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The Effect of a Sertoli Cell-Selective Knockout of the Androgen Receptor on Testicular Gene Expression in Prepubertal Mice

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To unravel the molecular mechanisms mediating the effects of androgens on spermatogenesis, testicular gene expression was compared in mice with Sertoli cell-selective androgen receptor knockout (SCARKO) and littermate controls on postnatal d 10. Microarray analysis identified 692 genes with significant differences in expression. Of these, 28 appeared to be down-regulated and 12 up-regulated at least 2-fold in SCARKOs compared with controls. For nine of the more than 2-fold down-regulated genes, androgen regulation was confirmed by treatment of wild-type mice with an antiandrogen (flutamide). Some of them were previously described to be androgen regulated or essential for spermatogenesis. Serine-type protease inhibitors were markedly overrepresented in this down-regulated subgroup. A time study (d 8–20), followed by cluster analysis, allowed identification of distinct expression patterns of differentially expressed genes. Three genes with a pattern closely resembling that of Pem, a prototypical androgen-regulated gene expressed in Sertoli cells, were selected for confirmation by quantitative RT-PCR and additional analysis. The data confirm that the SCARKO model allows identification of novel androgen-regulated genes in the testis. Moreover, they suggest that protease inhibitors and other proteins related to tubular restructuring and cell junction dynamics may be controlled in part by androgens. (*Molecular Endocrinology* 20: 321–334, 2006)

DESPITE THE OVERWHELMING evidence that androgens play a key role in the control of spermatogenesis (1–6), the cellular and molecular mechanisms underlying this control remain poorly understood. Germ cells, peritubular myoid cells, and Sertoli cells (SC) have been mentioned as potential androgen target cells, but their relative contributions to the control of spermatogenesis remain unclear. Germ cells probably do not express the classical androgen receptor (AR) (7), and they certainly do not need it for normal development (8, 9). However, alternative mechanisms of androgen action, for instance, via conversion into estrogens (10) or via nongenomic pathways (11, 12), cannot be excluded. Peritubular myoid cells are located in the vicinity of spermatogonia. They do express the AR (13); moreover, they depend on androgens for their normal development (14). Furthermore, androgen-modulated interactions between peritubular cells and SC have been described (15, 16). SC display intimate interactions with germ cells at all stages of spermatogenesis (17, 18) and express the AR in a stage-dependent manner (13). Therefore, they are usually considered to be the most likely mediators of androgen action on spermatogenesis.

Because attempts to use isolated and cultured SC to study the molecular mechanisms of androgen action in the control of spermatogenesis have been largely unsuccessful (19, 20), we and others have recently developed *in vivo* models in which the AR gene is selectively inactivated in SC during embryonic development (from d 15 post coitum), more than 1 wk

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Abbreviations: AMH-Cre, Cre expression under control of the anti-Mullerian hormone promoter; AR, androgen receptor; ARE, androgen response element; Dazl, deleted in azoospermia-like; DNase, deoxyribonuclease; Flut, flutamide; hpg, hypogonadal; Hsp, heat shock protein; MAS, Microarray Suite; PCI, protein C inhibitor; RMA, robust multiarray analysis algorithm; SC, Sertoli cell; SCARKO, Sertoli cell-selective androgen receptor knockout; TP, testosterone proprionate.

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before immunoexpression becomes evident (postnatal d 3–5) (21–23). In our laboratory, knockout of the AR gene was achieved by crossing mice with a floxed exon 2 of the AR with mice expressing Cre under control of the anti-Mullerian hormone promoter (AMH-Cre) (24). These mice (SCARKO mice) have normally descended testes, virtually absent levels of Pem (a prototypical gene expressed in SC under control of androgens), normal AR expression in Leydig and peritubular cells, and normal circulating levels of androgens and LH. Spermatogenesis, however, is arrested in meiosis.

In the present study we have used the GeneChip Mouse Expression Array 430a 2.0 to identify genes that are differentially expressed in the testis of SCARKO and control (AMH-Cre) littermates on d 10, a time point at which differences in AR expression have not yet resulted in noticeable differences in cellular composition of the testis. A subset of 692 genes, including the Pem gene, was differentially expressed in a statistically significant manner. Among these differentially expressed genes, serine-type protease inhibitors were clearly overrepresented. Analysis of testicular gene expression in SCARKO and control mice between d 8 and 20 allowed cluster analysis and identification of some typical gene expression patterns of androgen-regulated genes. A subset of genes with an expression pattern resembling that of Pem was selected for additional analysis. Furthermore, functional analysis of the data suggested that androgen action in SC may affect tubular remodeling and junction dynamics.

RESULTS

The microarray datasets generated in this study are available through National Center for Biotechnology Information via the Gene Expression Omnibus (GEO) data repository (http://www.ncbi.nih.gov/geo/), GEO accession nos. GSE2259 and GSE2260.

Identification of Differentially Expressed Transcripts in Control *vs.* SCARKO Testes on d 10

To avoid confounding effects of differences in testicular cell composition related to the meiotic arrest occurring in SCARKO mice, gene expression patterns in SCARKO and control littermates were compared on d 10. At this age, testicular weights were identical in SCARKO and control mice [mean $\pm$ SEM, 4.55 $\pm$ 0.18 mg (n = 25) and 4.58 $\pm$ 0.16 mg (n = 17), respectively]. Moreover, tubular and germ cell complements were still comparable, as confirmed by hematoxylin/eosin staining and immunostaining for Dazl, a germ cell-specific RNA-binding protein. Nonetheless, on d 10, the AR was clearly immunoexpressed in SC nuclei of control mice, whereas it was completely absent in SC of SCARKO mice (Fig. 1). As expected, AR staining in interstitial and peritubular myoid cells was comparable for SCARKO and control mice (21).

Statistical analysis of microarray data from five independent samples of 10-d-old SCARKO and control mice (see *Materials and Methods*) generated a list of 848 differentially expressed transcripts corresponding

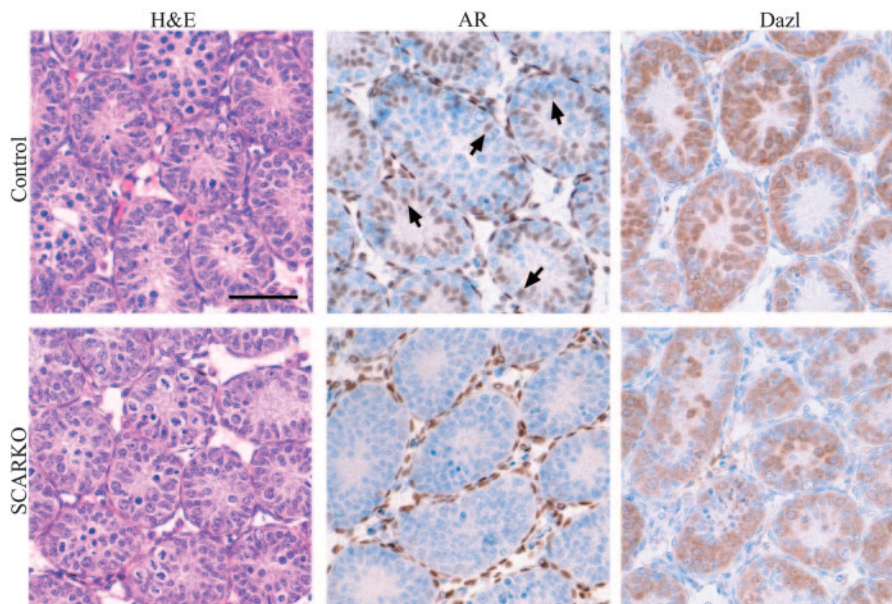

Fig. 1. Hematoxylin/Eosin (H&E) Staining and Immunoexpression Profiles for AR and Dazl in Testes of 10-d-Old Control and SCARKO Mice

H&E and Dazl staining show that testicular morphology and germ cell complement are still similar in control and SCARKO testes on d 10. Immunostaining of the AR is observed in SC nuclei of control mice (*arrows*) but is absent in SC nuclei of SCARKO mice. *Scale bar*, 50 μm.

to 692 genes after correction for the redundancy of oligonucleotide probes (supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Of these genes, 296 were expressed at a higher level in SCARKOs compared with controls (referred to as up-regulated in SCARKOs) and 396 were expressed at a lower level in SCARKOs compared with controls (referred to as down-regulated in SCARKOs). Forty of the identified genes showed a difference in expression level of at least 2-fold. The majority of the latter genes (n = 28) displayed lower expression levels in SCARKOs (strongly down-regulated) compared with controls (Table 2).

Using Onto-Express software (25), the list of 396 genes that were down-regulated in SCARKOs reflected a significant ($P < 0.01$) overrepresentation of genes related to MAPK activity (n = 4) and serine-type endopeptidase inhibitor activity (n = 6). Interestingly, most of these serine-type endopeptidase inhibitors [Eppin, Serpina3N, Serpina12, Pcskn1n, and protein C inhibitor (PCI)] were down-regulated more than 2-fold (Table 2). For the 296 genes that appeared up-regulated in SCARKOs, overrepresentation was observed for genes related to IGF binding (n = 5), Ca^{2+} binding (n = 16), Ca^{2+}-dependent phospholipid binding (n = 5), regulation of cell cycling (n = 12) and cell growth (n = 8), cell adhesion (n = 13), and signal transduction (n = 43).

Confirmation of Androgen Regulation and Tubular Localization for a Selection of Down-Regulated Genes

To clarify the contribution of androgens to the differences in testicular gene expression identified by the microarray experiments, 7-d-old wild-type mice were treated for 3 or 5 d with the antiandrogen flutamide (Flut) or with testosterone proprionate (TP), and the expressions of nine of the strongly down-regulated genes were measured by quantitative RT-PCR (Table 3). The selected genes comprised the four genes most strongly down-regulated in SCARKOs (Pem, Eppin, Galgt1, and DRD4), a testis-specific X-linked gene (Tsx), a gene encoding an enzyme involved in lipid and glucose metabolism (Gpd1), and genes encoding a microtubular component (Tubb3), a serine protease inhibitor (PCI), and a tumor protein (Tpd52l1). After 3 d of treatment with Flut (d 10), the expression of all studied genes tended to be decreased, but a significant decrease was observed only for Pem, Eppin, Drd4, and Gpd1. After 5 d of Flut (d 12), the expressions of all studied genes were significantly decreased. With the exception of Galgt1, genes that were most strongly down-regulated in SCARKOs tended to be more sensitive to inhibition by Flut. Interestingly, on d 10, TP still increased the expression of three of the studied genes, whereas on d 12, stimulatory effects were absent, and inhibitory effects were observed for five of the selected genes, suggesting that, already at that age, intratesticular androgen levels may be decreased by treatment with TP.

Enzymatic digestion, followed by quantitative RT-PCR, were used to study the interstitial or tubular localization of the same subset of selected genes in 10-d-old wild-type mice. Pem and SCP3 were used as markers of tubular localization (for SC and spermatocytes, respectively), and $P450_{c17}$ (Leydig cells) was used as a marker for interstitial localization. As shown in Table 4, the data point to a predominantly tubular localization for all selected genes.

Expression Patterns of Transcripts Differentially Expressed on d 10 in SCARKO and Control Mice during Prepubertal Development

To analyze the relationship of the differentially expressed genes, identified above, to the initiation of spermatogenesis and the development of the testis, gene expression was assessed in testis samples derived from SCARKO and control littermates at five different ages (d 8, 10, 12, 16, and 20).

The physiological relevance of the resulting data was supported by the expression pattern displayed by a number of typical meiotic marker genes (Fig. 2). Dmc1, for instance, and SCP3, which are known to be expressed during early stages of meiosis, showed a marked increase in expression from d 12 onward and displayed similar expression profiles in control and SCARKO testes. In contrast, genes such as heat

Table 1. Oligonucleotide Primers Used for Quantitative RT-PCR

Gene	5′ Primer (5′–3′)	3′ Primer (5′–3′)
Eppin	GCTTCTGCTCCAAGCTCTGTG	TTGCAGTGCTCAAAGTGCTCTC
Galgt1	TGGAGCACTACTTCATGCCCT	GGACACCGCCAGGTTCC
Drd4	GAAGGAGAGGCGCCAAGAT	GACTACCACCGGCAGGACTC
Tsx	ATTCCAGCCTGGGCCGT	CTCAGTGGCTCATCTGCCTG
Gpd1	TGCCTCACACACACTGGGA	TTTCCTGTGGCTGAAGTGCTT
Tubb3	CCCTTCGATTCCCTGGTCA	ACGGCACCATGTTCACAGC
PCI	TGACCCCCAAAAGGACCAC	TGGTCCAGGTAGTAGGAATACCCA
Tpd52l1	CACGACATGCAGACCACGAC	TTGCCTTCTGCCCTGCG
SCP3	ATGCTTCGAGGGTGTGGG	TTCCACCAGGCACCATCTTT

Accession number of gene sequences used for primer design can be found in Table 2.

Table 2. Genes with a More than 2-Fold Difference in Expression Level between SCARKO and Control (Ctrl)

Gene Name (Gene symbol)	Accession No.	Fold Difference
A. Genes with Lower Expression Level in SCARKO than in Control Testes		Ctrl/SCARKO
Placentae and embryos oncofetal gene (Pem)	BM210473	17.55
Serine protease inhibitor, with Kunitz and WAP domains 1 (Spinlw1)/Eppin	NM_029325	8.01
β-1,4-Acetylgalactosaminyltransferase (Galgt1)	U18975	7.07
Dopamine receptor 4 (Drd4)	NM_007878	5.61
Serine (or cysteine) proteinase inhibitor, clade A, member 3N (Serpina3N)	NM_009252	5.49
Chaperonin subunit 3γ (Cct3)/Matricin	L20509	5.09
Testis specific X-linked gene (Tsx)	NM_009440	5.01
Low-density lipoprotein receptor-related protein 4 (Lrp4)/Corin	NM_016869	3.92
Recombinant antineuramidase single chain Ig VH and VL domains (LOC56304)	NM_019633	3.53
Glycerol-3-phosphate dehydrogenase 1 (soluble) (Gpd1)	BC019391	3.42
Mcf.2 transforming sequence (Mcf2)/Diffuse B-cell lymphoma oncogene (Dbl)	NM_133197	2.83
Serine (or cysteine) proteinase inhibitor, clade A, member 12 (Serpina12)	AK014346	2.60
Expressed sequence AW125753 (AW125753)	BC002154	2.58
START domain containing 10 (Stard10)/Phosphatidylcholine transfer protein-like (PCTP-L)	NM_019990	2.56
RIKEN cDNA 1700112P19 gene (1700112P19Rik)	NM_026459	2.50
Ecotropic viral integration site 1 (Evi1)	NM_007963	2.46
Solute carrier family 22 (organic cation transporter), member 4 (Slc22a4)	BC010590	2.45
RIKEN cDNA 1500031H04 (1500031H04Rik)	BC017627	2.38
Proprotein convertase substilisin/kexin type 1 inhibitor (Pcsk1n)/Granin-like neuroendocrine peptide precursor	AF181560	2.36
RIKEN cDNA 2300002F06 (2300002F06Rik)	BC024546	2.35
Tubulin, β3 (Tubb3)	NM_023279	2.34
Serine (or cysteine) proteinase inhibitor, clade A, member 5 (Serpina5)/Protein C inhibitor (PCI)	NM_172953	2.33
Cysteine dioxygenase 1, cytosolic (Cdo1)	NM_033037	2.30
Calsyntenin 1 (Clstn1)	BG065300	2.25
Tumor protein D52-like (Tpd52l1)	NM_009413	2.19
N-acetylglutamate synthase (Nags)	AF462069	2.15
Butyrophilin related 1 (Butr1)	NM_138678	2.13
Carbonic anhydrase 5b, mitochondrial (Car5b)	NM_019513	2.09
B. Genes with Higher Expression Level in SCARKO than in Control Testes		SCARKO/Ctrl
Z-DNA binding protein 1 (ZBP1)/DLM-1	NM_021394	4.28
Lectin, galactose binding, soluble 7 (Lgals7)/Galectin-7	AF038562	4.01
Fatty acid binding protein 7, brain (Fabp7)	NM_021272	3.94
Regucalcin (Rgn)/Senescence marker protein 30 (SMP30)	NM_009060	3.46
—	S69212	3.42
RIKEN cDNA 1810010M01 (1810010M01Rik)	BF581690	2.79
Desmocollin 2 (Dsc2)	BC004663	2.44
Mus musculus, clone IMAGE:4188338, mRNA, partial cds/epiplakin 1 (Eppk1)	BC026387	2.25
Desmoglein 2 (Dsg2)	BG092030	2.18
Myeloblastosis oncogene (Myb)	NM_033597	2.18
6-Pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 α (TCF1/Pcbd)	NM_025273	2.02
Nectin-like 1 (Necl1)/Mus musculus Ig superfamily, member 4B (Igsf4b)	NM_053199	2.01

Differentially expressed genes with an average fold difference of 2 or more across the pairwise comparison of mRNA expression in testes of 10-d-old control *vs.* SCARKO mice. Statistical significance was confirmed by three different statistical tests (see *Materials and Methods*). For genes represented by more than one transcript in this list, only the transcript with the highest fold difference was retained.

shock protein 70-2 (Hsp70-2) and proacrosin-binding protein (Acrbp) displayed their steepest increase in expression later in meiosis (from d 16 on) and rose more markedly in controls than in SCARKOs, a finding compatible with the previously described progressive disruption of meiosis in SCARKO testes.

Cluster analysis of the transcripts found to be differentially expressed on d 10 resulted in the identification of 14 clusters encompassing 796 transcripts. Clusters containing at least 30 genes are shown in Fig. 3A, and the detailed data can be consulted in supplemental Table 2. The vast majority of the transcripts that showed a difference in expression level of at least 2-fold when comparing SCARKO and controls were found in clusters 3, 4, and 5. Clusters 1, 2, and 6 grouped genes with more limited differences in ex-

Table 3. Effect of (Anti)-Androgen Treatment on Testicular Gene Expression

Treatment	d 10			d 12		
	Control	Flut	TP	Control	Flut	TP
Pem	100 ± 26	11 ± 2[a]	432 ± 50[a]	100 ± 18	2 ± 1[a]	102 ± 22
Eppin	100 ± 9	35 ± 4[a]	143 ± 15[a]	100 ± 24	19 ± 4[a]	57 ± 15
Galgt1	100 ± 30	81 ± 16	226 ± 20[a]	100 ± 6	53 ± 7[a]	102 ± 9
Drd4	100 ± 10	12 ± 2[a]	102 ± 13	100 ± 30	23 ± 9[a]	36 ± 14[a]
Tsx	100 ± 15	84 ± 13	129 ± 10	100 ± 17	15 ± 3[a]	76 ± 17
Gpd1	100 ± 11	43 ± 7[a]	101 ± 9	100 ± 21	19 ± 5[a]	35 ± 9[a]
Tubb3	100 ± 10	83 ± 8	89 ± 8	100 ± 10	46 ± 9[a]	64 ± 6[a]
PCI	100 ± 8	77 ± 11	81 ± 7	100 ± 10	56 ± 5[a]	77 ± 6[a]
Tpd52l1	100 ± 23	72 ± 8	79 ± 5	100 ± 4	54 ± 4[a]	83 ± 5[a]

Seven-day-old male C57BL/6@Rj mice were treated for 3 or 5 d with TP (50 μg/animal/d), Flut (50 μg/animal/d), or vehicle (Control). At d 10 or 12, mice were killed and testes were removed. Luciferase mRNA was added to the testis samples as an external standard. RNA was extracted and cDNA was prepared as described in *Materials and Methods.* Each treatment group consisted of five animals. Results are expressed as the number of mRNA copies per 10^8 copies of luciferase mRNA, and values shown are means ± SEM from three independent experiments.
[a] $P < 0.05$, in comparison with respective control group.

pression level on d 10. Cluster 1 genes were characterized by a progressive decline in expression from d 8 to 20. The decline was more pronounced in controls than in SCARKOs. Cluster 2 genes displayed a marked decline in expression in control testes from d 12 onward, whereas expression in SCARKOs remained relatively stable. Cluster 6 genes were characterized by a late-onset (d 16) increase in expression that was more pronounced in controls than in SCARKOs.

Cluster 5 contained transcripts with a decreasing expression pattern from d 8–20 and with higher expression levels in SCARKOs than in controls. The time course of the most representative transcript (minimal distance between expression profile and cluster center) for this cluster (RIKEN cDNA 6530411B15) is illustrated in Fig. 4. Five of the 12 transcripts with a more than 2-fold higher expression level in SCARKO testes (Table 2 and supplemental Table 2) were found in this cluster.

Eight and 12 of the genes that had a more than 2-fold lower expression level in SCARKO testes than in control testes (29% and 43% of this entire subgroup) were found in clusters 3 and 4, respectively (Table 2 and supplemental Table 2). Prototypical genes of clusters 3 (phosphatidylinositol 4-kinase type 2β) and 4 (*N*-acetylglutamate synthase) are shown in Fig. 4. The expression of the transcripts found in cluster 3 increased over the study period, more so in controls than in SCARKOs (Fig. 4). Transcripts in cluster 4 were

Table 4. Gene Expression in Interstitial and Tubular Fractions in Testes at d 10

	Interstitial Compartment	Tubular Compartment
$P450_{c17}$	1335 ± 202	4 ± 2
SCP3	20 ± 3	128 ± 38
Pem	8 ± 2	85 ± 27
Eppin	81 ± 13	645 ± 91
Galgt1	21 ± 4	339 ± 86
Drd4	9 ± 0	21 ± 4
Tsx	69 ± 20	367 ± 129
Gpd1	9 ± 1	51 ± 6
Tubb3	277 ± 80	3579 ± 1553
PCI	1548 ± 343	8284 ± 2189
Tpd52l1	348 ± 76	2001 ± 451

Interstitial and tubular fractions were isolated by enzymatic digestion of testes from 10-d-old mice and gene expression levels were determined by quantitative RT-PCR. Results are expressed as the number of mRNA copies per 10^8 copies of 18S rRNA, and values shown are means ± SEM from three independent experiments. Pem and SCP3 are markers for the tubular compartment (SC and germ cells, respectively). $P450_{c17}$ is a marker for the interstitial compartment (Leydig cells).

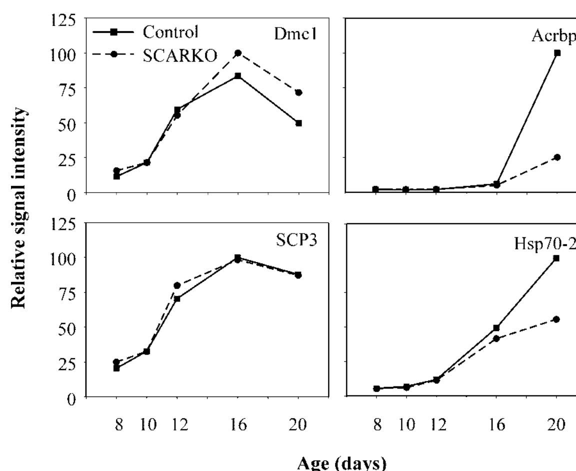

Fig. 2. Representative Expression Profiles (d 8–20) of Early (Dmc1 and SCP3) and Late (Acrbp and Hsp70-2) Meiotic Marker Genes

Microarray signal values (RMA normalized) were normalized to the highest signal value observed for the studied gene in SCARKO or control testes, arbitrarily set at 100. The resulting values are designated the relative signal intensity for the studied gene at the indicated time points.

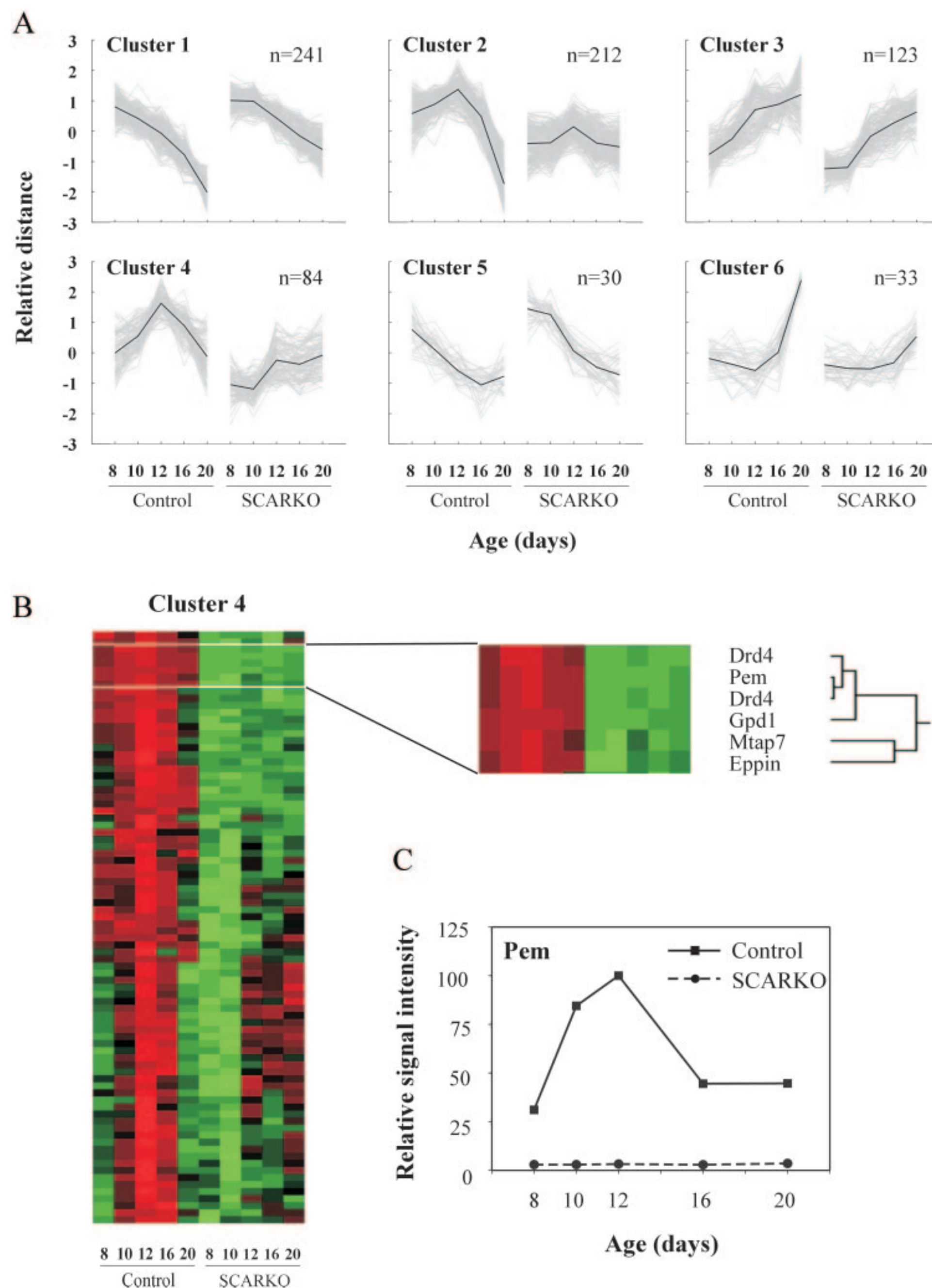

Fig. 3. Expression Patterns of Transcripts, Differentially Expressed on d 10 in SCARKO and Control Mice, between d 8 and d 20
A, Adaptive quality-based clustering (AQBC) of transcripts differentially expressed on d 10. Cluster analysis was used to identify subsets of transcripts with a similar expression pattern. Fourteen clusters were identified, containing 796 transcripts in total. Only clusters containing at least 30 transcripts are shown. For each cluster, the representative expression profile is represented in *bold*. B, *Left*, Hierarchical clustering of all transcripts present in cluster 4. *Columns* indicate different genotypes (control and SCARKO) and ages (d 8–20), and *rows* show the expression patterns for individual genes. Expression levels are visualized using a color code in which expression decreases from *dark red* (highest expression level) to *dark green* (lowest expression level). Genes are grouped based on the resemblance of their expression pattern to that of neighboring genes. The panel on the *right* gives a detailed view of the transcripts with an expression pattern most strongly resembling that of Pem. C, Expression pattern of Pem. Relative signal intensities were calculated as explained in Fig. 2.

characterized by a limited increase in the SCARKO testes, but a marked early increase in controls (up to d 12), followed by a slower decline (Fig. 4). Several of the genes with the largest decrease in expression level in SCARKO compared with controls (Pem, Eppin, Drd4, and Gpd1; Table 2) were found in cluster 4.

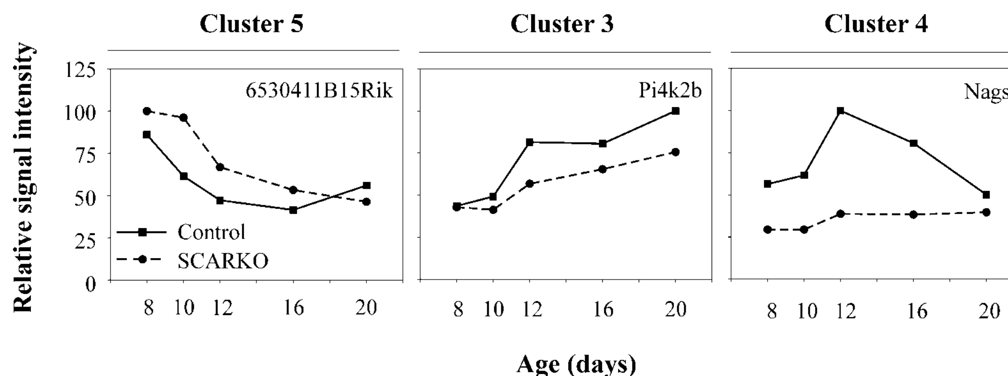

Fig. 4. Expression Patterns of the Most Representative Transcripts of Clusters 5, 3, and 4 in the AQBC Clustering of the Differentially Expressed Genes

RIKEN cDNA 6530411B15 gene (6530411B15Rik), phosphatidylinositol 4-kinase type 2 β (Pi4k2b), and *N*-acetylglutamate synthase (Nags) represent clusters 5, 3, and 4, respectively. Relative signal intensities were calculated as explained in Fig. 2.

Because Pem was previously shown to be directly regulated by androgens in SC, and the main aim of our study was to identify additional androgen-regulated genes in SC, we specifically searched for genes whose expression pattern most strongly resembled that of Pem (Fig. 3C). To this end, pairwise Euclidian distances between all profiles in cluster 4 were calculated and visualized using hierarchical clustering as implemented in Expression Profiler (EPCLUST) (26). Figure 3B shows that the expression profiles of Eppin, Drd4, and Gpd1 most strongly resemble that of Pem. Therefore, these genes were retained for additional analysis. Mtap7 was represented on the array by several transcripts, and because not all of them shared the same expression pattern, this gene was not included.

Genes with an Expression Pattern Resembling that of Pem

The expressions of Pem, Eppin, Drd4, and Gpd1 were studied from d 2–20 using quantitative RT-PCR. The data summarized in Fig. 5 confirm a similar expression pattern as a function of age for the four genes studied and noticeably higher expression in controls compared with SCARKOs at all ages. This difference in expression remained evident on d 50 (data not shown). In contrast to the expression pattern of these genes as studied by microarray analysis (illustrated for Pem in Fig. 3C) the expression pattern studied by quantitative RT-PCR did not show a decline after d 12. This may be explained by the fact that the quantitative RT-PCR data are normalized to exogenously added luciferase mRNA to obtain an estimate of the absolute number of transcripts per testis. The absence of such a normalization for the microarray data means that transcripts in a particular cell type (for instance, SC) will be diluted in the total amount of transcripts when another cell population (in this case, the germ cells) is making up an increasing proportion of the total testicular volume.

Effects of SC-Selective AR Inactivation on Genes Related to Tubular Remodeling and Junction Dynamics

Given the finding from the original d 10 study that transcripts encoding serine protease inhibitors and cell adhesion molecules are overrepresented in the list of genes differentially expressed in SCARKO and control testes, we explored whether androgen action in SC might play a role in tubular remodeling and junction dynamics. Time patterns were analyzed for transcripts differentially expressed on d 10 and encoding potentially relevant proteins, such as proteases and protease inhibitors, cytoskeletal elements, extracellular matrix molecules, and cell adhesion molecules. The data summarized in Fig. 6 illustrate that differentially expressed transcripts were found for each of these categories of molecules, and that for all transcripts, differential expression was observed at several time points. For many genes, including Eppin, PCI, tissue-type plasminogen activator (tPA), tubulin β3 (Tubb3), procollagen type IV α6 (Col4a6), brevican (Bcan), claudin 11 (Cldn11), and desmocollin (Dsc2), differential expression was already evident on d 8. For other genes, such as actinin α3 (Actn3), scinderin (Scin), thrombospondin 1 (Thbs1), and embigin (Emb), differential expression was absent or marginal on d 8, but increased toward d 12. As explained above, the decline in relative signal intensity observed in control testes after d 12 may be due at least in part to the increase in the relative proportion of germ cells in the testis.

DISCUSSION

Recently developed transgenic models in which the AR is selectively inactivated in SC support the contention that SC play a pivotal role in the control of spermatogenesis by androgens (21–23). In this study we used the

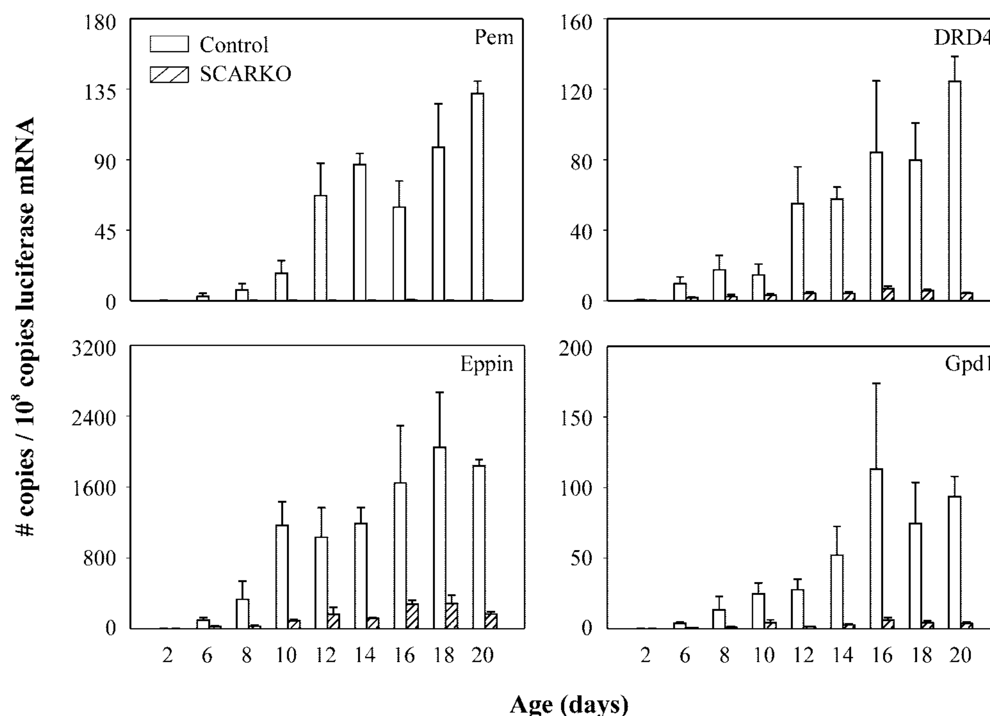

Fig. 5. mRNA Levels of Placentae and Embryos Oncofetal Gene (Pem), Dopamine Receptor 4 (Drd4), Serine Protease Inhibitor with Kunitz and WAP Domains 1 (Eppin), and Glycerol-3-Phosphate Dehydrogenase 1 (Gpd1) in Control and SCARKO Testes from d 2–20, as Determined by Quantitative RT-PCR

The results confirm differential expression between SCARKO and control testes and similarity of the expression patterns of the studied genes. Expression values were corrected using luciferase mRNA as an external standard and are represented as the mean ± SEM of three independent experiments with triplicate measurements. Note that the expression levels of Eppin are much higher than those of Pem, Drd4, and Gpd1.

combination of such a model, the SCARKO mouse, and microarray technology to analyze the molecular consequences of selective absence of androgen action in SC at the onset of spermatogenesis.

SCARKO mice develop spermatogenic arrest in meiosis. Accordingly, to avoid confounding effects due to the marked differences in tissue composition once spermatogenesis proceeds beyond the spermatocyte stage, we selected d 10 as the main time point to search for genes differentially expressed in SCARKO and control testes. Earlier experiments on the temporal mRNA expression of Pem suggested a marked increase between d 7 and 9, reflecting the onset of androgen action in SC (27). Moreover, the present data show that 1) there are no differences in testis weight between SCARKO testes and controls on d 10; 2) apart from the absence of AR expression in SC from SCARKO testes, the morphology of the testes is comparable in SCARKO and control mice; and 3) the expression of typical meiotic genes (Dmc1, SCP3, Hsp70-2, Acrbp, *etc.*) is identical in d 10 SCARKO and control testes.

Microarray analysis identified a subset of 692 genes differentially expressed in SCARKO and control testes. Of these genes, 396 were apparently down-regulated in SCARKOs, and 296 were up-regulated. Although the list covered a variety of functions, analysis using Onto-express software revealed overrepresentation of some potentially important genes/functions, including genes encoding serine-type protease inhibitors (down-regulated in SCARKOs) and genes related to cell cycling, cell growth, cell-cell adherence, and signal transduction (up-regulated in SCARKOs). When the list was limited to genes with an at least 2-fold difference in expression level between SCARKOs and controls, 28 down-regulated and 12 up-regulated genes were retained, with genes encoding serine-type endopeptidase inhibitors prominently present in the subset of genes down-regulated in SCARKOs.

The detailed mechanisms responsible for the differential expression of individual genes in SCARKOs and controls require further investigation. Because the primary difference between SCARKO and control testes, however, resides in the absence or presence of a functional AR in SC, genes that are down-regulated in SCARKOs may reasonably be expected to depend on androgen action in SC for their physiological expression, whereas genes that are up-regulated in SCARKOs may be repressed by androgen action. Physiologically relevant levels of androgens are present in the testis and the circulation on d 10 (28–32). Moreover, the two genes showing the most pronounced difference in expression in the present experiments, Pem and Eppin, have both previously been identified as

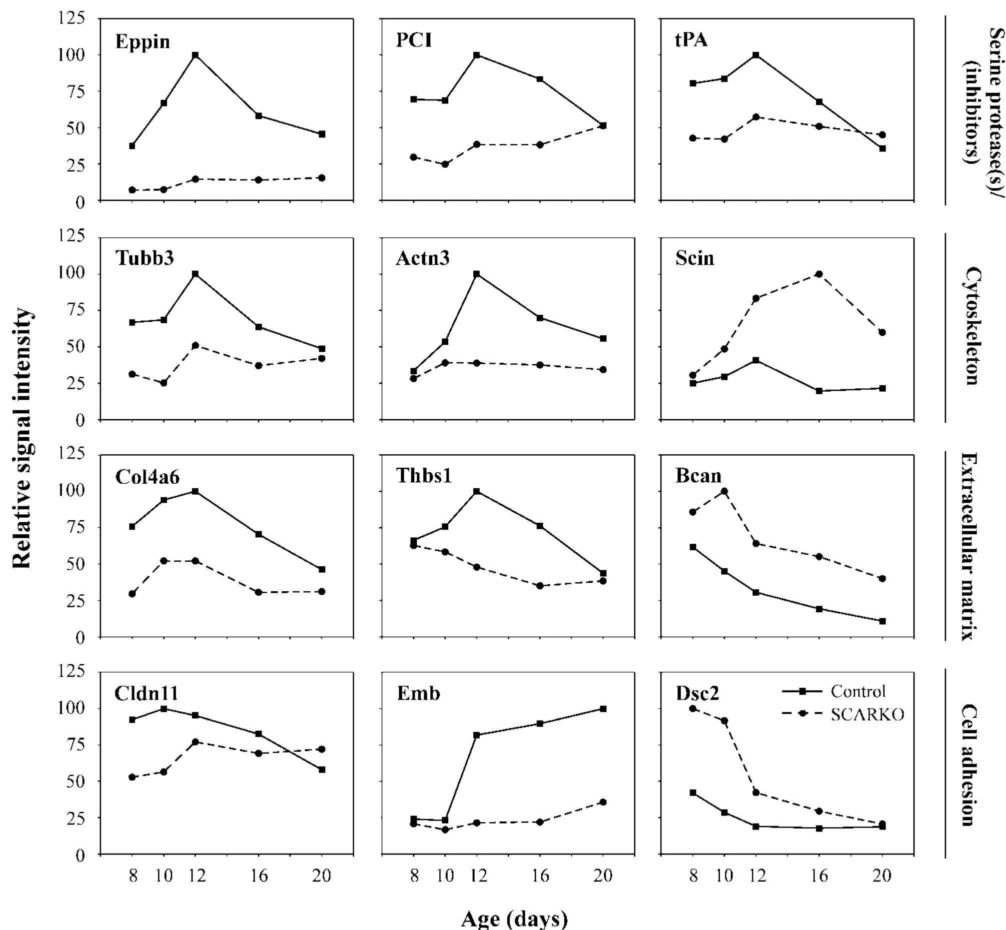

Fig. 6. Expression Pattern of Genes Related to Tubular Remodeling and Junction Dynamics: Eppin, PCI, Tissue-Type Plasminogen Activator (tPA), Tubulin β3 (Tubb3), Actinin α3 (Actn3), Scinderin (Scin), Procollagen Type IV α6 (Col4a6), Thrombospondin 1 (Thbs1), Brevican (Bcan), Claudin 11 (Cldn11), Embigin (Emb), and Desmocollin (Dsc2)

Relative signal intensities were calculated as explained in Fig. 2.

androgen target genes. Pem is a member of the Rhox cluster of homeobox genes (33). Its regulation by androgens in SC is well documented, and the androgen response elements (AREs) mediating regulation by the AR have been extensively characterized (34). Eppin is a member of a highly conserved family of protease inhibitors expressed in the testis and epididymis. The presence of an ARE in the promoter region and immunoexpression in SC from d 12 onward have been interpreted as indirect evidence of androgen regulation (35). Finally, for a subset of nine of the genes strongly down-regulated in SCARKO testes (Pem, Eppin, Galgt1, Drd4, Tsx, Gpd1, Tubb3, PCI, and Tpd52l1), we could demonstrate by quantitative RT-PCR that treatment with the antiandrogen flutamide for 5 d significantly decreased expression on d 12. Whether all of these genes are directly or indirectly regulated by androgens remains to be investigated. Similarly, the exact cell(s) expressing the identified genes remains to be identified. Quantitative RT-PCR measurements after enzymatic separation of tubular and interstitial tissue, however, suggest that the studied subset of nine genes is expressed predominantly in the tubular compartment.

Other experimental paradigms have been used to identify androgen-regulated genes in the testis. It is striking that apart from Pem, the differentially expressed genes detected in the present study show little overlap with androgen-regulated genes identified in two other studies recently performed in hypogonadal (*hpg*) and neonatal mice (36, 37). There may be several explanations for this apparent discrepancy. The SCARKO model may be expected to identify specifically genes whose expression depends on AR action in SC, whereas in the other models, effects induced by androgen administration may be mediated not only by SC, but also by other AR-positive testicular cells, such as peritubular myoid and Leydig cells. Moreover, in both the *hpg* model and the neonatal mouse studies, gene expression was studied early (4–24 h) after androgen administration. In our experimental setup, gene expression was studied on d 10. Because AR expression in SC is observed from d 3–5, it is conceivable that some of the observed effects are

indirect and do not necessarily imply direct interaction of the activated AR with an ARE in the relevant gene. Finally, in the SCARKO model we are looking at the effects of physiological amounts of endogenous androgens, whereas in the other models high amounts of exogenous androgens are administered. The absence of FSH could be an additional factor affecting the results in the *hpg* model.

The physiological relevance of the genes identified by use of the SCARKO model is underscored by the finding that three of the genes found to be markedly down-regulated in SCARKOs (Galgt1, Eppin, and PCI) have already been shown to be essential for normal spermatogenesis and fertility. Eppin is a serine protease inhibitor discussed above. Immunization of male monkeys against Eppin results in reversible contraception, but the mechanism underlying this effect remains to be defined (38). Gene inactivation of β1,4-*N*-acetylgalactosaminyltransferase (Galgt1) or PCI results in severe disruption of spermatogenesis. Galgt1 encodes an enzyme involved in the formation of complex gangliosides, and the infertility of Galgt1$^{-/-}$ mice may be related to a disturbance in the production of a specific class of fucosylated glycosphingolipids that are apparently essential for spermatogenesis (39, 40). PCI encodes a serine-type protease inhibitor, and spermatogenic failure in PCI$^{-/-}$ mice has been related to disruption of the blood-testis barrier (41).

To search for genes with a particular relevance to the onset of spermatogenesis and the presence or absence of AR activation in SC we also studied gene expression in SCARKO and control testes between d 8 and 20 using microarray techniques and cluster analysis. Cluster analysis of the subset of genes known to be differentially expressed on d 10 revealed three clusters that may be particularly relevant. Two of these (clusters 3 and 4) identified subgroups of genes displaying higher expression levels in mice with an active AR in SC, but showing a different pattern of change with time. Interestingly, these two clusters encompassed 71% of the genes down-regulated more than 2-fold in SCARKOs. Cluster 5 encompassed 42% of the genes identified as strongly up-regulated in SCARKOs. Pem, which can be considered a lead gene in the search for androgen-regulated genes in SC, was found in cluster 4. A more detailed analysis of cluster 4, using hierarchical clustering, revealed that several of the genes showing the highest up-regulation on d 10 (Eppin, Drd4, and Gpd1) clustered in the immediate vicinity of Pem. Quantitative RT-PCR confirmed that the expression of these genes strongly depended on activation of the AR in SC and that their expression pattern mimicked that of Pem. Eppin has been discussed above, and the present data are the first to show directly that androgen action in SC enhances its expression in the testis. We can only speculate about the roles of Drd4 and Gpd1 in the testis. Drd4 encodes a G protein-coupled receptor that is strongly expressed in the testis, but no testicular phenotype was reported in mice lacking Drd4 (42, 43). Gpd1 encodes a reduced nicotinamide adenine dinucleotide-dependent enzyme involved in glycerol synthesis. Gpd1 is ubiquitously expressed, but its promoter appears to be highly methylated, specifically in sperm (44). Additional research will be required to clarify the roles and the relationship of these genes to androgen action in SC.

There is increasing evidence that the interplay between proteases and protease inhibitors plays an important role in the restructuring of testicular tubules at the onset of spermatogenesis and in the assembly and functional dynamics of tight and adhesion junctions between SC and germ cells (45, 46). Our data seem to support this contention and provide preliminary evidence that the AR in SC may play a more prominent role in the control of these processes than previously suspected. Both protease inhibitors and cell adhesion molecules were overrepresented in the genes differentially expressed in SCARKO and control testes. Moreover, the time study showed that not only protease inhibitors and cell adhesion molecules, but also cytoskeletal and extracellular matrix elements, showed divergent expression patterns at the onset of spermatogenesis.

In conclusion, the present study has identified a subset of genes that are differentially expressed in d 10 testes with or without an active AR in SC. Some of the identified genes have previously been shown to be relevant for spermatogenesis or to be controlled by androgens. Of particular interest is the finding that some genes that may be related to tubular remodeling and junction dynamics are affected by androgen action in SC. Moreover, three genes with an expression pattern strongly resembling that of the lead gene, Pem, have been identified. These findings provide a basis for additional dissection of the molecular mechanisms by which androgen action in SC affects spermatogenesis.

MATERIALS AND METHODS

Tissue Sampling and RNA Extraction

To obtain SCARKO animals, female mice (129/Swiss) heterozygous for the floxed AR allele (AR$^{flox/+}$) (21) were mated to male mice (C57BL/6) homozygous for the AMH-Cre transgene (24). These AMH-Cre transgene animals were also used as a control for SCARKO animals. All animals were treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all experiments were approved by the local ethical committee. Whole testes were obtained from animals at different ages depending on the experiment. Immediately after removal, testis samples were snap-frozen and stored in liquid nitrogen. Before RNA extraction, testis samples were weighed and homogenized in a Dounce homogenizer (Kontes Co., Vineland, NJ). Thereafter, RNA was isolated with the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions, encompassing an on-column deoxyribonuclease I (DNase I) treatment of the RNA. For quantitative RT-PCR analyses, 5

ng luciferase mRNA (Promega Corp., Madison, WI) were added to each testis sample before RNA preparation as an external standard (47).

Immunohistochemistry

Standard procedures were used for hematoxylin and eosin staining. A rabbit polyclonal antibody (N20) raised against a peptide within the N-terminal domain of the human AR (sc-816, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a 1:500 dilution. Rabbit anti-Dazl antibody was a gift from Dr. H. J. Cooke (Medical Research Council Human Genetics Unit, Edinburgh, UK) and was used at a 1:1000 dilution. Testes from d 10 control and SCARKO males were removed, weighed, fixed in Bouin's fluid for 4–6 h, and transferred into 70% (vol/vol) ethanol before processing into paraffin wax using standard methods. Immunohistochemistry was performed on dewaxed 5-μm sections in conjunction with heat-induced antigen retrieval for 5 min in 0.01 M citrate buffer (pH 6.0; Sigma-Aldrich Corp., Dorset, UK) using a pressure cooker. A swine antirabbit biotinylated second antibody (E0353, DakoCytomation, Cambridge, UK) was used, and details of the immunohistochemistry protocol were reported previously (21).

To enable comparative evaluation of the immunostaining, sections of tissues from control and knockout animals were processed in parallel on at least three occasions to ensure reproducibility of results; on each occasion, tissue sections from four to six animals in each group were run. To ensure direct comparability of staining intensities, one section each from control and SCARKO mouse was mounted on the same slide for the immunostaining procedure.

Microarray Target Synthesis and Processing

The quality of all testis RNA samples was monitored by measuring the 260/280 and 260/230 nm ratios with a NanoDrop spectrophotometer (NanoDrop Technologies, Centreville, DE) and by means of the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). Only RNA showing no signs of degradation or impurities (260/280 and 260/230 nm ratios, >1.8) was considered suitable for microarray analysis.

Before target synthesis, RNA extracts from one testis of three control or three SCARKO littermates were pooled, creating paired control and SCARKO samples optimally adjusted for potential variation between litters. Five micrograms of polyadenylated RNA from each pool was reverse transcribed into double-stranded DNA with a polydeoxythymidine-T7 primer using SuperScript II RT, ribonuclease H, and DNA polymerase I (Invitrogen Life Technologies, Inc., Carlsbad, CA). From this double-stranded DNA, biotin-labeled target aRNA was generated in a T7 *in vitro* transcription reaction using the Affymetrix IVT Labeling Kit (Affymetrix, High Wycombe, UK). After purification (GeneChip Sample Cleanup Module, Affymetrix), yield (30–120 μg) and purity (260/280 and 260/230 nm ratios, >1.8) of the labeled amplified RNA were analyzed, 20 μg quality-controlled amplified RNA was fragmented by alkaline hydrolysis and resuspended with control spikes in 300 μl hybridization buffer (Eukaryotic Hybridization Control Kit, Affymetrix). Of this probe solution, 200 μl was used for hybridization in a rotisserie oven at 45 C.

The mouse expression array 430a 2.0 from Affymetrix was used in all hybridizations. This array contains 22,690 transcripts from *Mus musculus*, each represented by 11 probe pairs. Each pair consists of a perfect match and a mismatch (with a single base point mutation) 25-mer oligonucleotide probe.

After hybridization, gene chips were washed and stained in the GeneChip Fluidics Workstation 400 (Affymetrix) using the EukGE-WS2v4 protocol and subsequently scanned with the GeneChip Scanner 3000 (Affymetrix). Image analysis was performed by use of Affymetrix GeneChip operating software.

Microarray Data Analysis

A first experiment was set up to identify differentially expressed genes in the testis of 10-d-old control *vs.* SCARKO mice. To this end, five control and five SCARKO samples were compared. The data were considered paired because the samples consisted two-by-two of testicular RNA from littermate mice (see microarray target synthesis and processing section). In an initial phase, results were analyzed using Affymetrix Microarray Suite (MAS) 5.0 software. The amount of transcripts in a sample that scored present, absent, or marginally detectable was assessed. MAS 5.0 software was also used to generate expression ratios after comparison of a control dataset with its littermate SCARKO dataset. For additional statistical analysis, all data were first normalized using the robust multiarray analysis algorithm (RMA) (48, 49) as implemented in the BioConductor packages (www.bioconductor.org) (50). Three statistical approaches, all part of the BioConductor software, were used to identify differentially expressed genes in testis of control *vs.* SCARKO mice: 1) significance analysis for microarrays (51), 2) a paired *t* test controlling the false discovery rate using the Benjamini and Hochberg correction (52), and 3) the moderated *t* test as described in linear models for microarray data (53) also encompassing the Benjamini and Hochberg correction. A threshold was set for each test, generating three lists of differentially expressed transcripts (significance analysis for microarrays: false discovery rate, $P \leq 0.05$; paired *t* test and Benjamini-Hochberg correction, $P \leq 0.05$; linear models for microarray data and Benjamini-Hochberg correction, $P \leq 0.05$). A cross-section of these lists was obtained in Access (Microsoft Corp., Redmond, WA), yielding a summary of transcripts that were depicted as differentially expressed in a statistically significant way by all three tests. This list, which contained 848 transcripts, was used for further analysis. Onto-Express software (http://vortex.cs.wayne.edu/Projects.html#Onto-Express) (25) was used to check for statistical overrepresentation of certain molecular or biological functions among the differentially expressed transcripts.

In a second experiment, we tried to identify typical expression patterns during initiation of spermatogenesis for the list of differentially expressed transcripts generated. Hence, gene expression was assessed in one control and one littermate SCARKO sample at five different ages (d 8, 10, 12, 16, and 20). After a first data analysis using MAS 5.0 software, as described above, data were again RMA normalized for additional statistical analysis. An adaptive quality-based clustering using a heuristic iterative two-step algorithm (54), as implemented in the INCLUSive package of web-based tools (www.esat.kuleuven.ac.be/~dna/BioI/Software.html), was used in the search for expression patterns shared by genes that were differentially expressed in d 10 animals. This method is similar to k-means clustering, but has two advantages. First, no prior choice of the number of clusters is required, and second, not every gene is forced into a cluster. Only significantly coexpressed genes are retained in a cluster after derivation of its optimal radius. One of the resulting clusters was further studied by calculating and visualizing pairwise Euclidian distances between centered profiles using hierarchical clustering as implemented in Expression Profiler (http://ep.ebi.ac.uk) (26). Functional analysis of the clusters was performed based on Onto-Express statistics.

In Vivo Treatment with Androgens and Antiandrogens

Seven-day-old C57BL/6@Rj mice (Janvier, Le Genest-St-Isle, France) were treated for 3 or 5 d with TP (50 μg/animal·d), Flut (50 μg/animal·d), or vehicle only. TP and Flut were purchased from Fluka Chemie AG (Buchs, Switzerland)

and Sigma-Aldrich Corp. Stock solutions were made in ethanol and then further diluted in arachis oil for injection. In all conditions a volume of 50 μl containing 10% ethanol was injected sc. Treatment groups consisted of five animals. Mice were killed on d 10 or 12, and testes were removed and additionally processed for quantitative RT-PCR as described above.

Enzymatic Separation of Interstitial and Tubular Fractions

Interstitial and tubular fractions were prepared through enzymatic digestions essentially as described previously (55). Twenty testes, derived from 10-d-old C57BL/6@Rj mice (Janvier) were decapsulated and digested for 10 min with trypsin (0.67 mg/ml) and DNase (6.7 μg/ml) in a shaking water bath (120 oscillations/min) at 32 C. The action of trypsin was stopped by the addition of 2 mg/ml soybean trypsin inhibitor. After sedimentation of the tubular fragments, the supernatant was collected as the interstitial fraction, and the sedimented tubular fragments were treated for an additional 5 min at 32 C with collagenase (0.67 mg/ml), hyaluronidase (0.67 mg/ml), and DNase (10 μg/ml). The remaining tubular fragments were treated once more with hyaluronidase (0.67 mg/ml) and DNase (10 μg/ml) for about 15 min in a shaking water bath (120 oscillations/min) to remove the remaining peritubular cells. The resulting tubular fraction as well as the above-generated interstitial fraction were centrifuged, snap-frozen in liquid nitrogen, and stored at −80 C until RNA preparation. RNA was isolated using the Qiagen RNeasy kit with an on-column DNase I treatment as described above.

Quantitative RT-PCR

cDNA was synthesized using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen Life Technologies, Inc.). Primer sequences for Eppin, Galgt1, Drd4, Tsx, Gpd1, Tubb3, PCI, Tpd52l1, and SCP3 are described in Table 1. Pem primer and probe sequences and 17α-hydroxylase ($P450_{c17}$) primer sequences were described previously (21, 31). For quantification of gene expression, the ABI PRISM 7700 sequence detector PCR detection system (Applied Biosystems, Foster City, CA) was used with a two-step quantitative RT-PCR protocol. Components for real-time PCR, apart from primers and probes (Eurogentec, Sar-Tilman, Belgium) and SYBR Green (Sigma-Aldrich Corp.), were obtained from Applied Biosystems. For Eppin, Galgt1, Drd4, Tsx, Gpd1, Tubb3, PCI, Tpd52l1, SCP3, and $P450_{c17}$, each 25-μl real-time PCR contained 1× buffer A, 5 mM $MgCl_2$, 400 μM deoxy-NTPs, 200 nM of each primer, 0.4× SYBR Green, and 0.025 U/μl AmpliTaq Gold enzyme. The uniqueness of the amplified band was checked by PAGE. For Pem, no SYBR Green was used; instead, 100 nM of each primer and 400 nM probe were added to the reaction. The quantity of measured mRNA was expressed relative to the external luciferase mRNA standard added before RNA preparation or to the 18S rRNA present in the same sample. All samples and standard curves were run in triplicate.

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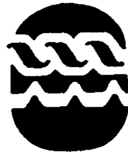

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