Loss of androgen receptor binding to selective androgen response elements causes a reproductive phenotype in a knockin mouse model

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Edited by Bert W. O'Malley, Baylor College of Medicine, Houston, TX, and approved February 5, 2007 (received for review December 6, 2006)

Androgens influence transcription of their target genes through the activation of the androgen receptor (AR) that subsequently interacts with specific DNA motifs in these genes. These DNA motifs, called androgen response elements (AREs), can be classified in two classes: the classical AREs, which are also recognized by the other steroid hormone receptors; and the AR-selective AREs, which display selectivity for the AR. For *in vitro* interaction with the selective AREs, the androgen receptor DNA-binding domain is dependent on specific residues in its second zinc-finger. To evaluate the physiological relevance of these selective elements, we generated a germ-line knockin mouse model, termed SPARKI (SPecificity-affecting AR KnockIn), in which the second zinc-finger of the AR was replaced with that of the glucocorticoid receptor, resulting in a chimeric protein that retains its ability to bind classical AREs but is unable to bind selective AREs. The reproductive organs of SPARKI males are smaller compared with wild-type animals, and they are also subfertile. Intriguingly, however, they do not display any anabolic phenotype. The expression of two testis-specific, androgen-responsive genes is differentially affected by the SPARKI mutation, which is correlated with the involvement of different types of response elements in their androgen responsiveness. In this report, we present the first *in vivo* evidence of the existence of two functionally different types of AREs and demonstrate that AR-regulated gene expression can be targeted based on this distinction.

DNA-binding domain | fertility | Rhox5 | transcription

Steroid receptors are ligand-dependent transcription factors that mediate steroid hormone signaling. They bind as homodimers to three-nucleotide-spaced palindromically repeated sequences of their monomer binding site (1–4). For all steroid hormone receptors, apart from the estrogen receptor, the consensus monomer binding motif is 5′-TGTTCT-3′. Their DNA-binding domains (DBDs) are highly conserved and consist of two zinc-fingers in which three α-helical structures are involved in their structural and functional integrity. The α-helix in the first zinc-finger is inserted into the major groove of the DNA, whereas the second zinc-finger forms the dimerization interface (5).

This high degree of structural and functional conservation implies that the steroid hormone receptors recognize virtually identical DNA motifs. Many androgen response elements (AREs), therefore, also function as glucocorticoid or progesterone response elements and vice versa (1, 6–8).

Next to these classical DNA elements, however, the androgen receptor (AR) has been shown to interact with other binding motifs that resemble a direct, rather than a palindromic, repeat of the 5′-TGTTCT-3′-hexamer (8, 9). The glucocorticoid receptor is unable to transactivate via these DNA motifs, which we therefore termed AR-specific AREs. Four amino acids in the AR-DBD (Fig. 1*A*, arrows) are crucial for its interaction with these motifs (9–11). In the 3D structure of a steroid receptor DBD interacting with DNA, none of these residues is directly involved in contacting the DNA major groove (5, 11). Their location within the structure, however, suggests that they are involved in the dimerization of the DBDs or participate in the stabilization of the DBD on the DNA by contacting the phosphate backbone in the minor groove of flanking DNA (9, 12, 13).

In *in vitro* experiments, a swap of the second zinc-finger between the AR and glucocorticoid receptor (GR) resulted in the loss of interaction of the chimeric receptor with AR-selective DNA motifs (9). In the mouse genome, this second zinc-finger is encoded by exon 3 of the mAR and exon 4 of the mGR gene. We have now generated a mouse model in which the second zinc-finger of the AR is replaced by that of the GR, resulting in a chimeric protein termed SPARKI (SPecificity-affecting AR KnockIn)-AR, which has a defect in binding to AR-specific DNA motifs while displaying normal interaction with the classical AREs.

We describe here the first characterization of this mouse model, which shows a reproductive phenotype but displays surprisingly little or no anabolic defects. We also demonstrate that the expression of androgen-responsive genes in the testis is affected differentially, reflecting the fact that their expression is driven by a different type of ARE.

Results

The SPARKI-AR Distinguishes Between Specific and Nonspecific Elements *in Vitro*. The swap introduced in the SPARKI-AR results in the exchange of 12 amino acids in the second zinc-finger as depicted in Fig. 1*A*. Transfection experiments show that the AR function via selective AREs (Fig. 1*B Upper*) is severely affected by the SPARKI mutation, whereas virtually no effect is seen on the classical nonselective AREs (Fig. 1*B Lower*). If anything, the SPARKI-AR displays a higher activity compared with the wild-type AR when transactivating via the nonspecific elements.

Author contributions: G.Verrijdt and F.C. contributed equally to this work; G. Verrijdt and F.C. designed research; K.S., K.D.G., P.T.K.S., N.A., A.H., L.C., U.M., J.V.S., and G. Verhoeven performed research; U.M. contributed new reagents/analytic tools; K.S., G. Verhoeven, G. Verrijdt, and F.C. analyzed data; and K.S. and G. Verrijdt wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: AIS, androgen insensitivity syndrome; AR, androgen receptor; ARE, androgen response element; DBD, DNA-binding domain; GR, glucocorticoid receptor; KO, knockout; SCARKO, Sertoli cell-specific KO of the AR; SPARKI, Specificity-affecting AR KnockIn.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0610814104/DC1.

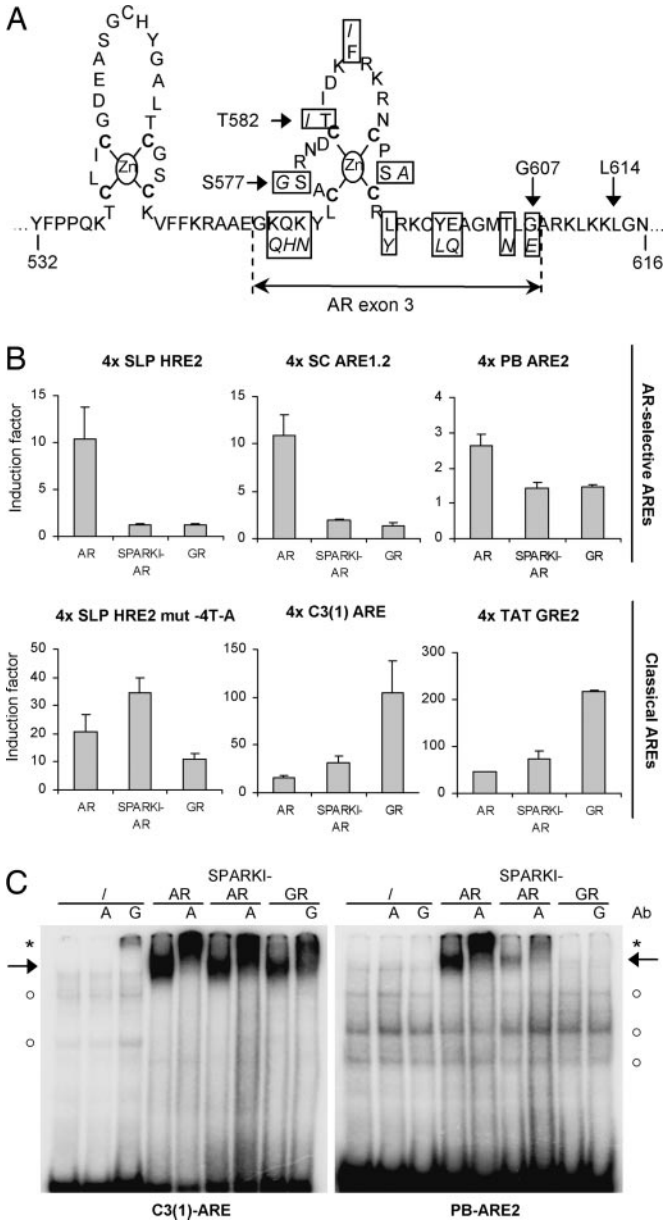

Fig. 1. Transfection experiments and *in vitro* binding assays comparing selective and nonselective DNA elements. (*A*) Schematic overview of the mAR-DBD. The amino acids in the exon 3-encoded region that differ between the AR-DBD and GR-DBD are boxed, and GR residues are italic. The four amino acids involved in binding to AR-specific elements are depicted by arrows. (*B*) Transient transfection experiments using reporter constructs containing AR-selective (*Upper*) or nonselective (*Lower*) AREs. Sequences and references for each DNA element are given in *SI Methods*. Results are presented as induction factors that are the averages ± SEM of at least three independent experiments performed in triplicate. (*C*) Band shift assays with full-size receptor constructs and using the PB-ARE2 and the C3(1)-ARE, identical to the motifs cloned in the luciferase reporter constructs in *B*, as radiolabeled probes. Anti-AR (A) or anti-GR (G) antibody was used as indicated. Circles, nonspecific binding of protein; arrows, DNA/receptor complex; asterisks, supershifted complex.

In contrast, all of the nonselective elements, but none of the selective motifs, are able to confer glucocorticoid responsiveness to the thymidine kinase promoter (Fig. 1*B*).

Band shift assays with the nonselective C3(1)-ARE (6) and the AR-specific PB-ARE2 (14) demonstrate that the functional specificity of each receptor is correlated with its specificity in DNA binding (Fig. 1*C*).

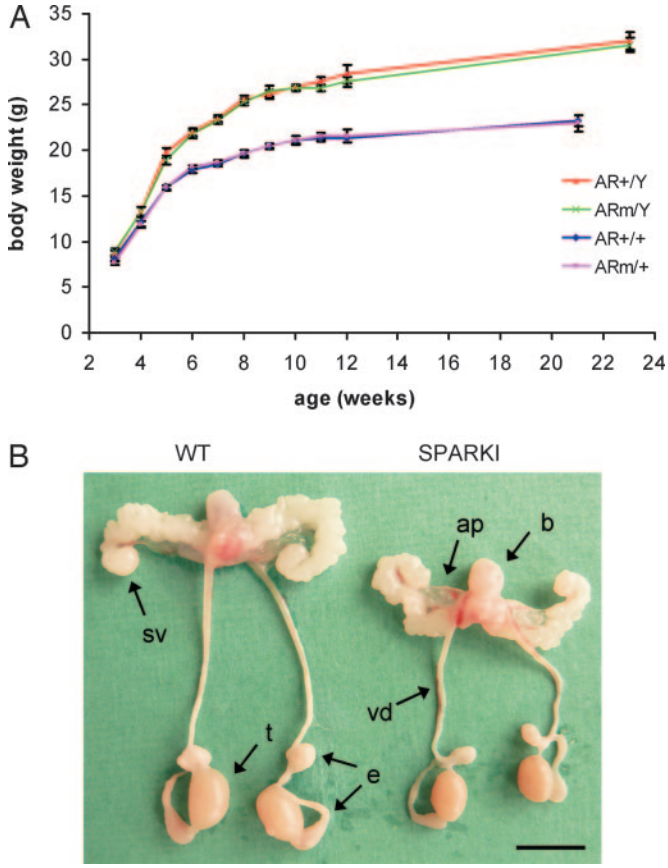

Fig. 2. Macroscopic phenotype of SPARKI animals. (*A*) Total body weight curves of wild-type (AR+/Y) vs. SPARKI (ARm/Y) males and wild-type (AR+/+) vs. heterozygous (ARm/+) mutant females. Each data point is the average of at least six individuals. Error bars represent SEM. (*B*) Dissection of the urogenital tracts of a wild-type (*Left*) and a SPARKI (*Right*) male. t, testis; e, epididymis; sv, seminal vesicle; vd, vas deferens; ap, anterior prostate; b, bladder. (Scale bar, 1 cm.)

Generation of the SPARKI Mouse Line. A detailed description of the generation of the SPARKI mouse line is shown in supporting information (SI) *Methods*. A depiction of the targeting vector and a schematic overview of the steps and controls performed in the generation of the germ-line knockin mouse line are shown in SI Fig. 6. Because the AR gene is located on the X chromosome, male mutant mice, by definition, have no wild-type allele and are therefore simply termed "SPARKI."

Macro- and Microscopic Evaluation of SPARKI Animals. Homo- or heterozygous SPARKI females do not show a distinct phenotype and display normal fertility (data not shown). The external phenotype of SPARKI males is indistinguishable from that of their wild-type littermates. Body weight curves of SPARKI versus wild-type males and heterozygous versus wild-type females are superimposable (Fig. 2*A*).

SPARKI males display reduced fertility: litters from SPARKI fathers are reduced 2- and 4-fold in size and frequency, respectively, compared with wild types (data not shown). At 11 weeks of age, their reproductive organs are reduced in size and weight: testis weight is down to 67% of wild type, epididymis to 56%, ventral prostate to 54%, anterior prostate to 67%, and seminal vesicles to 55% (Fig. 2*B* and SI Table 3). Testes have descended normally into the scrotum, and serum levels of testosterone and gonadotropins are not significantly altered (SI Table 4).

Analysis of H&E-stained cross-sections of testicular tissue of

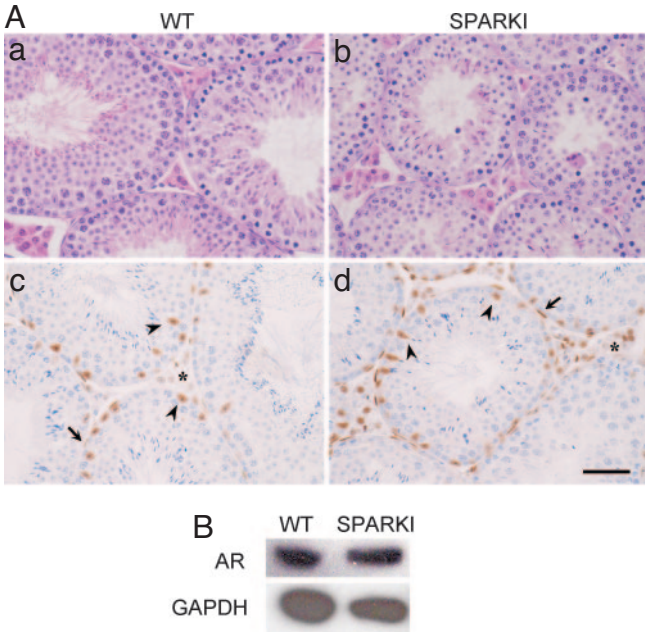

Fig. 3. AR expression and histochemical analysis of wild-type and SPARKI testes. (*A*) H&E-stained (*a* and *b*) and AR-stained (*c* and *d*) testicular cross-sections of wild-type (*Left*) and SPARKI (*Right*) animals. (Magnification, 40×.) Asterisks, arrows, and arrowheads indicate Leydig, peritubular myoid, and Sertoli cells, respectively. (Scale bar, 50 μm.) (*B*) Western blot analysis of AR expression in wild-type and SPARKI testes extracts probed with anti-AR (*Upper*). GAPDH (*Lower*) served as a loading control.

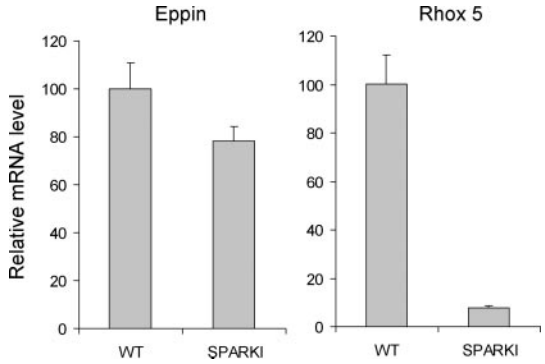

Fig. 4. Relative expression levels of Eppin and Rhox5 in wild-type and SPARKI testes. Expression of Eppin (*Left*) and Rhox5 (*Right*) was determined by quantitative RT-PCR on RNA from 10-day-old testes, as indicated. Values are the averages (± SEM) of experiments on six wild-type and six SPARKI littermates performed in triplicate. Values are depicted as relative to the expression level of the genes in wild-type testes (arbitrarily set at 100).

11-week-old animals (Fig. 3 *Aa* and *Ab*) reveals that the diameters of the seminiferous tubules are reduced, on average, to ≈85% of wild type. The AR is expressed in the Sertoli, peritubular myoid, and Leydig cells in both wild-type and SPARKI testes (Fig. 3 *Ac* and *Ad*). Western blot analysis on testicular extracts (Fig. 3*B*) shows that there is no significant difference in the levels of AR protein in wild-type versus SPARKI animals.

The total number of Sertoli cells, expressed as nuclear volume per testis (Table 1), is reduced to 68% of controls. The total number of germ cells is reduced to 55% of controls, with the most marked reduction in the numbers of round and elongate spermatids.

Expression of AR-Regulated Genes Is Differentially Affected in SPARKI Animals. We studied the expression of Sertoli cell-specific genes that were shown to be AR-dependent in a study of the Sertoli cell-specific KO of the AR (SCARKO) mouse model (15, 16) by quantitative RT-PCR analysis on RNA isolated from 10-day-old wild-type and SPARKI testes (Fig. 4). Eppin (Epididymal protease inhibitor), PCI (Protein C inhibitor), and Tubb3 (Tubulin β3) are not, or only marginally, reduced in SPARKI mice compared with wild-type littermates. The expression levels of Rhox5 (Reproductive homeobox X-linked gene 5), Tsx (Testis specific X-linked gene), and Drd4 (Dopamine receptor 4), in contrast, are reduced severely by 95%, 62%, and 56%, respectively. At 10 days of age, cellular composition of the testis is indistinguishable between wild-type and SPARKI mice (data not shown). At an older age, the subtle differences in the germ cell complement would compromise the results of a comparative quantitative RT-PCR analysis. Microarray analysis using mRNA of 10-day-old testes (data not shown) confirmed the quantitative RT-PCR results: expression levels of Rhox5, Tsx, and Drd4 were down 9.2-, 2.5-, and 2.5-fold, respectively ($P \leq 0.05$) in SPARKI mice compared with wild type. The expression levels of PCI, Tubb3, and Eppin were not affected or only 1.3- and 1.4- fold lower, respectively.

***In Vitro* Confirmation of a Direct Effect of the Zinc-Finger Change on Transactivation via the AREs in the Rhox5 and Eppin Genes.** The Rhox5 ARE-1 (5′-AGATCTCATTCTGTTCC-3′) is a selective ARE that has previously been shown to bind AR, but not GR, and to mediate responsiveness to androgens, but not glucocorticoids (17, 18). No promoter studies have been performed so far on the Eppin gene. A MatInspector search (19) of the mouse Eppin gene promoter region revealed the presence of a putative ARE (5′-AGAACTTGGTGTTCC-3′) at position −141 from the Eppin translational start site.

Upon cotransfection with SPARKI-AR, transcriptional response of a luciferase construct driven by the Eppin ARE was 2-fold higher compared with wild-type AR (Fig. 5*A*). When using a Rhox5 ARE-1 reporter construct, however, the activity of the SPARKI-AR was reduced to ≈50% of the wild-type receptor. In band shift assays, the full-length wild type and SPARKI-AR both bind the Eppin ARE, whereas the Rhox5 ARE-1 is only recognized by wild type but not SPARKI-AR (Fig. 5*B*).

Discussion

Two Types of ARE in AR Signaling. All steroid hormone receptors are known to bind their palindromic, three-nucleotide-spaced

Table 1. Total testis cell counts

	SC	Total GC	Spc/Spg	Rspd	Espd	Espd/Spc
WT ($n = 4$)	1.2 ± 0.08	18.3 ± 1.3	9.5 ± 1.7	6.2 ± 0.8	3.9 ± 0.1	0.5 ± 0.05
SPARKI ($n = 5$)	0.8 ± 0.07	10.1 ± 1.0	10.2 ± 1.6	2.9 ± 0.4	1.7 ± 0.3	0.3 ± 0.04
SP/WT (%)	68%*	55%*	108%	47%*	44%*	64%*

Values are the averages ± SEM of the nuclear volumes of each cell type per testis (in mm^3 per testis). SC, sertoli cell; GC, germ cell; Spg, spermatogonia; Spc, spermatocytes; Rspd, round spermatids; Espd, elongate spermatids. *SPARKI cell counts are significantly different ($P < 0.05$; Student's *t* test) from wild type.

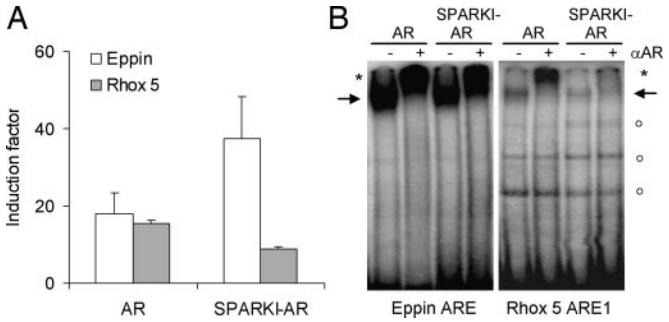

Fig. 5. Wild type and SPARKI-AR transactivation via, and binding to, the Rhox5 and Eppin AREs. (*A*) Transient transfections of luciferase reporter vectors containing four copies of the Eppin (white bars) and Rhox5 (shaded bars) AREs and cotransfected with wild-type and SPARKI-AR expression plasmids. Indicated are the induction factors ± SEM values mediated by each receptor after stimulation with 10 nM of R1881 for 24 h. Sequences and references for each DNA element are given in *SI Methods*. Results are shown as in Fig. 1*B*. (*B*) Band shift assays using the Eppin ARE and the Rhox5 ARE-1. Experiments were performed and are depicted as described previously (Fig. 1*C*).

response elements as a homodimer in a head-to-head conformation (20, 21). The AR, however, is able to recognize an additional set of response elements, in which the palindromic nature of the 5′-TGTTCT-3′ repeat is much less evident. Such androgen-selective motifs have now been described in several androgen-responsive genes (8, 17, 22–25). We have previously presented evidence that the type of DNA motif can, through allosteric effects, strongly determine the mechanism and outcome of an AR action. For example, the effects of sumoylation of the AR-NTD and the role of the polyglutamine stretch in the AR-NTD or NTD-LBD interaction are strikingly different for AR interacting with selective versus nonselective DNA motifs (26–28). These effects are examples of allosteric influences of the DNA on the functioning of nuclear receptors or transcription factors, similar to what has been described before for the AR (29) and GR (30, 31).

The SPARKI Model. The SPARKI mouse is the first *in vivo* model that allows the identification of genes or gene programs that are dependent on selective AREs for their responsiveness to androgens. The external phenotype of SPARKI males is remarkably similar, if not identical, to their wild-type littermates, yet they display a clear reproductive phenotype: they have approximately eight times less pups, and their reproductive organs are reduced in size and weight (Fig. 2*B* and SI Table 3). Their levels of circulating steroid and gonadotropic hormones, however, are within the normal range (SI Table 4). Elevated testosterone and luteinizing hormone levels caused by a failing AR-dependent negative feedback mechanism are hallmarks for partial androgen insensitivity syndrome (AIS) (32). In AIS, which is usually caused by the expression of a dysfunctional AR, androgen-dependent tissues are to various degrees insensitive to androgen stimulation, resulting in phenotypes ranging from a fully female external phenotype (complete AIS) to an undervirilized male phenotype.

Total body weight (Fig. 2*A*), the weights of organs outside the reproductive system (SI Table 3), and the relative amounts of body fat, muscle, and bone (data not shown) are not affected by the SPARKI mutation, despite the fact that these parameters display a clear gender difference. The SPARKI mutation, therefore, seems to selectively affect the reproductive system. This finding indicates that not only genes, but also different physiological programs, can be affected differentially by targeting either class of response elements.

Changes in Testicular Cell Composition in SPARKI Mice. The process of generation and maturation of spermatozoa takes place in the seminiferous tubuli that consist of germ cells at different stages of development, in close contact with the Sertoli cells. Although SPARKI testes are ≈35% smaller compared with wild type, histological analysis reveals only a discrete change in testicular architecture and cell composition of the seminiferous epithelia (Fig. 3*A* and Table 1). The reduction in the total number of Sertoli cells was also seen in an AR knockout (KO) mouse model (33), but not in an SCARKO mouse model (15), indicating that the Sertoli cell number is controlled by a process outside of the Sertoli cell that is dependent on selective AREs. Testis size is known to be strongly correlated with the number of Sertoli cells that are formed during fetal and early postnatal life (34).

Sertoli cells are an essential component of the seminiferous epithelium, guiding and supporting the differentiating germ cells during the different stadia of spermatogenesis. Germ cells do not express the AR (35). Therefore, Sertoli cells are considered to mediate, to a large extent, the influence of androgens on spermatogenesis (15, 36, 37).

The ratio of spermatocytes to spermatogonia in SPARKIs (Table 1) suggests that there is no block in entry to meiosis. The decreased ratio of elongate spermatids to spermatocytes indicates that the Sertoli cells fail to support the cells to complete meiosis. This fact is also reflected by the clear reduction in the ratio of round and elongate spermatids to Sertoli cells, with normal ratios of spermatogonia and spermatocytes to Sertoli cells (Table 2). These findings demonstrate that the reduction in total germ cells cannot completely be attributed to reduced Sertoli cell numbers, but is also due to a decreased ability of the Sertoli cells to support maturation of meiotic and postmeiotic germ cells.

AR-Dependent Genes Are Differentially Affected by the SPARKI-AR. The study of the SCARKO mouse model revealed a series of AR-dependent genes expressed in the Sertoli cells (15, 16). Of these genes, Rhox5 and Eppin are affected the most in SCARKO animals. Rhox5 is a member of the Rhox family of homeobox genes that control a variety of embryonic and postnatal developmental processes (38). After birth, Rhox5 is expressed in the Sertoli cells and in the epithelial cells of the epididymis (38). Although a KO of the Rhox5 gene has little effect on male fertility (39), a mouse model overexpressing Rhox5 in Sertoli

Table 2. Supporting capacity of Sertoli cells

	Total GC/SC	Spg/SC	Spc/SC	Rspd/SC	Espd/SC
WT ($n = 4$)	15.5 ± 1.1	0.7 ± 0.1	6.2 ± 0.4	5.2 ± 0.6	3.3 ± 0.3
SPARKI ($n = 5$)	12.6 ± 0.8	0.6 ± 0.1	6.1 ± 0.3	3.7 ± 0.4	2.1 ± 0.3
SP/WT (%)	81%*	89%	98%	71%*	63%*

Values are the averages ± SEM of the nuclear volumes of each cell type per testis (in mm^3 per testis) of *n* samples. SC, sertoli cell; GC, germ cell; Spg, spermatogonia; Spc, spermatocytes; Rspd, round spermatids; Espd, elongate spermatids.
*Ratios are significantly different ($P < 0.07$; Student's *t* test) between SPARKI and wild-type controls.

cells in a nonstage-dependent manner shows an increase in DNA strand breaks in germ cells, suggesting that Rhox5 alters the expression of Sertoli cell genes involved in germ cell regulation (40). Androgen- versus glucocorticoid-specificity of up-regulation of Rhox5 expression has been thoroughly studied (12, 17, 18). A 300-bp Rhox5 proximal promoter fragment confers Sertoli cell- and epididymis-selective as well as androgen-regulated expression to a reporter gene in a transgenic mouse model (41, 42). An androgen-specific ARE (Rhox5 ARE-1) is responsible for the androgen-dependence of Rhox5 expression (17).

In contrast, very little is known about the androgen regulation of the expression of Eppin, a serine protease inhibitor, expressed in Sertoli cells and in the ciliated cells in the epididymis (43). Eppin is an important factor in fertility because Eppin immunization of male *Macaca radiate* monkeys resulted in reversible contraception (44).

In the SPARKI model, Rhox5 expression is decreased >10-fold, whereas the expression of Eppin is only mildly affected (Fig. 4). Transient transfection experiments confirmed that wild type and SPARKI-AR could both transactivate via the Eppin ARE, but SPARKI-AR was much less successful in stimulating transcription from the Rhox5 ARE-1 compared with the wild-type AR (Fig. 5). The wild-type AR versus SPARKI-AR effect on Rhox5 ARE-1 function was less dramatic *in vitro* than could be expected from quantitative RT-PCR and microarray data. However, transient transfection experiments do not fully reflect the *in vivo* physiological situation. They probably do not take into account the effects of chromatin packaging on the regulation of gene expression. In addition, putative enhancer elements are absent in the reporter constructs, which may result in quantitative differences in transcriptional responses.

Conclusion

Our results on the androgen responsiveness of Rhox5 and Eppin in the SPARKI mouse model have confirmed the regulation of Rhox5 by an AR-selective ARE, while Eppin is regulated by a classical, nonselective ARE. This finding demonstrates that androgen-responsive genes can be differentially targeted depending upon the nature of their response elements. With the SPARKI mouse model, we thus have created a tool to clearly assess the physiological relevance of the ability of the AR to act via selective AREs. This observation could be of major importance in the development of new selective androgen receptor modulators, which can target one of two types of genes to maximize desired effects and minimize unwanted side effects.

Methods

Animal Care. Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). All animals had ad libitum access to water and standard chow and were housed at 20°C with a 12-h light/dark cycle in standard cages. All procedures involving animals were conducted in accordance with the National Institutes of Health guidelines and with approval of the Katholieke Universiteit Leuven ethical committee.

Histochemical Analysis. Urogenital systems from 11-week-old wild-type and SPARKI mice were fixed in Bouin's fluid for 6 h and then transferred to 70% ethanol. Testes were dissected and processed into paraffin wax by standard methods. Five-micrometer sections were stained with H&E. Cell counts were determined on testicular cross-sections of four wild-type and five SPARKI animals at 7 weeks of age essentially as described (45–47). Immunohistochemical demonstration of AR was performed on dewaxed sections after heat-induced antigen retrieval for 5 min in 0.01 M citrate buffer, pH 6.0 (Sigma–Aldrich, St. Louis, MO) by using a pressure cooker. A rabbit anti-AR antiserum (sc-816; Santa Cruz Biotechnology, Santa Cruz, CA) was used in conjunction with a swine anti-rabbit biotinylated secondary antibody (E0353; DAKO, Carpinteria, CA). Bound antibodies were visualized by incubation with R.T.U. VECTASTAIN Elite ABC-HRP reagent (PK-7100; Vector Laboratories, Burlingame, CA), followed by color development with 3,3′-diaminobenzidine tetrahydrochloride chromogenic substrate (K3468; Liquid DAB+ kit, DAKO) and monitored microscopically. Sections were counterstained with hematoxylin, and images were captured with a Canon D10 microscope (Canon Inc, Tokyo, Japan) equipped with a Kodak DCS330 camera (Eastman Kodak, Rochester, NY).

RT-PCR. Testes were removed from 10-day-old wild-type or SPARKI mice, snap-frozen in liquid nitrogen, weighed, and homogenized in RNeasy lysis buffer (RNeasy kit, QIAGEN, Chatsworth, CA) with a Dounce homogenizer. RNA was extracted, cDNA was synthesized, and a two-step quantitative RT-PCR was performed on an ABI PRISM 7700 sequence detector PCR detection system (Applied Biosystems, Foster City, CA) as described (16). The Rhox5 and Eppin cDNA-specific primers are described in *SI Methods*. Eppin amplicons were detected by the incorporation of Sybr Green in the amplified fragment, and Rhox5 amplicons were detected using a molecular beacon (see *SI Methods*). Duplicate samples were assayed for the expression of 18S RNA as an internal control. The quantity of measured mRNA was expressed relative to the internal 18S RNA standard. All samples and standard curves were run in triplicate, and six different samples of each group were tested.

Western Blotting. For Western blotting, testes from 11-week-old mice were snap-frozen in N_2 and homogenized in a sample buffer [110 mM SDS, 100 mM DTT, 80 mM Tris (pH 6.9), 10% glycerol, 0.002% bromophenol blue] using a Dounce homogenizer. Thirty micrograms of each sample was run on a 4–12% Bis-Tris gel (NuPAGE, Invitrogen, Carlsbad, CA). After transfer to a PVDF membrane, the blot was probed with either an in-house polyclonal AR antibody [1:2000 dilution (48)] or a monoclonal GAPDH antibody (6C5; Santa Cruz Biotechnology).

Cell Culture Experiments. The luciferase reporter vectors used in this study were derived from the pGL3 basic vector (Promega, Madison, WI) and contained four tandem copies of the indicated response elements upstream of a minimal thymidine kinase promoter driving the luciferase cassette. The ARE sequences that were used are listed in *SI Methods*. The CMV-driven mammalian expression vectors containing full-length wild-type mAR and mGR are described in ref. 49. The vector expressing the SPARKI mutant of mAR was identical to the wild-type expression plasmid, apart from the exchange of a peptide region encoded by the mAR exon 3 with a fragment encoded by the mGR exon 4.

HeLa cells were transfected with the GeneJuice transfection reagent (Novagen, Madison, WI) in 96-well plates with 100 ng per well luciferase reporter vector, 0.1 ng of the appropriate receptor expression plasmid, and 10 ng of CMV-β-galactosidase plasmid as a control for transfection efficiency. For each reporter–receptor combination, three wells were and three wells were not stimulated 24 h after transfection with the corresponding hormone (10 nM R1881 for the AR-transfected samples or 10 nM dexamethasone for the GR-transfected wells). Induction factors are the ratios of the luciferase values of each of the hormone-stimulated samples over the average value of the three nonstimulated samples.

Band Shift Assays. A full-length receptor protein for use in band shift assays was obtained by transfecting 175-cm^2 cell culture plates (COS7 cells) with 7 μg of the appropriate expression vector. After 24 h, medium was changed and the appropriate hormone (10 nM R1881 or dexamethasone) was added for 24 h. The cells were

washed twice with ice-cold PBS and resuspended in 200 μl of extraction buffer [20 mM Hepes KOH (pH 7.8), 450 mM NaCl, 0.4 mM EDTA, 25% glycerol, 0.5 mM PMSF, 0.5 mM DTT]. After three freeze–thaw cycles in liquid N_2, the lysate was centrifuged at 9,000 $\times$ *g*, and the supernatant was stored at -80°C. Typically, 5 μl of total cell extract was used in a band shift experiment. Band shift experiments were performed essentially as described (10). The oligonucleotides that were used as radiolabeled probes are listed in *SI Methods*. Anti-AR or anti-GR antibody was added to the binding mixture to prove the specific interaction of each receptor.

We thank Hilde Debruyn, Rita Bollen, Kathleen Bosmans, Ludo Deboel, Hilde Geeraerts, Arantza Esnal, and Michael Millar for excellent technical assistance; Dr. Richard Sharpe for useful comments; and Dr. Diane M. Robins for the kind gift of the pCMV-mAR. This work was partly supported by a Concerted Research Grant (Research Fund of the Katholieke Universiteit Leuven), Fonds Voor Wetenschappelijk Onderzoek-Vlaanderen Grant 1.5173.04N, and an Association for International Cancer Research grant (to U.M.). K.S. is a holder of a fellowship of the Instituut voor de Aanmoediging van Innovatie door Wetenschap en Technologie–Vlaanderen, and G. Verrijdt, K.D.G., A.H., and L.C. are holders of postdoctoral fellowships of the Fund for Scientific Research–Vlaanderen.

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