androgen production, followed by 24-h incubation with the synthetic androgen R1881, resulted in 45-, 5-, 19-, and 6-fold induction of mRNA levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4*, respectively. However, noticeable differences in control of the studied genes were observed. *Rhox5* and *Spinlw1* were fully induced by R1881 in the continuous (48 h) presence of ketoconazole, whereas only marginal effects were observed on expression of *Gpd1* and *Drd4*. Similarly, FSH only marginally affected expression of *Rhox5* and *Spinlw1*, whereas it markedly increased *Gpd1* and *Drd4* expression. Explant cultures of SCARKO testes confirmed the differential effects of FSH on the studied genes and, for *Gpd1*, showed that the effect did not depend on a functional androgen receptor in SC, whereas this was essential for the effects of FSH on *Drd4*. In conclusion, organotypic cultures represent the first in vitro approach to preserving androgen responsiveness of putative SC-expressed genes. This approach facilitates detailed analysis of their regulation in ways not possible in vivo.

androgen receptor, bicalutamide, cell-selective knockout, follicle-stimulating hormone, ketoconazole

INTRODUCTION

There is an overwhelming amount of evidence indicating that optimal spermatogenesis requires both androgens and follicle-stimulating hormone (FSH) [1–3]. Under a number of conditions, however, androgens are able to support complete germ cell development in the virtual absence of FSH. In mice that are hypogonadal due to a major deletion in the *Gnrh1* gene (*hpg* mice), for instance, androgen administration restores qualitatively normal spermatogenesis and even fertility [4]. Similarly, androgens maintain fertility in mice with a knockout of the *Fshb* gene and in men and mice with inactivating mutations of the FSH receptor gene (*Fshr*) [5–8].

The mechanisms by which androgens affect spermatogenesis remain incompletely understood, but the generation of mice with a cell-selective knockout of the androgen receptor (AR) either in germ cells [9], in peritubular myoid cells [10], or in Sertoli cells [11–13] indicates that Sertoli cells are the major targets of androgen action in the control of germ cell development. Animals with a complete and selective knockout of the AR in Sertoli cells (SCARKO), such as the SCARKO mice, develop a spermatogenic block in meiosis [12, 13].

In rats and mice, Sertoli cells acquire androgen responsiveness during the first week of postnatal life [14], and AR expression becomes detectable by immunohistochemical techniques between Days 3 and 5 postpartum [15, 16]. In contrast, peritubular cells stain positive for the AR already from Day 15 postcoïtum onwards [17]. Expression of mRNA encoded by *Rhox5*, a gene known to be selectively expressed in Sertoli cells under the control of specific androgen response elements located in its promoter region, was barely detectable on Day 7 but dramatically upregulated from Day 9 postpartum onwards [18]. Notably, microarray analysis reveals that, on Postnatal Day 10, as many as 692 genes are differentially expressed in SCARKO and control testes, suggesting that a relatively large number of genes are affected by ablation of the AR in Sertoli cells [19]. However, for only 40 of these genes is the difference in expression level larger than 2-fold. Twenty eight of the latter are downregulated in SCARKOs (suggesting direct or indirect stimulation via the AR in Sertoli cells), and 12 of them are upregulated (suggesting inhibition via the AR in Sertoli cells). *Rhox5* displays the largest difference in expression level (18-fold higher in control as compared to SCARKO testis). Interestingly, cluster analysis of the expression profiles of the differentially expressed genes between Days 8 and 20 revealed 14 different clusters, but 43% of the genes with a more than 2-fold lower expression in SCARKOs are found in one cluster (cluster 4) that also includes the *Rhox5* gene [19]. Since *Rhox5* is known to be directly regulated by androgens in Sertoli cells and is also the only gene that has been identified in all transcriptional profiling studies searching for androgen-regulated genes in Sertoli cells [19–22], we used hierarchical clustering of the genes in cluster 4 to identify additional genes that might be regulated by androgens in Sertoli cells [19]. The three genes for which the expression pattern most strongly resembled that of *Rhox5* were those

[1]Correspondence: Guido Verhoeven, Laboratory for Experimental Medicine and Endocrinology, Gasthuisberg, Onderwijs en Navorsing, Herestraat 49, bus 902, B-3000 Leuven, Belgium. FAX: 32 16 34 59 34; e-mail: guido.verhoeven@med.kuleuven.be

Received: 27 April 2009.
First decision: 19 May 2009.
Accepted: 1 July 2009.
© 2009 by the Society for the Study of Reproduction, Inc.
This is an Open Access article, freely available through *Biology of Reproduction's* Authors' Choice option.
eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

encoding serine protease inhibitor-like protein with Kunitz and WAP domains 1 (*Spinlw1*, previously known as Eppin), with well-described effects on fertility in monkeys [23]; glycerol-3-phosphate dehydrogenase 1 (*Gpd1*), an enzyme involved in glycerol and lipid metabolism [24]; and dopamine receptor 4 (*Drd4*), a G-protein-coupled receptor that might modulate responsiveness to hormones using cAMP as second messenger [25]. Enzymatic separation of the testis into interstitial and tubular tissue confirmed that, at least on Day 10, all of these genes are mainly expressed in the tubular compartment [19].

Since Sertoli cells rapidly lose the expression of androgen target genes such as *Rhox5* and lose androgen responsiveness after their isolation and culture [26, 27], we explored whether organotypic cultures can be used to circumvent these problems. Organotypic cultures of fetal, neonatal, and adult testis tissue derived from rodent, monkey, and man were developed more than 40 yr ago [28] and have been used to study testicular development, spermatogonial proliferation and differentiation, Sertoli cell proliferation, and germ cell progression [29–34]. To the best of our knowledge, however, this is the first attempt to use this experimental paradigm to study androgen-dependent gene regulation in Sertoli cells. Here we used organotypic cultures to study and compare the regulation of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* in the prepubertal testis in more detail. Our data confirm androgen regulation of all studied genes but also show that, despite a very similar behavior in cluster analysis, each of these genes displays its own specific pattern of regulation.

MATERIALS AND METHODS

Organotypic Cultures of Prepubertal Mouse Testes

Testes and epididymides were removed "en bloc" from 8-day-old C57BL6/J@Rj mice or from 8-day-old SCARKO and control (wild type, AMHCre$^{+/-}$, or AR^{fl}/Y) mice [12]. The tissue clumps were disinfected for 10 sec in 50% (v/v) tincture of iodine (1%) in PBS (Invitrogen, Carlsbad, CA) and subsequently washed twice in PBS. The testes were separated from the epididymides in PBS, carefully decapsulated with fine forceps and scissors, and divided into four equal parts using needle tips. The tissue pieces derived from one testis were placed at the medium/air interface in a 15-mm Netwell insert with a 74-μm mesh (Corning, Amsterdam, The Netherlands). Each well contained 800 μl Dulbecco modified Eagle medium/F12 medium (Invitrogen) supplemented with vehicle, R1881 (NEN, now Perkin Elmer, Waltham, MA), hCG (Sigma, St. Louis, MO), ovine FSH (oFSH; NIDDK-oFSH-20; obtained from Dr. Parlow through the NIDDK program), recombinant human FSH (rhFSH; Puregon, Organon, Oss, The Netherlands), bicalutamide (kindly provided by AstraZeneca, Ukkel, Belgium), ketoconazole (MP Biomedicals, Solon, OH), or combinations of these agents as indicated. Tissue cultures were maintained in 12-well plates in a sealed incubator at 37°C. The latter incubation temperature was selected because between Days 8 and 10, in vivo testes are still located intra-abdominally and as such are kept at body temperature. Since initial experiments revealed necrosis in the center of the tissue clumps when the incubator was gassed with air, a 95% O_2/5% CO_2 mixture was used in all subsequent experiments. Tissues were cultured for 48 h, and media and gas were changed after 24 h. Conditioned media collected after 24 and 48 h were stored at −20°C for subsequent analysis of testosterone production, as described below. Fresh decapsulated testis tissue from 8- and 10-day-old mice was used as a reference. All animal experiments were approved by the Ethical Committee of the Katholieke Universiteit Leuven and conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

At the end of the culture period, the tissue clumps were removed from the culture mesh and snap-frozen in liquid nitrogen for RNA extraction. Tissues destined for immunohistochemical analysis were fixed by replacing the medium with Bouin fluid for 6 h at room temperature. Thereafter, the testis fragments were carefully removed from the mesh, dehydrated, and further processed into paraffin blocks using standard procedures.

Detection and Quantification of Apoptotic Cells

Apoptotic cells were identified based on DNA fragmentation detected by in situ TUNEL as described in detail previously [35]. Briefly, DNA 3′-end labeling was carried out on tissue sections using terminal transferase and digoxygenin-11-dUTP (both from Roche Diagnostics GmbH, Mannheim, Germany). Digoxygenin labeling was detected immunohistochemically (see below). Sections from three explant samples were analyzed using a Zeiss AxioImager microscope (Carl Zeiss Ltd., Welwyn Garden City, U.K.) fitted with a Hitachi HV-C20 camera (Hitachi Denshi Europe, Leeds, U.K.) and a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, U.K.). Image-Pro 6.2 software with Stereologer plug-in (MagWorldwide, Wokingham, U.K.) was used to select 70 random fields per sample for counting of all apoptotic cells within each field.

Immunohistochemical Staining

Specific proteins were detected by immunohistochemistry using standard methods described in detail previously [36, 37]. Briefly, detection of AR only required prior antigen retrieval in 0.01 M citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubation of slides in 3% (vol/vol) H_2O_2 in methanol. Slides were incubated overnight at 4°C with primary antibodies as follows: anti-digoxygenin (Roche Diagnostics) used at a dilution of 1:100; anti-HSD3B (Santa Cruz Biotechnology, Santa Cruz, CA) used at a dilution of 1:800; anti-CYP11A1 (Chemicon, Chandlers Ford, U.K.) used at a dilution of 1:300; and anti-AR (SC816; Santa Cruz) used at a dilution of 1:400. Slides were then incubated with appropriate biotinylated secondary antibody, followed by incubation with streptavidin-conjugated horseradish peroxidase (Dako, Ely, U.K.) and visualization of immunostaining using diaminobenzidine (Liquid DAB+; Dako). To ensure reproducibility of results and accurate comparison of immunostaining between treatment groups, sections from all groups were run in parallel on at least two occasions. Representative sections were photographed using a Provis AX70 microscope (Olympus Optical, London, U.K.) fitted with a Canon DS6031 camera (Canon Europe, Amsterdam, The Netherlands). Images were compiled using Photoshop CS2 (Adobe Systems Inc., Mountain View, CA).

Testosterone Measurements in Conditioned Media

Conditioned medium (500 μl/testis culture) was extracted with four volumes of cyclohexane/ethylacetate (1:1, v/v). Extraction efficiency as measured by addition of tritiated testosterone averaged 85% ± 2% (mean ± SEM, n = 25) and did not differ depending on treatment conditions. A 0.25-ml aliquot of the extract was evaporated in a vacuum-evaporator, the residue resuspended in charcoal-stripped human serum, and the concentration of testosterone determined using the Testo-RIA-CT kit (Biosource International, Camarillo, CA) according to the instructions of the manufacturer.

Quantitative PCR Analysis

RNA was extracted with the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, including an on-column DNaseI-treatment (Qiagen). Total RNA (1 μg) was reverse transcribed with the Superscript II RNase H$^-$ reverse transcription kit (Invitrogen) including RNaseOUT (Invitrogen), according to the manufacturer's instructions, using 150 ng random primers (Invitrogen) per reaction.

The 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA) was used for sample cDNA quantification. *Spinlw1*, *Gpd1*, and *Drd4* were assayed using SybrGreen as a fluorescent dye. The relevant primers were described previously [19]. The quantitative PCR (qPCR) two-step protocol was 2 min at 50°C followed by 2 min at 95°C. Subsequently, 40 cycles of 3 sec at 95°C and 30 sec at 60°C were performed. Each 10-μl real-time PCR reaction contained 1× Platinum SybrGreen qPCR Supermix-UDG (Invitrogen), 1× ROX dye (Invitrogen), 150 nM of each primer, and 2 μl of a 1/10 dilution of the cDNA reaction. Dissociation curves confirmed uniqueness of each amplicon. For the *Rhox5* gene, a labeled probe was used as described previously [12]. In this case, the PCR protocol was 2 min at 95°C followed by 40 cycles of 3 sec at 95°C and 30 sec at 60°C. The reaction mixture contained 1× Taqman Fast Universal Master Mix (Applied Biosystems), 1 μM of each primer, 400 nM probe, and 2 μl of a 1/10 dilution of the cDNA reaction.

To create standards for the quantitative PCR, gene-specific cDNAs were generated by RT-PCR. The fragments were then cloned into pGEM-T Easy (Promega, Madison, WI), sequenced to confirm their identity, and quantified by spectrophotometry. All samples and standard curves were run in triplicate. Data were analyzed with the 7500 Fast System SDS Software, version 1.4 (Applied Biosystems). 18S rRNA was used as an internal control, and expression levels were calculated as copy number per 10^8 copies 18S rRNA.

Statistical Analysis

Gene expression levels in cultured explants and testosterone levels in conditioned media were compared by ANOVA followed by Fisher LSD test

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

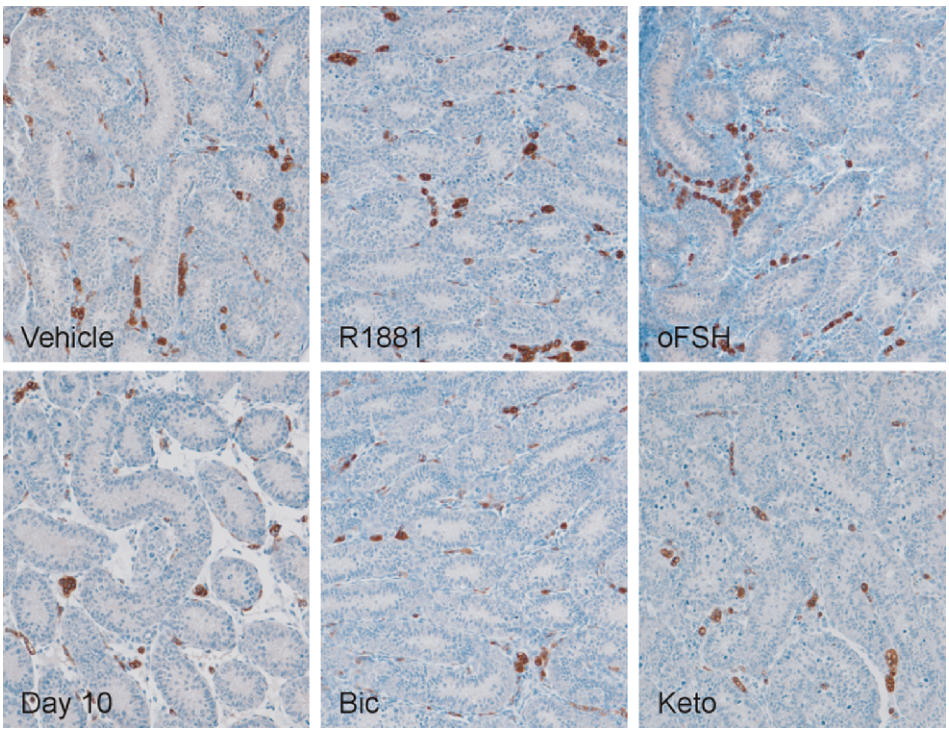


FIG. 1. Immunohistochemical staining for HSD3B in testicular explants treated for 48 h with vehicle, R1881 (10^{-8} M), oFSH (20 ng/ml), bicalutamide (Bic; 10^{-5} M), or ketoconazole (Keto; 2.10^{-5} M). Testicular tissue derived from 10-day-old mice (Day 10) was included as a reference. Original magnification ×200.

using NCSS 2000 software (NCSS, Statistical Analysis and Data Analysis Software, Keysville, UT).

RESULTS

Tissue Preservation, Leydig Cell Function, and Androgen Receptor Expression in Organotypic Cultures

Routinely, testes derived from 8-day-old mice (~4 mg/testis) were divided into four equal parts and were maintained in culture for 48 h as described in the *Materials and Methods* section. Examination of the explants by hematoxylin and eosin staining (data not shown) and by immunostaining for 3β-hydroxysteroid dehydrogenase (HSD3B) at the end of the 48-h culture period and comparison with testis tissue freshly derived from 10-day-old mice revealed that general testis architecture was well preserved and that culturing did not affect the number or size of HSD3B immunopositive (Leydig) cells per cross section (Fig. 1). Comparable results were obtained after immunostaining for CYP11A1 (data not shown). Staining for apoptotic cells revealed that 48 h of culture in control medium (vehicle) resulted in a limited (approximately two-fold) increase in the number of apoptotic cells in the interstitial and tubular compartments as compared to freshly removed testes of 10-day-old controls (Table 1).

Testosterone levels in media derived from vehicle-treated cultures after the first 24 h of incubation reached a mean value of 4.33 ng/ml, apparently reflecting active androgen secretion (see further). These levels dropped to 1.01 ng/ml in media collected during the second half of the 48-h culture period (Table 2).

As illustrated in Figure 2, AR immunoexpression in vehicle-treated cultures was comparable to that observed in freshly derived testis tissue from 10-day-old control mice, with distinct staining in nuclei from interstitial cells, peritubular myoid cells, and Sertoli cells.

Organotypic Cultures Confirm Androgen Regulation of Rhox5*,* Spinlw1*,* Gpd1*, and* Drd4

In a first series of experiments, we compared the effects of the synthetic androgen R1881, the antiandrogen bicalutamide, oFSH, and rhFSH in the organotypic cultures. None of these treatments had noticeable effects on general testis architecture or Leydig cell number/size per explant cross section (Fig. 1). Ovine FSH slightly increased the number of apoptotic cells in

TABLE 1. Number of apoptotic cells in control testes and in testicular explants.*

Experimental condition	Interstitial compartment	Tubular compartment
Control (Day 10)	0.02 ± 0.03^{a}	0.54 ± 0.29^{a}
Explant + vehicle	0.06 ± 0.03^{a}	$1.13 \pm 0.36^{a,c}$
Explant + R1881 (10^{-8} M)	0.07 ± 0.05^{a}	$1.79 \pm 0.49^{c,d}$
Explant + oFSH (20 ng/ml)	0.07 ± 0.04^{a}	2.18 ± 0.14^{d}
Explant + hCG (10 ng/ml)	0.05 ± 0.05^{a}	$1.10 \pm 0.30^{a,b}$
Explant + bicalutamide (10^{-5} M)	0.10 ± 0.10^{a}	$1.31 \pm 0.23^{b,c}$
Explant + ketoconazole (2.10^{-5} M)	0.41 ± 0.15^{b}	4.75 ± 0.64^{e}

* Apoptotic cells were counted in three explant samples (70 fields per sample); data represent counts per field (mean ± SD, n = 3).
[a–e] Data that differ significantly ($P < 0.05$) are indicated by different superscript letters.

TABLE 2. Testosterone levels (ng/ml) in culture media after 24 or 48 h of incubation.*

Treatment	24 h (n)	48 h (n)
Control (vehicle)	4.33 ± 0.43^{c} (6)	$1.01 \pm 0.24^{b,c}$ (5)
Bicalutamide (10^{-5} M)	2.01 ± 0.36^{b} (4)	$0.43 \pm 0.11^{b,c}$ (4)
Ketoconazole (2.10^{-5} M)	0.37 ± 0.10^{a} (5)	0.03 ± 0.01^{a} (3)
oFSH (20 ng/ml)	14.84 ± 2.68^{d} (3)	11.95 ± 2.51^{d} (3)
rhFSH (20 ng/ml)	4.29 ± 0.09^{c} (3)	1.60 ± 0.74^{c} (3)

* Values are the mean ± SEM of the number of independent experiments indicated between brackets. In each experiment, media from 5 organotypic cultures treated as indicated were assayed.
[a–d] Values that differ significantly ($P < 0.05$) are indicated by different superscript letters.

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

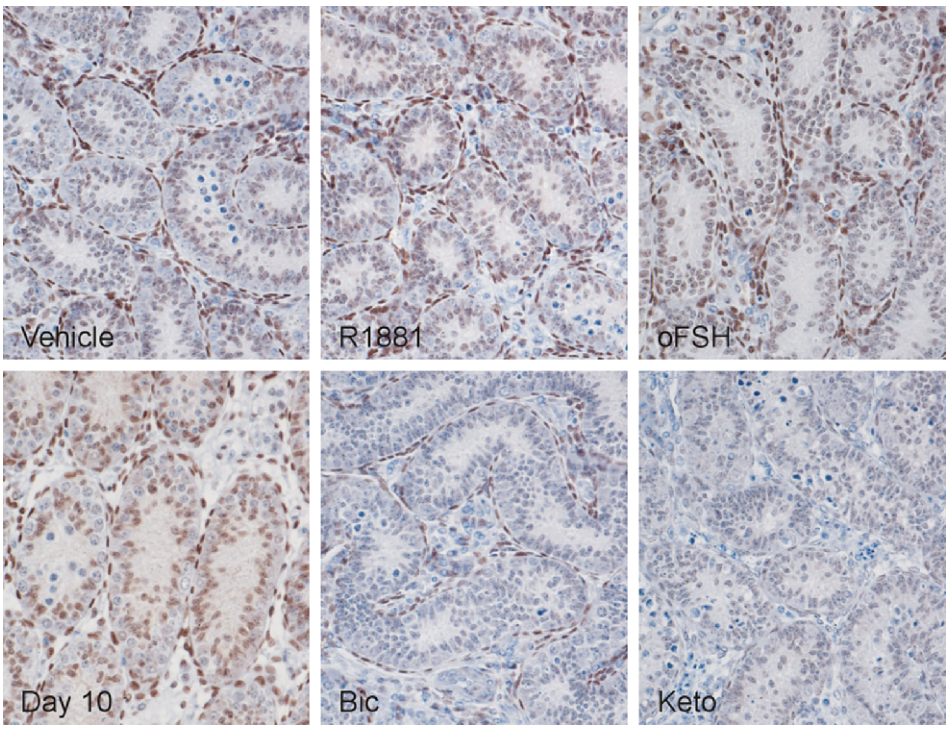


FIG. 2. Immunohistochemical staining for the AR in testicular explants treated for 48 h with vehicle, R1881 (10^{-8} M), oFSH (20 ng/ml), bicalutamide (Bic; 10^{-5}M), or ketoconazole (Keto; 2.10^{-5} M). Testicular tissue derived from 10-day-old mice (Day 10) was included as a reference. Original magnification ×400.

the tubular compartment (Table 1). Bicalutamide reduced testosterone levels in explant-conditioned media by approximately 50%, whereas oFSH had the opposite effect, causing a 3-fold increase (Table 2). The latter effect was not observed with rhFSH, suggesting contamination of the oFSH preparation with luteinizing hormone. AR immunoexpression was not affected by R1881 or oFSH. Bicalutamide caused a marked reduction of AR immunostaining in nuclei of interstitial cells and Sertoli cells, whereas immunostaining in peritubular myoid cells was not visibly affected (Fig. 2).

Transcript levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* were studied by quantitative PCR. To allow comparison of expression levels observed in culture with those observed during prepubertal development in vivo, samples derived from organotypic cultures were processed in parallel with testis samples freshly derived from 8- and 10-day-old mice. In the in vivo situation, the transcript levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* displayed a 17.0 ± 3.5, 2.8 ± 0.3, 3.1 ± 0.3, and 2.8 ± 0.5-fold increase between Days 8 and 10 (mean ± SEM of seven independent experiments), confirming the observations in control animals reported previously [19]. In testis explants cultured in control medium for 48 h (vehicle), the expression levels were generally higher than those observed on Day 8 in vivo, but lower than those found in vivo on Day 10 (Fig. 3).

Treatment of the organotypic cultures with the synthetic androgen R1881 did not significantly affect the expression of the studied genes. Bicalutamide [38], however, markedly reduced the expression of all the studied transcripts, suggesting that the maintenance and/or increase in the level of gene expression observed during the 48-h culture period depends on endogenous androgens. Neither oFSH nor rhFSH significantly affected the transcript levels of *Rhox5* or *Spinlw1*. Recombinant human FSH significantly stimulated the transcript levels of *Gpd1* and *Drd4*. For oFSH the effect was significant for *Gpd1* only. Transcript levels in the presence of FSH (ovine or recombinant human) and bicalutamide were comparable to those observed in the presence of bicalutamide only. The combination of FSH and R1881 tended to be more effective than FSH only, but the effect was variable and often not significant.

In a second series of experiments, the antiandrogen bicalutamide was replaced by ketoconazole, an inhibitor of steroidogenesis [39]. As expected, ketoconazole markedly reduced the levels of testosterone in the culture media (Table 2). This drop in testosterone was accompanied by a marked reduction in AR immunostaining in interstitial, peritubular, and Sertoli cells (Fig. 2). Although ketoconazole did not affect the tissue architecture of the cultured explants, a 4.2-fold increase in apoptotic activity was noted in the tubular compartment, and a 6.8-fold increase in the interstitium (Table 1).

In confirmation with the above-described experiments, treatment with R1881 did not increase the transcript levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* above those observed in the vehicle-treated control (Fig. 4). Treatment with ketoconazole for 48 h markedly reduced the expression levels of all the studied genes. Interestingly, for *Rhox5* and *Spinlw1*, the effect of ketoconazole could be neutralized completely by simultaneous treatment with exogenous androgens (R1881), whereas this was obviously not the case for *Gpd1* and *Drd4*. Both oFSH and rhFSH stimulated the expression of *Gpd1* and *Drd4* above that observed in the vehicle-treated control. For *Rhox5* and *Spinlw1*, the effects of FSH were limited and generally not significant. For all four genes, the transcript levels in the combined presence of FSH and ketoconazole did not differ significantly from those observed in the presence of ketoconazole only. Also, in the presence of FSH, R1881 restored the expression levels of *Rhox5* and *Spinlw1* to those observed in vehicle-treated controls. A similar restoration was not seen for *Gpd1* and *Drd4*.

Improved Responses to Androgens by Avoiding Prolonged Exposure to Ketoconazole

Since androgens were apparently unable to restore *Gpd1* and *Drd4* expression in the continuous presence of ketoconazole, we explored whether transient exposure to ketoconazole

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

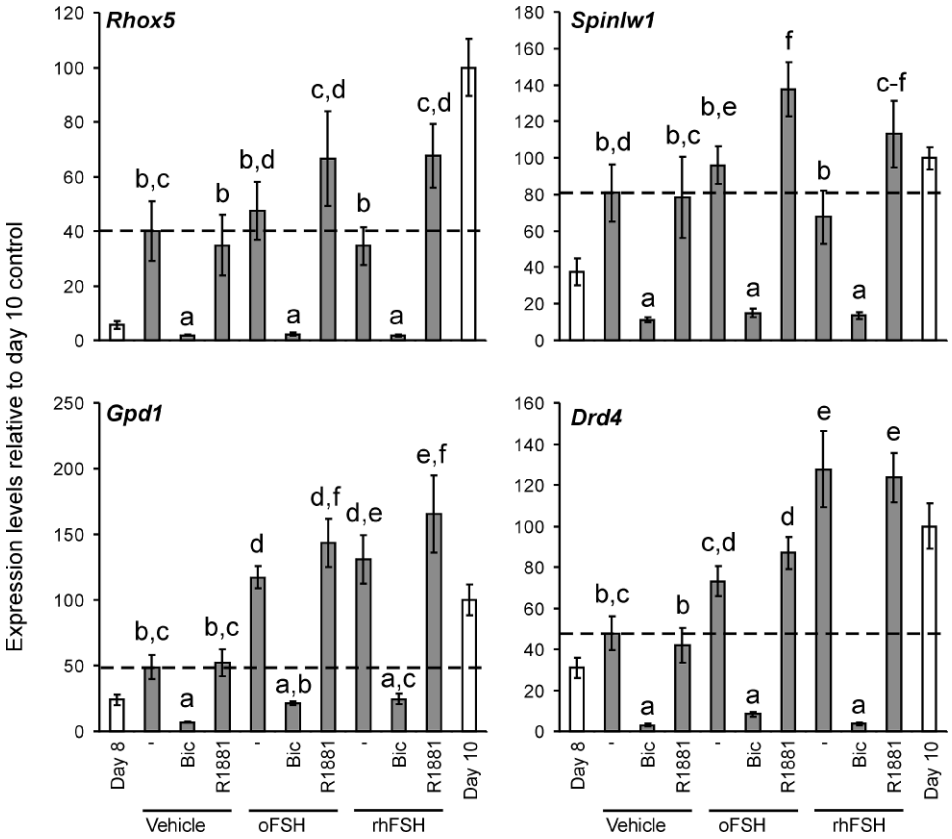


FIG. 3. Hormonal control of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* in organotypic cultures. Testicular explants from 8-day-old mice were cultured for 48 h in control medium (Vehicle) or in medium supplemented with R1881 (10^{-8} M), bicalutamide (Bic; 10^{-5} M), oFSH (20 ng/ml), rhFSH (20 ng/ml), or combinations of these agents as indicated. Expression levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* were measured by qPCR (gray bars). As a control, expression levels were also measured in testis samples derived from 8-day-old (Day 8) and 10-day-old (Day 10) mice processed in parallel (open bars). All measurements were normalized for the level of 18S rRNA and were expressed as a percentage of the values measured in testes from 10-day-old mice, arbitrarily set at 100. Values indicated are the mean ± SEM of five independent samples. ANOVA followed by Fisher LSD test was used to compare expression levels in cultured explants. Values derived from freshly prepared Day 8 and Day 10 testes were not included in the statistical analysis. Values that differ significantly ($P < 0.05$) are indicated by different lowercase letters (a–f). For comparison, the value of the vehicle-treated control is indicated as a dashed line throughout the graph.

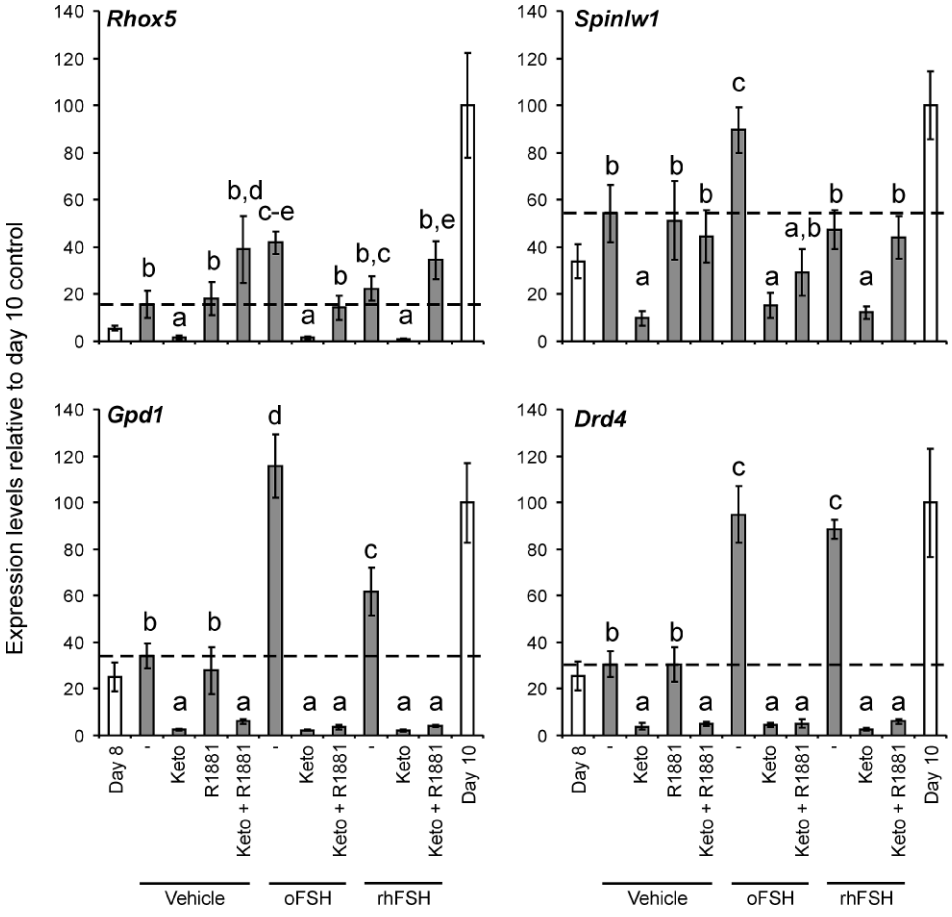


FIG. 4. Effects of ketoconazole, an inhibitor of steroidogenesis, on the ability of androgens to stimulate the expression of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* in the presence or absence of FSH. Testicular explants from 8-day-old mice were cultured for 48 h in control medium (Vehicle) or in medium supplemented with R1881 (10^{-8} M), ketoconazole (Keto; 2.10^{-5} M), oFSH (20 ng/ml), rhFSH (20 ng/ml), or combinations of these agents as indicated. Expression levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* were measured by qPCR (gray bars). As a control, expression levels were also measured in testis samples derived from 8-day-old (Day 8) and 10-day-old (Day 10) mice processed in parallel (open bars). Transcript levels were normalized, expressed, and analyzed as explained in Figure 3. Values indicated are the mean ± SEM of five independent samples. Values that differ significantly ($P < 0.05$) are indicated by different lowercase letters (a–e). For comparison, the value of the vehicle-treated control is indicated as a dashed line throughout the graph.

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

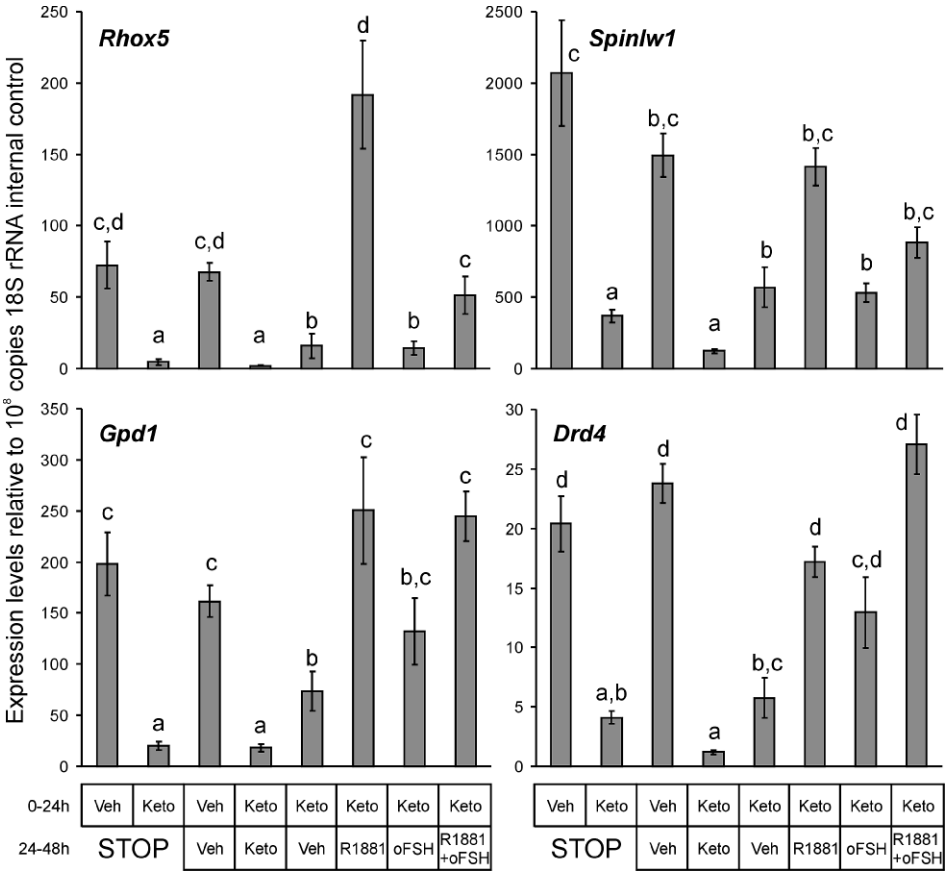


FIG. 5. Hormonal control of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* in organotypic cultures pretreated for 24 h with ketoconazole. Cultures were pretreated for 24 h with control medium (Veh) or with ketoconazole (Keto; 2.10^{-5} M) as indicated. Thereafter incubations were either stopped (STOP) or continued after replacement of the medium by control medium or by medium supplemented with ketoconazole (2.10^{-5} M), R1881 (10^{-8} M), oFSH (20 ng/ml), or the combination of R1881 and oFSH and incubation was continued for another 24 h. Expression levels were expressed relative to 10^8 copies of 18S rRNA as an internal control, and statistical analysis was performed as explained in Figure 3. Values indicated are the mean ± SEM of four or five independent samples. Values that differ significantly ($P < 0.05$) are indicated by different lowercase letters (a–d).

might be a better experimental approach to study the effects of androgens in organotypic cultures. As illustrated in Figure 5, a 24-h treatment of cultures with ketoconazole was sufficient to cause a drastic reduction in the expression of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4*. Removal of ketoconazole from the cultures after 24 h and subsequent incubation for another 24 h with control medium resulted in a significant increase in transcript levels, demonstrating the reversibility of the effects of ketoconazole (Fig. 5). Addition of R1881 to cultures previously exposed for 24 h to ketoconazole restored the expression levels of all four transcripts essentially to those observed in cultures maintained for the entire 48 h period in control medium. Except for *Rhox5*, oFSH also restored gene expression to levels that were not significantly different from those observed in cultures maintained for 48 h in control medium, but the stimulation tended to be less pronounced than that observed with R1881. Combined exposure to oFSH and R1881 had no greater effect than R1881 alone (except for *Rhox5* where, for unexplained reasons, the combined effect was lower; Fig. 5). In comparison to the starting conditions (i.e., after 24 h of ketoconazole treatment), addition of R1881 caused a 45-fold induction of *Rhox5* expression and a 5-fold, 19-fold, and 6-fold induction of expression for *Spinlw1*, *Gpd1*, and *Drd4*, respectively. A time study at 6, 12, and 24 h under the same conditions revealed that significant effects of androgens could already be observed after 12 h of androgen treatment for each of the four genes studied (data not shown).

Effects of oFSH and rhFSH on the Expression Levels of Rhox5*,* Spinlw1*,* Gpd1*, and* Drd4

Dose-response curves were performed to explore the effects of FSH on *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* expression in more detail. In accordance with the data summarized in Table 2, oFSH (5–50 ng/ml) caused a dose-dependent increase in testosterone production, whereas such an increase was not observed for rhFSH (Supplemental Fig. S1; all Supplemental Figures are available online at www.biolreprod.org). As shown in Figure 6, oFSH tended to increase *Rhox5* and *Spinlw1* expression. In line with the observations summarized in Figures 3 and 4, however, the effects were limited and not always statistically significant. Moreover, no obvious dose-response relationship could be observed. In contrast, a significant and dose-dependent stimulatory effect of oFSH was noted on the transcript levels of *Gpd1* and *Drd4*. *Gpd1* was stimulated up to 4.4-fold and *Drd4* up to 3.0 fold. The effects of oFSH were strongly reduced by bicalutamide and completely blocked by ketoconazole (Fig. 6). A similar dose-response study for rhFSH revealed similar (limited and variable) results on *Rhox5* and *Spinlw1* but a dose-dependent (up to 2.8-fold) stimulation for *Gpd1* and (up to 2.4-fold) stimulation for *Drd4* (Supplemental Fig. S2).

To explore whether oFSH has effects on the expression of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* that are not dependent on the Sertoli cell AR, testes derived from SCARKO animals and control testes were treated for 48 h with control medium, R1881, oFSH, or ketoconazole. As illustrated in Figure 7, the organotypic cultures confirmed the differential expression of all four genes in SCARKO and control testes observed also in vivo [19]. R1881 did not affect gene expression levels either in control testes or in SCARKO testes. In control testes, oFSH caused a 2.1-fold and 2.7-fold increase in the expression of *Gpd1* and *Drd4*, respectively. In SCARKO testes a similar increase was observed for *Gpd1* (2.7-fold) but not for *Drd4* (which even showed a 1.6-fold decrease). Ketoconazole

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

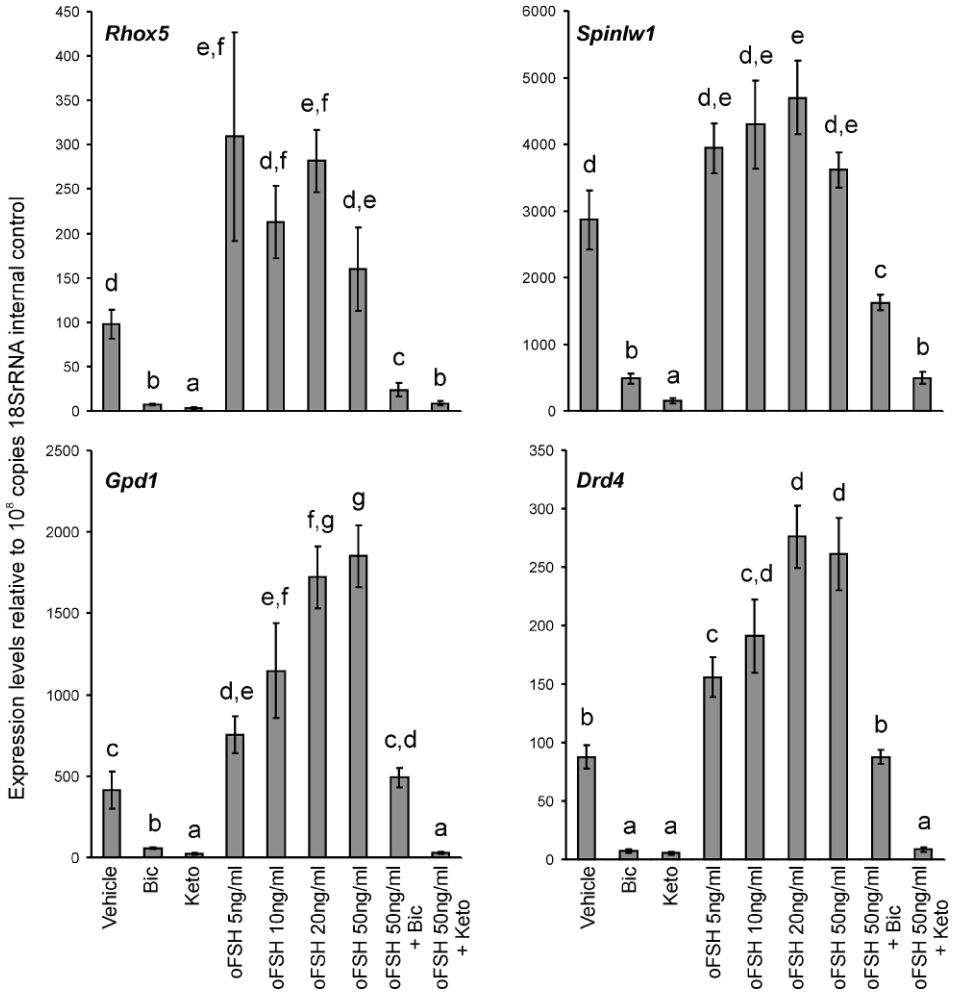


FIG. 6. Effect of increasing doses of oFSH on the expression of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4*. Testicular explants from 8-day-old mice were treated with control medium (Vehicle), bicalutamide (Bic; 10^{-5} M), ketoconazole (Keto; 2.10^{-5} M), oFSH (5–50 ng/ml), or with the indicated combinations of these agents for 48 h. Expression levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* were measured by qPCR (gray bars). As a control, expression levels were also measured in testis samples derived from 8-day-old (Day 8) and 10-day-old (Day 10) mice processed in parallel (open bars). Transcript levels were normalized, expressed, and analyzed as explained in Figure 3. Values indicated are the mean ± SEM of five independent samples. Values that differ significantly ($P < 0.05$) are indicated by different lowercase letters (a–f).

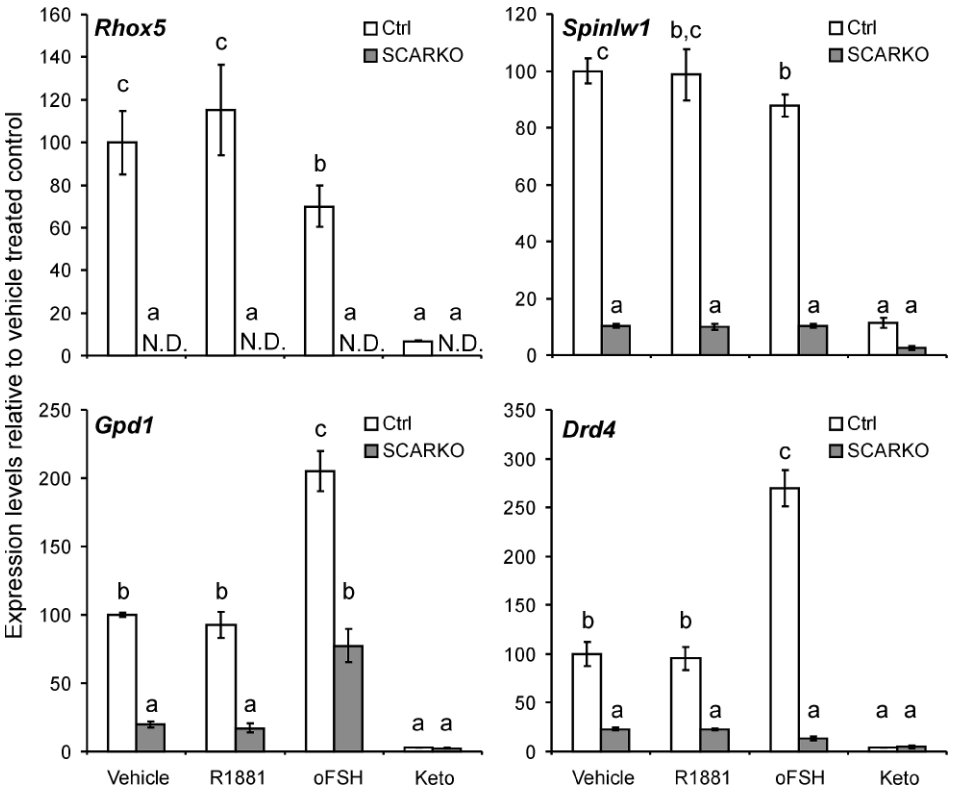


FIG. 7. Effect of vehicle, R1881, oFSH and ketoconazole on gene expression in organotypic cultures of SCARKO and control testes. Testicular explants from 8-day-old SCARKO and control mice (Ctrl) were cultured for 48 h in control medium (Vehicle) or in medium supplemented with R1881 (10^{-8} M), oFSH (50 ng/ml), or ketoconazole (Keto; 2.10^{-5} M). Expression levels of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* were measured by qPCR (control: white bars; SCARKO: gray bars). All measurements were normalized for the level of 18S rRNA and were expressed as a percentage of the values measured in control vehicle-treated testes, arbitrarily set at 100. Values indicated are the mean ± SEM of three independent samples. Statistical analysis was performed as described in Figure 3. Values that differ significantly ($P < 0.05$) are indicated by different lowercase letters (a–c).

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

decreased the expression levels of all four genes in both control and SCARKO testes (Fig. 7).

DISCUSSION

Recent microarray studies searching for differentially expressed genes in testes of hypogonadal [20] or prepubertal [21] mice treated or not treated with androgens, and in testes of mice with ubiquitous [40] or Sertoli cell-selective [19, 22] knockouts of the AR and controls, have disclosed a whole series of putative androgen-regulated genes. The limited degree of concurrence between the identified genes, however, points to the need to investigate the role of androgens in the control of these genes in more detail. Studies on isolated Sertoli cells have proven of limited use for this purpose because such cells apparently lose the expression of typical androgen-regulated genes such as *Rhox5* and also display a marked decrease in androgen responsiveness [26, 27]. In the present series of experiments we demonstrated that organotypic cultures of testes derived from prepubertal mice present an excellent alternative.

The data summarized above indicate that under the described culture conditions, and even in the absence of exogenously added hormones and growth factors, the morphology of testis tissue derived from 8-day-old mice is well preserved for at least 48 h. No obvious changes were noted in the number or size of interstitial cells showing positive immunostaining for HSD3B or in the intensity of nuclear AR immunoexpression in interstitial cells, peritubular cells, and Sertoli cells at the end of the 48-h culture period when compared to testis tissue freshly derived from 10-day-old mice. Moreover, although after the neonatal surge of androgens the prepubertal testis is considered to enter a relatively quiescent period [41], androgen production in the organotypic cultures—as reflected by testosterone levels in the conditioned media—remained high even in the absence of added gonadotropins (approximately 4 ng/ml after the first 24 h of culture and 1 ng/ml after the subsequent 24 h). These levels are apparently sufficient to maintain the expression of the studied androgen-regulated genes (*Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4*). In fact, addition of the synthetic androgen R1881 (10^{-8} M) did not result in significant further stimulation of expression of these genes. A similar absence of effect was observed when R1881 was replaced by testosterone (10^{-8} M) or by hCG (1–50 ng/ml; data not shown). A disadvantage of organotypic culture systems is that, due to the use of tissue fragments derived from different animals, experimental variation—particularly when comparing experiments performed on different days—tends to be larger than in experiments using cultures of freshly isolated cells or cell lines (Supplemental Fig. S3). With the high number of repeats used in most experiments, however, the observed treatment effects proved very consistent.

The dependence of the expression of *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* on endogenous androgen production and action in vehicle-treated organotypic cultures was convincingly demonstrated by the fact that transcript levels were undetectable (*Rhox5*) or markedly decreased (*Spinlw1*, *Gpd1*, *Drd4*) in cultured SCARKO testes and by the observation that both the AR antagonist bicalutamide and the inhibitor of androgen synthesis ketoconazole markedly decreased transcript levels after 48 h of incubation. These effects are consistent with earlier observations [19] and with the fact that *cis*-acting sequences able to act as androgen response elements have been described at least for *Rhox5* [42], *Spinlw1* [43], and *Gpd1* [44]. As expected, ketoconazole caused a pronounced drop in testosterone levels in the conditioned media. Surprisingly, a less dramatic decrease in testosterone secretion (down to approximately 40% of the control levels) was also observed with bicalutamide. The mechanism of the latter effect is unclear. Androgens are known to affect adult Leydig cell differentiation and function both directly (via the Leydig cell AR) and indirectly (via their actions on Sertoli cells) [45, 46], but their effects on early prepubertal Leydig cells remain to be studied in more detail. As expected, the decrease in testosterone levels observed after 48 h of treatment with ketoconazole was accompanied by a marked decrease in nuclear AR immunostaining. Bicalutamide also reduced nuclear AR immunostaining, presumably reflecting both its effects on androgen production and its ability to prevent stable receptor DNA binding [47].

Cotreatment during 48 h with R1881 completely overcame the reduction in *Rhox5* and *Spinlw1* expression caused by ketoconazole but caused only a limited increase in the transcript levels of *Gpd1* and *Drd4*, a difference that is puzzling and unexplained. Exposure of the organotypic cultures to ketoconazole for 48 h increased cell apoptosis, but this was a small effect, and if general Sertoli cell toxicity/loss had occurred, comparable effects on the inducibility of all four genes would have been expected, rather than just for *Gpd1* and *Drd4*. Therefore, at the present time, the most likely explanation for the differential effects of ketoconazole on the inducibility of *Rhox5* and *Spinlw1* gene expression, on the one hand, and on *Gpd1* and *Drd4* gene expression on the other hand, is that prolonged treatment with ketoconazole (48 h) affects the expression of the latter genes by a mechanism that is not related to its well-known effects on androgen production, but that could be related to its wider inhibition of P450 enzymes [48]. Such a mechanism might involve inhibition of the production of stimulatory factors required for *Gpd1* and *Drd4* expression or stimulation of inhibitory factors involved in the control of the same genes. The nature of these ketoconazole-affected factors warrants further investigation. Candidates might include androgen metabolites including estrogens (produced via the cytochrome P450-containing enzyme CYP19A1 [aromatase]) but also unrelated steroid derivatives and nonsteroidal molecules, such as vitamin A and its derivatives. In the context of the use of organotypic cultures to study androgen regulation of transcript levels, it is important to note that these confounding effects of ketoconazole can largely be avoided by limiting ketoconazole exposure to the first 24 h of culture and subsequent treatment for another 24 h with R1881 (Fig. 5).

Rhox5 and *Spinlw1* also differed from *Gpd1* and *Drd4* with respect to their response to FSH, which tended to increase the expression of *Rhox5* and *Spinlw1* in several independent experiments, but these effects were limited and variable. For oFSH (20 ng/ml), mean stimulation for *Rhox5* and *Spinlw1*, as compared to vehicle-treated controls, averaged 1.8 $\pm$ 0.3-fold and 1.3 $\pm$ 0.1-fold, respectively (mean $\pm$ SEM; n = 8). Comparable values for rhFSH were 1.2 $\pm$ 0.5-fold and 1.0 $\pm$ 0.2-fold (mean $\pm$ SEM; n = 3). Moreover, no obvious dose-response relationship could be documented. The nature of these variable effects could not be defined further. Some contribution of FSH-induced effects on endogenous androgen production cannot be excluded, since the effects were more pronounced with oFSH than with rhFSH. The possibility may also be considered, however, that the observed effects are indirect and that, for instance, in the absence of any exogenously added growth factors, FSH may stimulate the production of endogenously produced growth factors, such as IGF or transferrin, that improve the viability and functionality of the cultured explants [49–51].

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

In contrast, *Gpd1* and *Drd4* displayed consistent and dose-dependent increases in transcript levels in the presence of FSH. This increase was observed in the presence of oFSH (which increases endogenous testosterone production, Supplemental Fig. S1) as well as rhFSH (which did not affect testosterone production), supporting the contention that it is a genuine FSH effect. Mean stimulation for *Gpd1* and *Drd4* with oFSH (20 ng/ml) was 2.9 ± 0.3-fold and 2.7 ± 0.2-fold, respectively (n = 8). Corresponding values with rhFSH were 2.3 ± 0.8-fold and 2.7 ± 0.7-fold, respectively (n = 3). In line with the above-discussed effects of ketoconazole on the expression of these genes, the effects of FSH were completely blocked in the presence of ketoconazole. Surprisingly, however, bicalutamide also interfered with the effects of FSH on *Gpd1* and *Drd4* expression, suggesting that androgens may play a permissive role in the observed FSH effects. To explain the apparent paradox that increased endogenous androgen production does not seem to play a role in the effects of FSH on *Gpd1* and *Drd4* expression, but that interference with androgen action largely or completely blocks these effects, the role of the Sertoli cell AR in the effects of FSH on *Gpd1* and *Drd4* responses was investigated by comparing the effects of FSH in organotypic cultures derived from wild-type and SCARKO mice. The data summarized in Figure 7 clearly show that an active AR in Sertoli cells is required for oFSH-dependent stimulation of *Drd4* but not for *Gpd1*. We can only speculate on the mechanism by which bicalutamide also reduces the effect of FSH on *Gpd1*. This effect might be due in part to the bicalutamide-induced reduction in androgen production and/or to the inhibition by bicalutamide of androgen-controlled factors permissive for FSH-induced *Gpd1* expression produced by non-Sertoli cells (such as peritubular myoid cells). An alternative explanation for the above-mentioned paradox might be that FSH increases the maximally achievable response to androgens in Sertoli cells (e.g., by increasing AR levels), as previously described in Sertoli cell-enriched cultures [52]. At present we can only state that the immunohistochemical results summarized in Figure 2 do not show a noticeable increase in Sertoli cell AR concentration in FSH-treated explants. It is obvious, however, that more research will be needed to clarify the molecular mechanisms responsible for the observed FSH effects.

In conclusion, organotypic culture systems such as the one described here represent an excellent tool to study the control of putative androgen-regulated genes in more detail. By preserving normal testis architecture and thus allowing normal cell-cell interactions, the organotypic testis cultures increase the chance of detecting physiologically relevant effects and cell interactive effects. In this way, the system overcomes many of the limitations (loss of gene expression, loss of androgen responsiveness) observed with Sertoli cell-enriched cultures. Preincubation for 24 h with ketoconazole to block endogenous androgen production, followed by a period of treatment with exogenous androgens, may be an optimal setup to study the androgen responsiveness of putative target genes. The system can also successfully be used to study the interaction of androgens with other hormones (FSH) or factors controlling the expression of the relevant genes. The present data clearly confirm that *Rhox5*, *Spinlw1*, *Gpd1*, and *Drd4* are targets for androgen action, and they also stress that the regulation of *Rhox5* and *Spinlw1* differs considerably from that of *Gpd1* and *Drd4*, despite a very closely related behavior in cluster analysis. FSH has only limited effects on the expression of *Rhox5* and *Spinlw1* but markedly increases the transcript levels of *Gpd1* and *Spinlw1*. For *Gpd1* the latter effect does not depend on the simultaneous activation of the Sertoli cell AR, whereas for *Drd4*, oFSH is inactive in the absence of a functional AR in Sertoli cells.

ACKNOWLEDGMENTS

The technical assistance of Ludo Deboel is greatly appreciated. We thank Dr. Parlov, the National Hormone and Pituitary Program, and the NIDDK for providing oFSH-20 (AFP7028D) and AstraZeneca for bicalutamide.

REFERENCES

1. Sharpe RM. Regulation of spermatogenesis. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd ed. New York: Raven Press; 1994: 1363–1434.
2. McLachlan RI, O'Donnell L, Meachem SJ, Stanton PG, de Kretser DM, Pratis K, Robertson DM. Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog Horm Res 2002; 57:149–179.
3. Holdcraft RW, Braun RE. Hormonal regulation of spermatogenesis. Int J Androl 2004; 27:335–342.
4. Singh J, Oneill C, Handelsman DJ. Induction of spermatogenesis by androgens in gonadotropin-deficient (Hpg) mice. Endocrinology 1995; 136:5311–5321.
5. Kumar TR, Wang Y, Lu NF, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 1997; 15:201–204.
6. Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi IT. Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. Nat Genet 1997; 15:205–206.
7. Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P. Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci U S A 1998; 95:13612–13617.
8. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 2000; 141:1795–1803.
9. Tsai MY, Yeh SD, Wang RS, Yeh S, Zhang C, Lin HY, Tzeng CR, Chang C. Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells. Proc Natl Acad Sci U S A 2006; 103:18975–18980.
10. Zhang CX, Yeh SY, Chen YT, Wu CC, Chuang KH, Lin HY, Wang RS, Chang YJ, Mendis-Handagama C, Hu LQ, Lardy H, Chang CS. Oligozoospermia with normal fertility in male mice lacking the androgen receptor in testis peritubular myoid cells. Proc Natl Acad Sci U S A 2006; 103:17718–17723.
11. Holdcraft RW, Braun RE. Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. Development 2004; 131:459–467.
12. De Gendt K, Swinnen JV, Saunders PTK, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C, Heyns W, Carmeliet P, et al. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 2004; 101:1327–1332.
13. Chang CS, Chen YT, Yeh SD, Xu QQ, Wang RS, Guillou F, Lardy H, Yeh SY. Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc Natl Acad Sci U S A 2004; 101:6876–6881.
14. Sharpe RM. Sertoli cell endocrinology and signal transduction: androgen regulation. In: Skinner MK, Griswold MD (eds.), Sertoli Cell Biology. San Diego: Elsevier Academic Press; 2006:199–216.
15. Bremner WJ, Millar MR, Sharpe RM, Saunders PTK. Immunohistochemical localization of androgen receptors in the rat testis—evidence for stage-dependent expression and regulation by androgens. Endocrinology 1994; 135:1227–1234.
16. Willems A, De Gendt K, Allemeersch J, Smith LB, Welsh M, Swinnen JV, Verhoeven G. Early effects of Sertoli cell-selective androgen receptor ablation on testicular gene expression. Int J Androl, published 12 April 2009; PMID: 19392831.
17. Merlet J, Racine C, Moreau E, Moreno SG, Habert R. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. Proc Natl Acad Sci U S A 2007; 104:3615–3620.
18. Lindsey JS, Wilkinson MF. Pem: a testosterone- and LH-regulated

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use

homeobox gene expressed in mouse Sertoli cells and epididymis. Dev Biol 1996; 179:471–484.
19. Denolet E, De Gendt K, Allemeersch J, Engelen K, Marchal K, Van Hummelen P, Tan KAL, Sharpe RM, Saunders PTK, Swinnen JV, Verhoeven G. The effect of a Sertoli cell-selective knockout of the androgen receptor on testicular gene expression in prepubertal mice. Mol Endocrinol 2006; 20:321–334.
20. Sadate-Ngatchou PI, Pouchnik DJ, Griswold MD. Identification of testosterone-regulated genes in testes of hypogonadal mice using oligonucleotide microarray. Mol Endocrinol 2004; 18:422–433.
21. Zhou Q, Shima JE, Nie R, Friel PJ, Griswold MD. Androgen-regulated transcripts in the neonatal mouse testis as determined through microarray analysis. Biol Reprod 2005; 72:1010–1019.
22. Eacker SM, Shima JE, Connolly CM, Sharma M, Holdcraft RW, Griswold MD, Braun RE. Transcriptional profiling of androgen receptor (AR) mutants suggests instructive and permissive roles of AR signaling in germ cell development. Mol Endocrinol 2007; 21:895–907.
23. O'Rand MG, Widgren EE, Sivashanmugam P, Richardson RT, Hall SH, French FS, VandeVoort CA, Ramachandra SG, Ramesh V, Rao AJ. Reversible immunocontraception in mate monkeys immunized with Eppin. Science 2004; 306:1189–1190.
24. Brown LJ, Koza RA, Marshall L, Kozak LP, MacDonald MJ. Lethal hypoglycemic ketosis and glyceroluria in mice lacking both the mitochondrial and the cytosolic glycerol phosphate dehydrogenases. J Biol Chem 2002; 277:32899–32904.
25. Van Tol HHM, Wu CM, Guan HC, Ohara K, Bunzow JR, Civelli O, Kennedy J, Seeman P, Niznik HB, Jovanovic V. Multiple dopamine-D4 receptor variants in the human-population. Nature 1992; 358:149–152.
26. Denolet E, De Gendt K, Swinnen JV, Verrijdt G, Deboel L, Roskams T, Verhoeven G. Transfection with steroid-responsive reporter constructs shows glucocorticoid rather than androgen responsiveness in cultured Sertoli cells. J Steroid Biochem Mol Biol 2006; 98:164–173.
27. Verhoeven G, Denolet E, Swinnen JV, Willems A, Claessens F, Saunders PTK, Sharpe RM, De Gendt K. Contribution of recent transgenic models and transcriptional profiling studies to our understanding of the mechanisms by which androgens control spermatogenesis. Immunol Endocr Metab Agents Med Chem 2008; 8:2–13.
28. Steinberger A, Steinberger E, Perloff WH. Mammalian testes in organ culture. Exp Cell Res 1964; 36:19–27.
29. Steinberger A, Steinberger E. Replication pattern of Sertoli cells in maturing rat testis in vivo and in organ culture. Biol Reprod 1971; 4:84–87.
30. Boitani C, Politi MG, Menna T. Spermatogonial cell proliferation in organ culture of immature rat testis. Biol Reprod 1993; 48:761–767.
31. Boitani C, Stefanini M, Fragale A, Morena AR. Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. Endocrinology 1995; 136:5438–5444.
32. Schlatt S, Zhengwei Y, Meehan T, de Kretser DM, Loveland KL. Application of morphometric techniques to postnatal rat testes in organ culture: insights into testis growth. Cell Tissue Res 1999; 298:335–343.
33. Lambrot R, Coffigny H, Pairault C, Donnadieu AC, Frydman R, Habert R, Rouiller-Fabre V. Use of organ culture to study the human fetal testis development: effect of retinoic acid. J Clin Endocrinol Metab 2006; 91: 2696–2703.
34. Roulet V, Denis H, Staub C, Le Tortorec A, Delaleu B, Satie AP, Patard JJ, Jegou B, Dejucq-Rainsford N. Human testis in organotypic culture: application for basic or clinical research. Hum Reprod 2006; 21:1564–1575.
35. Sharpe RM, Atanassova N, Mckinnell C, Parte P, Turner KJ, Fisher JS, Kerr JB, Groome NP, Macpherson S, Millar MR, Saunders PTK. Abnormalities in functional development of the Sertoli cells in rats treated neonatally with diethylstilbestrol: a possible role for estrogens in Sertoli cell development. Biol Reprod 1998; 59:1084–1094.
36. Mahood IK, Hallmark N, Mckinnell C, Walker M, Fisher JS, Sharpe RM. Abnormal Leydig cell aggregation in the fetal testis of rats exposed to di (n-butyl) phthalate and its possible role in testicular dysgenesis. Endocrinology 2005; 146:613–623.
37. Welsh M, Saunders PTK, Marchetti NI, Sharpe RM. Androgen-dependent mechanisms of Wolffian duct development and their perturbation by flutamide. Endocrinology 2006; 147:4820–4830.
38. Furr BJ, Valcaccia B, Deegan B, Chesterton G, Tucker H. ICI 176,334—a novel nonsteroidal, peripherally selective antiandrogen. J Endocrinol 1986; 111:156.
39. Santen RJ, Vandenbossche H, Symoens J, Brugmans J, Decoster R. Site of action of low-dose ketoconazole on androgen biosynthesis in men. J Clin Endocrinol Metab 1983; 57:732–736.
40. O'Shaughnessy PJ, Abel M, Charlton HM, Hu B, Johnston H, Baker PJ. Altered expression of genes involved in regulation of vitamin A metabolism, solute transportation, and cytoskeletal function in the androgen-insensitive tfm mouse testis. Endocrinology 2007; 148:2914–2924.
41. Ketelslegers JM, Hetzel WD, Sherins RJ, Catt KJ. Developmental changes in testicular gonadotropin receptors—plasma gonadotropins and plasma testosterone in rat. Endocrinology 1978; 103:212–222.
42. Barbulescu K, Geserick C, Schüttke I, Schleuning WD, Haendler B. New androgen response elements in the murine pem promoter mediate selective transactivation. Mol Endocrinol 2001; 15:1803–1816.
43. Schauwaers K, De Gendt K, Saunders PT, Atanassova N, Haelens A, Callewaert L, Moehren U, Swinnen JV, Verhoeven G, Verrijdt G, Claessens F. Loss of androgen receptor binding to selective androgen response elements causes a reproductive phenotype in a knockin mouse model. Proc Natl Acad Sci U S A 2007; 104:4961–4966.
44. Cheng JD, de Vellis J. Oligodendrocytes as glucocorticoids target cells: functional analysis of the glycerol phosphate dehydrogenase gene. J Neurosci Res 2000; 59:436–445.
45. Hardy MP, Kelce WR, Klinefelter GR, Ewing LL. Differentiation of Leydig-cell precursors in vitro—a role for androgen. Endocrinology 1990; 127:488–490.
46. De Gendt K, Atanassova N, Tan KAL, de Franca LR, Parreira GG, Mckinnell C, Sharpe RM, Saunders PTK, Mason JI, Hartung S, Ivell R, Denolet E, et al. Development and function of the adult generation of Leydig cells in mice with sertoli cell-selective or total ablation of the androgen receptor. Endocrinology 2005; 146:4117–4126.
47. Farla P, Hersmus R, Trapman J, Houtsmuller AB. Antiandrogens prevent stable DNA-binding of the androgen receptor. J Cell Sci 2005; 118:4187–4198.
48. De Coster R, Wouters W, Bruynseels J. P450-dependent enzymes as targets for prostate cancer therapy. J Steroid Biochem Mol Biol 1996; 56: 133–143.
49. Cailleau J, Vermeire S, Verhoeven G. Independent control of the production of insulin-like growth factor-I and its binding protein by cultured testicular cells. Mol Cell Endocrinol 1990; 69:79–89.
50. Skinner MK. Cell-cell interactions in the testis. Endocr Rev 1991; 12:45–77.
51. Gnessi L, Fabbri A, Spera G. Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and environment. Endocr Rev 1997; 18:541–609.
52. Verhoeven G, Cailleau J. Follicle-stimulating hormone and androgens increase the concentration of the androgen receptor in Sertoli cells. Endocrinology 1988; 122:1541–1550.

Downloaded From: https://bioone.org/journals/Biology-of-Reproduction on 29 Mar 2024
Terms of Use: https://bioone.org/terms-of-use