Journal of Andrology, Vol. 29, No. 4, July/August 2008

Molecular Profiling of the Human Testis Reveals Stringent Pathway-Specific Regulation of RNA Expression Following Gonadotropin Suppression and Progestogen Treatment

ROSEMARY A. L. BAYNE,* THORSTEN FORSTER,‡ STEWART T. G. BURGESS,‡ MARIE CRAIGON,‡ MELANIE J. WALTON,† DAVID T. BAIRD,† PETER GHAZAL,‡ AND RICHARD A. ANDERSON†

*From *MRC Human Reproductive Sciences Unit and the †Division of Reproductive and Developmental Sciences, University of Edinburgh Centre for Reproductive Biology, The Queen's Medical Research Institute, Edinburgh, United Kingdom; and the ‡Division of Pathway Medicine, University of Edinburgh, Edinburgh, United Kingdom.*

ABSTRACT: Gonadotropin withdrawal induces changes in gene expression in all 3 major cell types of the testis. Knowledge of the genes affected, in both the presence and absence of additional progestogen, will give insight into the regulation of human testicular function and aid development of novel contraceptive methods. We have undertaken a whole-genome analysis of RNA expression in testicular biopsies from normal men and after 4 weeks of gonadotropin suppression induced by gonadotropin-releasing hormone antagonist plus testosterone administration sufficient to cause marked suppression of spermatogenesis. Microarray analysis shows that interindividual variability is markedly low, and the response to treatment is focused on a small subset of genes particularly related to pathways in steroidogenesis and cholesterol biosynthesis or metabolism, the Leydig cell gene *INSL3*, and genes involved in early meiosis or Sertoli–germ cell junctions. These changes in expression were confirmed by quantitative reverse transcriptase polymerase chain reaction. No major changes in gene expression were identified in men additionally treated with a progestogen, although *FLJ35767*, an expressed sequence tag that is expressed in the germ cell compartment, did show a small but significant additional effect of progestogen. Overall, the results of this investigation disclose a remarkably stringent regulation of testicular gene expression, revealing the genes most sensitive to gonadotropin withdrawal, and might reflect the most labile pathways in the regulation of testicular function.

Key words: Microarray analysis, steroidogenesis, testicular function.

J Androl 2008;29:389–403

The molecular mechanisms by which the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) support the testicular production of steroids and gametes in humans are poorly understood. However, such knowledge is important for improved understanding of the regulation of testicular function and for the development of safe and efficacious contraceptive methods.

LH receptors are present on Leydig cells, where they control steroid biosynthesis and thus the production of testosterone. Testosterone and FSH receptors are found on Sertoli and peritubular cells, but there are no receptors for any of these hormones on germ cells (Themmen and Huhtaniemi, 2000; Collins et al, 2003), with the exception of mature human sperm, which have recently been shown to have functional androgen receptors (Aquila et al, 2007). This indicates that the effects of these hormones on immature germ cells must be indirect, in that all are required for quantitatively normal spermatogenesis (Matsumoto et al, 1983, 1984).

Gonadotropin suppression results in suppression to azoospermia in most but not all men (Anderson and Baird, 2002). Suppression can be achieved by the administration of testosterone alone or with a gonadotropin-releasing hormone (GnRH) antagonist, but the addition of a progestogen increases spermatogenic suppression and is currently the most promising approach toward a hormonal male contraceptive (Nieschlag et al, 2003). It has been suggested that the degree of spermatogenic suppression in some progestogen regimens is greater than can be accounted for by gonadotropin suppression, which could indicate that progestogens act directly on the testis (McLachlan et al, 2004). Both nuclear and membrane progesterone receptors have been found on spermatozoa and on Sertoli and some Leydig cells in the human testis (Shah

Supported by grants from the United Kingdom Medical Research Council and Department for International Development (G9523250), the Wellcome Trust, and Scottish Funding Council.

Correspondence to: Dr Rosemary A. L. Bayne, MRC Human Reproductive Sciences Unit, Centre for Reproductive Biology, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, United Kingdom (e-mail: r.bayne@hrsu.mrc.ac.uk).

Received for publication September 12, 2007; accepted for publication December 11, 2007.

DOI: 10.2164/jandrol.107.004168

et al, 2005) and progestogens have direct inhibitory effects on Leydig cell function in a murine cell line (El-Hefnawy et al, 2000). We have recently provided direct evidence for a progestogenic effect on 5α-reductase expression in the testis (Walton et al, 2006) that might reduce the amplifying effect of conversion of testosterone to dihydrotestosterone and thus contribute to greater suppression of spermatogenesis.

To identify a more comprehensive picture of the effects in humans of suppression of gonadotropins by administration of a GnRH antagonist and of the addition of progestogen, we performed microarray analysis of gene expression on testis biopsies and show that the pharmacological effects are focused on a select number of pathway-specific genes indicating stringent regulation of testicular gene expression.

Methods

Study Design, Drug Treatment, and Testis Biopsy

The study protocol has been described in detail previously (Walton et al, 2006). In brief, 30 men (mean age 38 years) requesting vasectomy were recruited, and written informed consent was obtained. The study had ethical approval from the Lothian Regional Ethics Committee. Pretreatment investigations demonstrated that all had normal semen analysis and reproductive hormone concentrations. Subjects were randomized by sealed envelopes into 3 groups. Controls received no drug treatment before testis biopsy. A second group (CT) received cetrorelix 3 mg SC (Cetrotide, Serono Europe Ltd, London, United Kingdom) twice each week and testosterone enanthate 200 mg IM (Cambridge Laboratories, Wallsend, United Kingdom) repeated after 2 weeks; the third group (CTD) took the progestogen desogestrel 300 μg orally (Cerazette, 4 × 75 μg, Organon NV, Oss, The Netherlands) each day for the 28-day duration of the treatment period in addition to the other agents.

One subject withdrew for personal reasons. After 4 weeks of drug treatment, further blood and semen samples were analyzed, and testis biopsies were carried out on the remaining 29 men under local anesthetic at the time of vasectomy with a 14-gauge needle (Tru-Cut, Allegiance Healthcare Corporation, McGraw Park, Illinois). Tissue samples were immediately frozen and stored at −80°C.

RNA Samples

Total RNA was extracted from testis biopsies as described (Walton et al, 2006). Quality was confirmed on RNA 6000 Nanochips in the Agilent 2100 Bioanalyzer (both from Agilent Technologies UK Ltd, West Lothian, United Kingdom). Only very high quality RNA (RNA integrity number >7.5) preparations were considered for microarray screening. Five RNA samples from each of the control, CT, and CTD groups were used for screening the microarrays, along with the 5 "nonsuppressors" from the treatment groups (3 CT, 2 CTD) who did not show a reduction in sperm concentrations to below the reference range (Walton et al, 2006).

Microarray Hybridization and Analysis

Affymetrix Human genome U133 plus 2.0 microarrays (Affymetrix UK Ltd, High Wycombe, United Kingdom) containing 54 675 probes representing approximately 39 000 genes, were hybridized to 15 μg fragmented biotin-labeled cRNA prepared from each RNA and spiked with eukaryotic hybridization control in a volume of 200 μL for 16 hours. After hybridization, the arrays were washed on the Genechip Fluidics Station 450 and scanned with the Genechip Scanner 3000 all according to the manufacturer's protocols (Affymetrix UK Ltd).

Raw microarray data were processed and analyzed with R 2.1.1 statistical environment (http://www.R-project.org) and Bioconductor 1.6 microarray libraries (Gentleman et al, 2004). Numerical quality control steps were applied to each of the 20 samples. Arrays were normalized and converted to probe-level data with Robust Multi-Array Average (RMA; Irizarry et al, 2003). To reduce statistical multiple testing and algorithmic problems in downstream analysis, a nonspecific filter to remove genes expressed at low levels (signal $< \log_2 100$) in all of the samples was applied. This nonspecific filter is set quite stringently to remove low-signal genes and thus reduce the number of false positives, given the relatively low sample size in each group. It reduces the number of genes available for comparison from about 19 000 that pass the basic Affymetrix quality criteria (MAS5 Present/Marginal/Absent calls) to 5824 after nonspecific filtering but gives more reliable results.

To identify candidate genes that change in response to the various treatments, differential expression (by at least 2-fold) and its statistical significance for each gene was calculated on the basis of an empirical Bayes test (Smyth, 2004), which is robust for small sample sizes. Analysis involved 3 different stages. Stage 1 testing compared gene expression between controls and each of the treatment groups (control n = 5, CT n = 7, CTD n = 7), as well as between the 2 treatment groups. Stage 2 testing repeated these comparisons with nonsuppressed patients removed (CT-ns n = 4, CTD-ns n = 5). Stage 3 testing explicitly compared all nonsuppressed patients (ns n = 5) to the "suppressed" patients in each of the 2 treatment groups (CT-s n = 4, CTD-s n = 5). A reduction in the potential number of false positive results was achieved by correcting the significance test P values in relation to the number of genes tested simultaneously (n = 5824) (Benjamini and Yekutieli, 2005).

Functional characterization of significantly expressed transcripts was performed with the resource tool DAVID from the National Institute for Allergy and Infectious Diseases (NIAID) (http://niaid.abcc.ncifcrf.gov/). DAVID examines the overrepresentation of gene ontology pathways within a set of transcripts and provides statistical evaluation of the presence of these transcripts. Statistical significance was based on $P \leq .05$. Analyses were made with the use of the filtered set of 5824 genes.

Clustering analysis was next performed: By tagging the statistically significant genes and then isolating genes that

cluster alongside them, it is possible to identify additional genes that are potentially coregulated. These can be missed by statistical group means testing but could be biologically interesting. This was carried out with the open source Java application Biolayout Express^{3D} (Goldovsky et al, 2005; Freeman et al, in press), which is specifically designed for the construction, visualization, clustering, and analysis of transcription networks generated from microarray datasets. The networks created by the software consist of nodes (representing transcripts) that are connected by edges (which represent similarities in expression profiles across multiple conditions). The analysis was performed on the entire RMA normalized data set (54 675 gene probes) from all of the sample arrays (n = 20). The statistically significant genes in the original differential expression list were flagged, allowing them to be identified in the network graphs produced in Biolayout Express^{3D}. Clustering graphs where then produced with a Pearson threshold of 0.9 and a Markov Clustering Algorithm (MCL) inflation of 1.7, and those containing the flagged gene set were identified. Additional coregulated genes were thus identified, and the extended probe lists from these clusters were then analyzed with DAVID pathway analysis software to identify enrichment of gene ontologies within the lists.

Finally, an additional nonparametric means of verification of the data was obtained by assessing differential gene expression of the original data with the independent rank product method (Breitling et al, 2004). Briefly, this technique ranks each gene in each of the replicate samples. The product of these ranks is then calculated. Statistical significance is assigned by comparing the observed rank product value with a bootstrapped null distribution of rank product values for each gene, and the genes are sorted, with the highest ranking genes being placed at the top of the list.

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

First-strand cDNA (+/− reverse transcriptase), prepared from all testis biopsy samples as described previously (Walton et al, 2006), was used to confirm array results by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). This was performed with the Lightcycler (Roche Diagnostics, East Sussex, United Kingdom). Reverse-transcribed RNA samples were diluted 1:10 in nuclease-free water (Promega Ltd, Southampton, United Kingdom). Diluted first strand cDNA (1 μL) was added to a final volume of 10 μL containing 50 μg/mL BSA and 0.5 μM each of forward and reverse primer in 1× Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Paisley, United Kingdom) in duplicate. Primers (Table 1) were either previously published or designed with the use of online Primer3 software. mRNA concentration was calculated relative to that of the ribosomal protein RPL32. *RPL32* had previously proved to be the most consistent reference gene for these samples (Walton et al, 2006).

Results of these analyses are presented ($\bar{x} \pm$ SEM). Treatment effects on gene expression data were initially compared by analysis of variance (ANOVA). Significant treatment group effects suggested by ANOVA were further investigated by unpaired *t* tests, with cube root transformation to normalize the distribution. For all comparisons, $P < .05$ was considered significant.

Results

Spermatogenic Suppression

Marked and similar suppression of spermatogenesis was seen in both treated groups, although there was some interindividual heterogeneity. Median sperm concentration fell to 0.6×10^6/mL in the CT group and to 2.6×10^6/mL in the CTD group (both $P < .01$ compared with pretreatment). However 3 subjects in the CT group and 2 in the CTD group maintained sperm concentrations within the reference range ($>20 \times 10^6$/mL) at day 28, whereas sperm concentrations were less than 5×10^6/mL in all others. This allowed classification of 14 men as "suppressors" and 5 as nonsuppressors (Walton et al, 2006).

Preliminary Array Analysis

Five high-quality RNA samples were chosen from each treatment group (control, CT, and CTD) along with RNA from each of the 5 nonsuppressors for analysis on Affymetrix microarrays; thus, a total of 20 samples were analyzed. After data processing and analysis, 1 sample from the CT group showed a poor RNA digestion plot and was excluded from further analysis. After data quality control, normalization, and nonspecific filtering, 5824 probes—10.6% from the total 54 675 probes on the chip—showed expression of greater than $\log_2(100)$ for at least 1 sample.

These 5824 probes (supplementary Table 1) represent the predominant genes expressed in the adult human testis. Assignment of gene ontology pathways to each gene allowed them to be grouped and for biological processes statistically overrepresented in the testis to be identified (Table 2). Not surprisingly, these included genes involved in sexual reproduction, with cell death genes also highly represented. Other significant processes were growth regulation, enzyme regulation, cell physiology, and metabolism.

A striking finding on initial analysis was that interindividual variability was remarkably low. The median coefficient of variance within each group was 6.5% for controls, 6.7% for the CT group, and 7.8% for the CTD group, with 95th percentile CV values of 14%, 12.9%, and 15.2%, respectively. The average correlation between samples in a group was greater than 0.99 for control and CT groups and greater than 0.98 for the CTD group. This level of variation is in marked contrast to other tissue biopsy types, which show far greater variability (Whitney et al, 2003; Radich et al, 2004;

Table 1. *Sequences of primers used for quantitative reverse transcriptase polymerase chain reaction*

Gene	Forward Primer	Reverse Primer	Product Size, bp	GenBank Accession	Reference
SYCP1	GCAAAAGCCCTTTGCATTGTTC	ATGTTTTCCCCATTTTTGGAGAG	177	NM_003176	Primer 3
INSL3	CCCAGAGATGCGTGAGAAGT	CCAGCGTGAGATTACTGTCG	185	NM_005543	"
WISP2	CTGTATCGGGAAGGGGAGA	GGAAGAGACAAGGCCAGAAA	246	NM_003881	"
DDR1	CGTGTACCTCAACGACTCCA	TGCTCCATCCCACATAGTCA	158	NM_013994	"
CST9L	GACTGGGGCACATCTTGAAT	GTGCTGATGGTGAAGAAGCA	178	NM_080610	"
FLJ35767	GAAGACAACTGGGACCCTGA	CCTCCTGAGGCCATAGTTCA	220	NM_207459	"
PROK1	ACAAGGTCCCCTTCTTCAGGA	GCAAGCGCCTAAAAATTGATGTT	133	NM_032414	BioTrove
PGRMC1	ATCAACGGCAAGGTGTTCGAT	AAATGTGGCAAGGCCCCTG	108	NM_006667	BioTrove
RHOB	CCCACCGTCTTCGAGAACTA	CACCGAGAAGCACATGAGAA	153	NM_004040	Primer 3
APOE	GGTCGCTTTTGGGATTACCT	TTCCTCCAGTTCCGATTTGT	150	NM_000041	"
TAF4B	CAGAAGACGATGCCAGTGAA	GACCCCTGCCATAGAAGTCA	201	XM_290809	"

Critchley et al, 2006; P.G., unpublished data) and supports the biological significance of the array data.

Analysis of the Effect of Gonadotropin Suppression

Expression of very few genes was altered between the groups, as demonstrated by volcano plots (Figure 1a and b). Few genes showed significant 2-fold or greater differences between controls and treatment groups, although a greater number of genes showed significant differences less than 2-fold. No significant differences were identified between CT and CTD treatment groups at this stage (Figure 1c).

Stage 1 testing identified only 15 genes that showed at least a 2-fold change in gene expression between controls and 1 or both of the treatment groups (Figure 2). Many of these genes are known to be involved in steroidogenesis and cholesterol metabolism (*CYP17A1*, *DHCR24*, *HSD17B6*, *STAR*, *CYP11A1*, *HMGCS2*). One gene, *CYP17A1*, had already been shown to be down-regulated in the CT and CTD groups by qRT-PCR (Walton et al, 2006). However, other genes in that study showing changes in expression level in the treatment groups were not highlighted by this screen because of sensitivity differences between the 2 methods, as discussed below. Nevertheless, several novel genes (*INSL3*, *SYCP1*, *WISP2*, *CST9L*, *FLJ35767*, *DDR1*) were identified for which the function or site of expression suggested that they might be of particular interest for further examination. The array analysis indicated approximately 10-fold down-regulation of *INSL3* between controls and treated samples, with 2–3-fold changes for *FLJ35767*, *SYCP1* (only in the CTD group), *WISP2*, and *CST9L* (only in the CT group) and about 2-fold up-regulation of *DDR1* in both groups. Quantitative RT-PCR analysis (Figure 3A) on the complete set of biopsy RNA samples (Walton et al, 2006) showed degrees of down-regulation similar to those in the arrays for *INSL3* ($P < .001$), *SYCP1* ($P < .01$), *WISP2* ($P < .05$), and *FLJ35767* ($P < .01$) in both treatment groups. *FLJ35767* was the only gene identified for which expression was lower in the CTD group than in the CT group ($P = .03$). *INSL3* was lower in suppressors than nonsuppressors in the CT group (18.7 ± 3.5% compared with 36.5 ± 0.6% relative to *RPL32*, $P < .012$) but not in the CTD group. On the basis of qRT-PCR, *CST9L* expression was actually increased to a small but significant extent in both treatment groups compared with controls, whereas *DDR1* showed no significant change.

The remaining genes identified as down-regulated from this first stage of testing were *CT45-1* and *-3*, closely related cancer-testis antigens; *PAPSS*, 3′-phosphoadenosine 5′-phosphosulfate synthase 2, which is involved in purine metabolism; and *DPEP3* (*MDB3*), a

Table 2. *Biological processes statistically overrepresented in the testis*

Gene Ontology Term (Biological Processes)	Count	%[a]	Enrichment *P*
Growth	20	0.37	.0055
Regulation of growth	44	0.81	.0048
Regulation of enzyme activity	71	1.31	.0414
Regulation of cellular process	842	15.57	.0314
Regulation of cell size	16	0.30	.0388
Death	159	2.94	.0430
Sexual reproduction	87	1.61	2.41×10^{-8}
Negative regulation of biological process	221	4.09	.0018
Cellular physiological process	2708	50.07	7.02×10^{-67}
Cellular morphogenesis	16	0.30	.0388
Metabolism	2151	39.77	1.45×10^{-41}
Cell organization and biogenesis	16	0.30	.0388
Cell growth	58	1.07	.000112
Locomotion	84	1.55	.000087
Localization	756	13.98	1.65×10^{-6}
Response to endogenous stimulus	91	1.68	.0000879

[a] Number of gene hits out of 5824 genes in the filtered set.

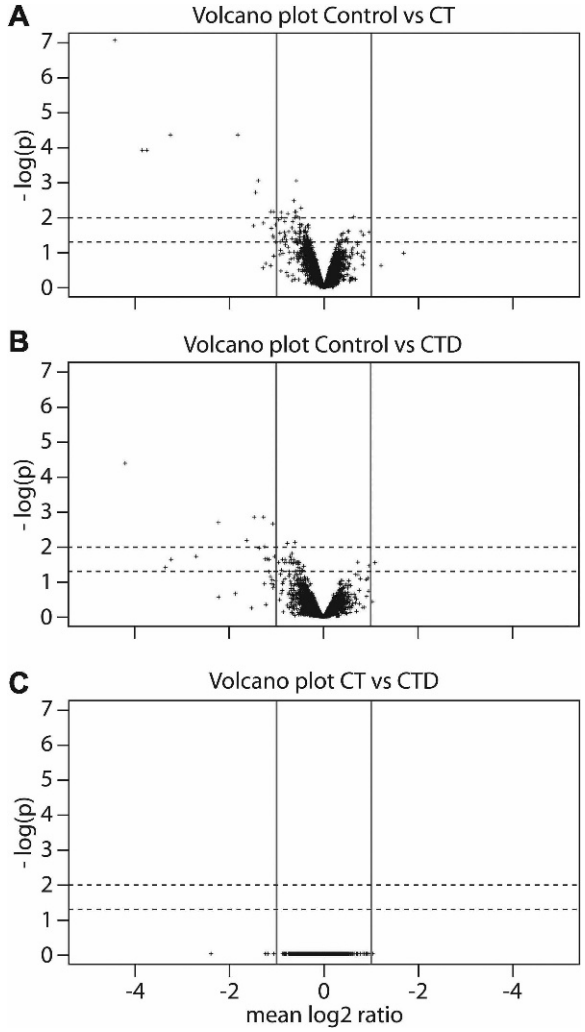

Figure 1. Volcano plots of the filtered gene set representing both differential expression and its statistical significance. The *x*-axis shows differential expression between 2 groups, and the *y*-axis shows the statistical significance. Genes above the lower horizontal dotted line have an adjusted $P \leq .05$. Genes above the upper horizontal dotted line have an adjusted $P \leq .01$. Differences in expression between CT (cetrorelix and testosterone) and CTD (cetrorelix, testosterone, and desogestrel) groups were not significant. Compared with control samples, only a small number of genes showed differential expression and statistical significance.

testis-specific dipeptidase involved in glutathione degradation (Habib et al, 2003).

Analysis by Degree of Spermatogenic Suppression

Stage 2 testing (excluding men showing poor suppression of spermatogenesis) identified a further 5 genes (*SC4MOL*, *GSTA2*, *CHGA*, *PRPS2*, *CYB5*; Table 3, upper section) that were significantly down-regulated at least 2-fold in the CT group relative to controls. Similar down-regulation in the CTD group did not reach significance, but there was no difference between CT and CTD groups.

No statistically significant differences were identified by the stage 3 test comparing sperm nonsuppressors and suppressors in the 2 treatment groups. However, 4 genes (*PROK1*, *PGRMC1*, *APOE*, *RHOB*; Table 3, lower section) that had shown small (<2-fold) but statistically significant reduction in expression between controls and the CT group, but not the CTD group, after stage 1 testing also showed slightly higher expression in CTD sperm suppressed samples compared with nonsuppressed samples. Although these array-based differences were not significant, this potentially intriguing effect of added progestogen was investigated further by qRT-PCR analysis on the full set of 29 samples (Figure 3B). This demonstrated that *PROK1*, *PGRMC1*, and *RHOB* in fact showed significant 2–3-fold down-regulation between control and all treated samples from both groups, with no difference between nonsuppressors and suppressors in either. *APOE* showed a 2–3-fold down-regulation that was only significant in the CT group ($P = .04$), as in the arrays, but did not differ significantly between nonsuppressors and suppressors within the CTD group ($P = .8$).

Given the dominance of GnRH suppression over progestogen-mediated effects and the absence of differences between the CT and CTD groups in terms of global gene expression, a final round of testing compared control samples with pooled CT and CTD groups. This identified no additional genes.

TAF4B is a germ cell–specific transcription factor that has recently been shown in the ovary to regulate expression of a number of genes, including *INHA* and *RHOB* (Geles et al, 2006). Because both *INHA* (Walton et al, 2006) and *RHOB* (this study) were down-regulated in the treatment groups, it seemed a likely possibility that it occurs through regulation of *TAF4B*. Although not identified in the array analysis (it neither passed the nonspecific filter nor showed an at least 2-fold change between groups), with the use of qRT-PCR (Figure 3C), we demonstrated a small (30%) but significant reduction in *TAF4B* expression in both treatment groups.

Clustering Analysis

Clustering analysis was also performed on the entire RMA-normalized dataset with Biolayout Express^{3D} (Goldovsky et al, 2005; Freeman et al, in press). The networks produced by the software consist of nodes that represent individual transcripts connected by edges representing similarities in their expression profiles across multiple conditions. This approach allows the rapid identification of biological relationships that could be missed by conventional analysis techniques (ie, statistical 2-sample tests). Several small cluster graphs and 1 major graph of genes that contained 19 out of the

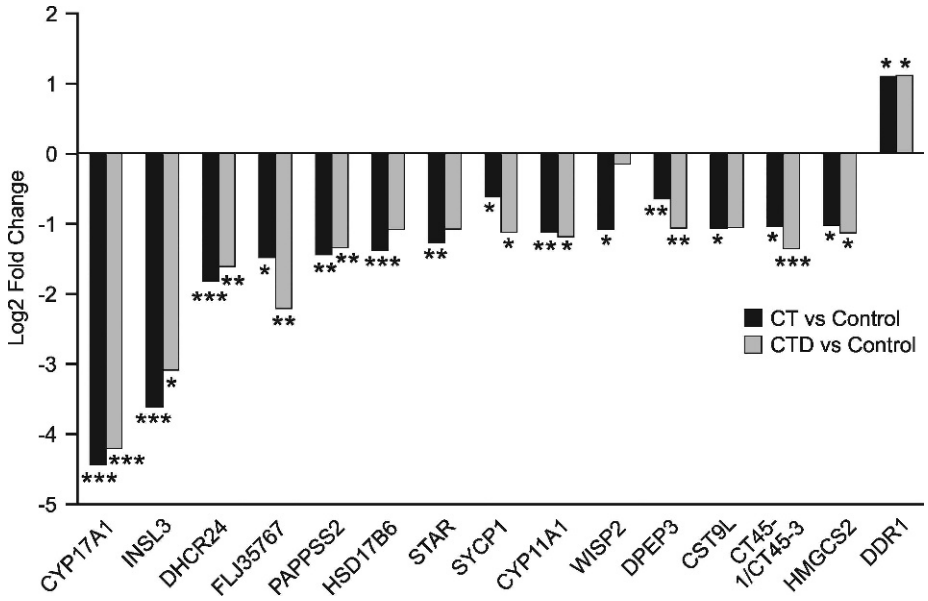

Figure 2. Graphical representation of $\log_2$-fold changes in expression in the CT (cetrorelix and testosterone; black bars) and CTD (cetrorelix, testosterone, and desogestrel; gray bars) groups relative to controls for each gene identified in the stage 1 test of array data. Bars below the line represent *n*-fold decreases, whereas those above the line represent increases in expression. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with controls.

20 probes from the original gene list were produced. Following clustering with MCL, the statistical hits ($\times 20$ probes) separated out into 2 distinct clusters. The first cluster (cluster 1, Table 4) consisted of 66 probes, including 4 of the original statistically significant probes (*CT45-1* [$\times 2$], *SYCP1*, and *DPEP3*). The second cluster (cluster 2, Table 5) consisted of 44 probes, including 9 of the original statistically significant probes (*INSL3* [$\times 3$], *DHCR24*, *PAPSS2*, *CYP11A1*, *STAR*, *CYP17A1*, and *HSD17B6*). With the use of DAVID pathway analysis software, cluster 1 showed enrichment for genes involved in spermatogenesis, male gamete generation, and gametogenesis, including early meiotic pathways (Table 6), whereas cluster 2 showed enrichment for genes involved in steroid, lipid, and cholesterol biosynthesis/metabolism (Table 7).

Rank Product Analysis

Finally, as an independent means of assessing differential gene expression, the original data were subjected to rank product analysis (Breitling et al, 2004). The gene list generated by this method (data not shown) was very similar to the expanded gene lists generated from Biolayout Express^{3D}, indicating that the additional genes identified are likely to have some biological significance. A summary of the notable genes identified in the screens, along with potential functions and sites of expression within the testis, is provided in Table 8.

Discussion

Microarray comparisons between biopsies of men treated with GnRH antagonist plus or minus progestogen and controls yielded a remarkably small subset of genes that were altered between the 3 groups, with most reflecting changes in Leydig cells. Cluster analysis suggests that the genes fall into 2 distinct groups, with one set of genes involved in gametogenesis and spermatogenesis and the other involved in steroid and cholesterol biosynthesis/metabolism. The significance of the biological functions in the original gene lists are increased in the expanded lists generated after cluster analysis, suggesting a genuine biological relationship between them. Further confirmation of the biological significance of these expanded gene lists comes from the very similar results obtained by the alternative rank product method (Breitling et al, 2004).

The striking homogeneity both between individuals and between treatment groups could paradoxically reflect heterogeneity within the testis. Marked histological variation in spermatogenic activity between nearby tubules has been observed in men treated with regimens similar to those here (McLachlan et al, 2002). Thus, the biopsies will contain tubules with varying suppression that are then averaged during processing, potentially leading to smaller apparent changes in gene expression within the tubule compartments. Sertoli cell and spermatogonial genes are also likely to be highly

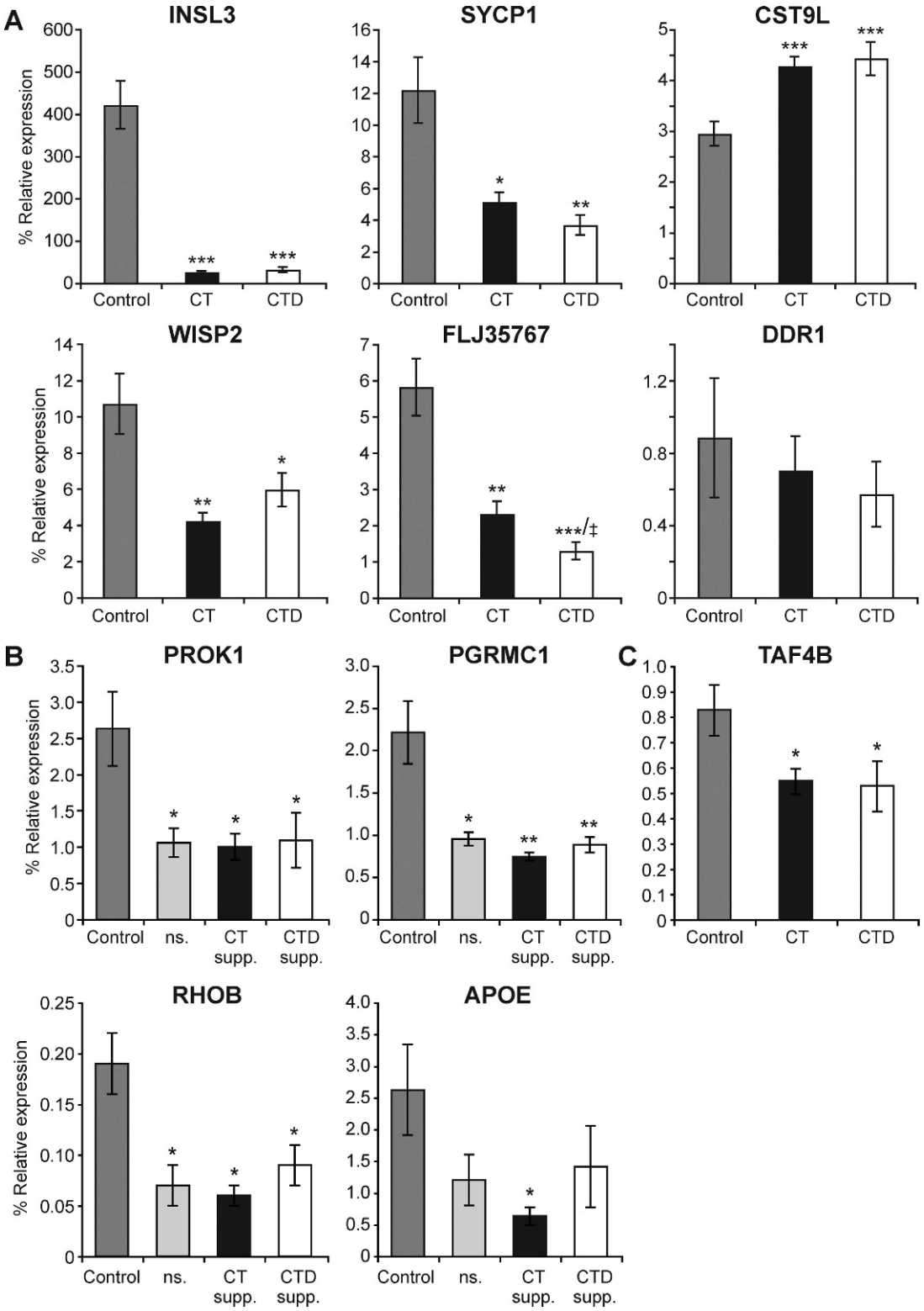

Figure 3. Quantitative reverse transcriptase polymerase chain reaction analysis of expression of **(A)** significant genes of interest identified after stage 1 testing, **(B)** genes identified as showing notable expression changes between suppressor (CT supp. and CTD supp.) and nonsuppressor (ns., light grey bars) subgroups after stage 3 testing, and **(C)** *TAF4B*, an associated transcription factor of *RHOB*, in controls (dark grey bars) and men in CT (cetrorelix and testosterone; black bars) and CTD groups (cetrorelix, testosterone, and desogestrel; white bars) ($\bar{x} \pm$ SEM). Gene expression is relative to *RPL32*. * $P < .05$, ** $P < .01$, *** $P < .001$ compared with controls; ‡ $P < .05$ CTD vs CT group (n = 7–10 per group except nonsuppressors, in which n = 5).

Table 3. *Log_2-fold changes in expression and P values of stage 2 and 3 genes of interest* [a]

		Stage 1 Testing				Stage 2 Testing			
		Control vs CT		Control vs CTD		Control vs CT[b]		Control vs CTD[b]	
Probe	Symbol	Log_2-Fold Change	*P*	Log_2-Fold Change	*P*	Log_2-Fold Change	*P*	Log_2-Fold Change	*P*
209146_at	***SC4MOL***	0.96	.011	1.01	.092	**1.04**	**.028**	0.98	0.141
203924_at	***GSTA2***	0.93	.05	0.73	.205	**1.15**	**.048**	0.7	0.254
204697_s_at	***CHGA***	0.93	.028	0.9	.061	**1.11**	**.028**	0.89	0.096
203401_at	***PRPS2***	0.9	.007	0.66	.221	**1.06**	**.008**	0.61	0.272
215726_s_at	***CYB5***	0.89	.01	0.82	.028	**1.01**	**.021**	0.81	0.061
						Stage 3 Testing CTD_{supp} vs ns Log_2-Fold Change			
229124_at	***PROK1***	**0.88**	**.044**	0.5	.445	**0.75**			
201120_s_at	***PGRMC1***	**0.83**	**.013**	0.69	.291	**0.51**			
203382_s_at	***APOE***	**0.8**	**.044**	0.4	.554	**0.81**			
212099_at	***RHOB***	**0.59**	**.009**	0.43	.47	**0.51**			

Abbreviations: CT, cetrorelix and testosterone; CTD, cetrorelix, testosterone, and desogestrel; ns, nonsuppressors; supp, suppressors.

[a] Data in bold are referred to in the text.

[b] Without nonsuppressors.

underrepresented in RNA from total testis biopsies because the major cellular component will be postmeiotic germ cells, thus reducing the sensitivity to changes in these cells. This is a limitation of the use of whole-testis biopsies. Meanwhile, more consistent changes will be observed in genes confined to Leydig cells and thus will be more pronounced. In addition, some genes might be expressed in many cell types but only regulated by gonadotropins in, for example, Leydig cells. This will lead to a robust expression level that partially masks changes in one cell type between control and treated samples.

Previously (Walton et al, 2006), we identified a small number of genes by qRT-PCR in which expression was reduced in at least 1 of the treatment groups, but only 1 of these (*CYP17A1*) overlapped with the genes identified here by array analysis. Probes for all of the other genes were present on the arrays, but in a number of cases (*SRD5A1*, *HSD3B2*, *MAGEA4*), their expression level was below the filtering threshold; therefore, they were excluded from the analysis. The remaining genes (*INHA*, *PEPP-2*, *ACRBP*) were present in the filtered gene set but did not show a greater than 2-fold change in hybridization and did not show statistical significance better then $P \leq .05$. Although a very useful tool, it is well known that arrays are less sensitive than qRT-PCR in detecting small changes in gene expression, especially with modest numbers of clinical samples, and many of the changes we observed by qRT-PCR were only around 2-fold and therefore close to the cut-off level. In addition, these genes are expressed in the tubule compartment so intertubule variation in suppression as mentioned above could have had an effect here.

Overall, the most significant changes identified were in expression of genes involved in cholesterol biosynthesis/metabolism and steroidogenesis, which are directly sensitive to the concentration of LH acting on Leydig cells. Also within this group, *SC4MOL*, which is involved in cholesterol biosynthesis (Li and Kaplan, 1996), and *CYB5*, the product of which binds and allosterically modulates CYP17 in Leydig cells (Dharia et al, 2004), showed small changes in expression. *APOE* has many functions, but its role in cholesterol homeostasis (Levi et al, 2005) could also be relevant in this context. The observation that *APOE* was significantly down-regulated in the CT but not the CTD group suggests that progestogens, known to regulate Leydig cell steroidogenesis (El-Hefnawy et al, 2000), might affect its expression.

The only other gene observed to show a dramatic reduction in expression was *INSL3*, which is also expressed in Leydig cells. Serum concentrations were not affected by hCG administration to normal men (Bay et al, 2005); however, LH suppression resulted in reduced INSL3 concentrations (Bay et al, 2006). *INSL3* is known to be induced by the transcription factor NR4A1 (Nur77) (Robert et al, 2006), which is expressed at low levels but is rapidly and robustly induced by LH and cyclic adenosine monophosphate analogs and is thought to mediate the dynamic expression of *INSL3* in response to LH (Robert et al, 2006). The steroidogenic enzyme genes *3BHSD2*, *CYP17A1*, and *STAR* are also activated by NR4A1 (Zhang and Mellon, 1997; Martin and Tremblay, 2005). Thus, reduced LH will lead to reduced NR4A1 (expression of which was below the threshold to detect changes in the array), which will produce lower levels

Table 4. *Cluster analysis: expanded list of genes showing expression profiles similar to genes identified as statistically significant—cluster 1*[a]

Probe	Gene Symbol	Probe	Gene Symbol
1553285_s_at	*RAD9B*	213404_s_at	*RHEB*
1553599_a_at	*SYCP3*	**216917_s_at**	***SYCP1***
1554768_a_at	*MAD2L1*	218370_s_at	*S100PBP*
1556554_at	*TRIM50*	218683_at	*PTBP2*
1560912_at	*LOC389043*	218768_at	*NUP107*
1564688_a_at	*RAD9B*	220126_at	*TSP50*
1567912_s_at	***CT45-1***	**220179_at**	***DPEP3***
200996_at	*ACTR3*	221035_s_at	*TEX14*
201291_s_at	*TOP2A*	221259_s_at	*TEX11*
201292_at	*TOP2A*	221521_s_at	*GINS2*
201614_s_at	*RUVBL1*	222848_at	*CENPK*
201697_s_at	*DNMT1*	223542_at	*ANKRD32*
201738_at	*EIF1B*	223861_at	*HORMAD1*
201965_s_at	*SETX*	223906_s_at	*TEX101*
202033_s_at	*RB1CC1*	225783_at	*UBE2F*
203062_s_at	*MDC1*	226779_at	No class
203362_s_at	*MAD2L1*	227085_at	*H2AFV*
203755_at	*BUB1B*	227818_at	*CCDC21*
203947_at	*CSTF3*	227894_at	*WDR90*
204033_at	*TRIP13*	228171_s_at	*PLEKHG4*
204900_x_at	*SAP30*	230236_at	*LOC732253*
205292_s_at	*HNRPA2B1*	231210_at	*LOC283129*
205995_x_at	*IQCB1*	233514_x_at	*TEX11*
206052_s_at	*SLBP*	234296_s_at	*TEX11*
207909_x_at	*DAZ1/DAZ3/DAZ2/DAZ4*	235557_at	*LOC150763*
208282_x_at	*DAZ1/DAZ3/DAZ2/DAZ4*	**235700_at**	***CT45-1***
208382_s_at	*DMC1*	236811_at	*DMRTC2*
208821_at	*SNRPB*	236852_at	*FBXO43*
209674_at	*CRY1*	237170_at	...
210410_s_at	*MSH5*	237781_at	*HORMAD2*
211707_s_at	*IQCB1*	238508_at	*DBF4B*
211953_s_at	*RANBP5*	239824_s_at	*TMEM107*
212666_at	*SMURF1*	240546_at	*LOC389043*

[a] Probes in bold represent those identified in the initial analysis as statistically significant, with novel probe hits in normal font.

Table 5. *Cluster analysis: expanded list of genes showing expression profiles similar to genes identified as statistically significant—cluster 2*[a]

Probe	Gene Symbol	Probe	Gene Symbol
1553594_a_at	***INSL3***	209146_at	*SC4MOL*
200862_at	***DHCR24***	209366_x_at	*CYB5A*
200969_at	*SERP1*	210130_s_at	*TM7SF2*
201120_s_at	*PGRMC1*	210367_s_at	*PTGES*
201121_s_at	*PGRMC1*	212297_at	*ATP13A3*
201790_s_at	*DHCR7*	**214400_at**	***INSL3***
201791_s_at	*DHCR7*	**214572_s_at**	***INSL3***
202068_s_at	*LDLR*	215506_s_at	*DIRAS3*
202539_s_at	*HMGCR*	215726_s_at	*CYB5A*
202540_s_at	*HMGCR*	217014_s_at	*AZGP1*
203058_s_at	*PAPSS2*	222102_at	*GSTA3*
203060_s_at	***PAPSS2***	223734_at	*OSAP*
203924_at	*GSTA1*	225305_at	*SLC25A29*
204309_at	***CYP11A1***	225373_at	*C10orf54*
204526_s_at	*TBC1D8*	226613_at	*LOC652968*
204548_at	***STAR***	227506_at	*SLC16A9*
205428_s_at	*CALB2*	228754_at	*SLC6A6*
205502_at	***CYP17A1***	228885_at	*MAMDC2*
205700_at	***HSD17B6***	229789_at	*TIGD3*
207695_s_at	*IGSF1*	230352_at	*PRPS2*
207843_x_at	*CYB5A*	231948_s_at	*UBE2F*
207935_s_at	*KRT13*	37512_at	*HSD17B6*

[a] Probes in bold represent those identified in the initial analysis as statistically significant, with novel probe hits in normal type.

of *3BHSD2*, *CYP17A1*, *STAR*, and *INSL3*, all of which we have observed here or reported previously (Walton et al, 2006). Recently, it has been demonstrated that serum INSL3 concentrations are higher in nonsuppressors than suppressors on male contraceptive regimens (Amory et al, 2007). In this context, it is notable that we also observed higher levels of *INSL3* transcripts in nonsuppressors compared with suppressors in the CT group (but not the CTD group).

The receptor for INSL3, LGR8, is expressed in both Leydig cells and in meiotic and postmeiotic germ cells but not in Sertoli or peritubular cells (Kawamura et al, 2004; Anand-Ivell et al, 2006). *Insl3* knockout mice show cryptorchidism and INSL3 regulates apoptosis in germ cells in both male and female mice (Kawamura et al, 2004). Because germ cells do not express gonadotropin receptors, germ cell genes cannot be directly regulated by gonadotropins. INSL3 might therefore provide a link between Leydig cells and spermatogenesis, which could contribute to the changes in gene expression levels observed here in the germ cell compartment, such as for *SYCP1*, a gene essential for chromosomal synapsis at prophase I of meiosis and detected in the stage 1 screen, as well as *SYCP3*, *DMC1*, *DAZ1*,*MSH5*, *TEX11*, *TEX14*, *TEX101*, and *HORMAD1* and *2*, which were identified from the cluster analysis. We detected previously (Walton et al, 2006) a reduced expression of *ACRBP*, a spermatocyte-specific marker in the CTD and suppressor groups, and of *MAGEA4*, which is present in spermatogonia and primary spermatocytes, in the suppressor group. In contrast, expression of *PRM1*, a postmeiotic germ cell marker, remained unchanged. Together with these new early meiotic genes, this further implicates an effect of the treatments on early stages of spermatogenesis. What is currently less clear is whether the reduced expression of germ cell markers after treatment is due to small changes in gene expression within germ cells or loss of a percentage of early germ cells, as has been observed at the histological level under similar regimens (McLachlan et al, 2002). Both would yield the same result. This could be resolved by histochemical analysis of biopsies for these gene products, but insufficient material was available to us for this.

Table 6. *Enrichment of gene ontologies in expanded gene lists: DAVID pathway analysis of cluster 1*[a]

Gene Ontology Term	No. of Genes	%[b]	Enrichment *P*	Gene Symbol
DNA metabolism	11	18.64	2.30×10^{-6}	*MSH5, DNMT1, TOP2A, GINS2, DMC1, RUVBL1, SYCP1, CRY1, H2AFV, MDC1, RAD9B*
Biopolymer metabolism	20	33.90	4.81×10^{-6}	*HNRPA2B1, SLBP, GINS2, DMC1, RUVBL1, H2AFV, BUB1B, UBE2F, MSH5, SNRPB, DNMT1t, TOP2A, CRY1, SYCP1, TEX14, SMURF1, MDC1, CSTF3, RAD9B, PTBP2*
Nucleobase, nucleoside, nucleotide, and nucleic acid metabolism	20	33.90	1.19×10^{-4}	*HNRPA2B1, SLBP, GINS2, TRIP13,DMC1, RUVBL1, H2AFV, RB1CC1, MSH5, SNRPB, DNMT1, TOP2A, CRY1, SYCP1, SAP30, MDC1, CSTF3, RAD9B, DMRTC2, PTBP2*
Macromolecule metabolism	23	38.98	1.68×10^{-4}	*HNRPA2B1, SLBP, GINS2, DMC1, RUVBL1t TSP50, H2AFV, EIF1B, BUB1B, UBE2F,MSH5, SNRPB, DNMT1, TOP2A, TEX14, CRY1, SYCP1, SMURF1, MDC1, CSTF3, RAD9B, PTBP2, DPEP3*
Male gamete generation	**5**	**8.47**	$\mathbf{3.33 \times 10^{-4}}$	***DAZ1/DAZ3/DAZ2/DAZ4, DMC1, RUVBL1, SYCP1***
Spermatogenesis	**5**	**8.47**	$\mathbf{3.33 \times 10^{-4}}$	***DAZ1/DAZ3/DAZ2/DAZ4, DMC1, RUVBL1, SYCP1***
Gametogenesis	**5**	**8.47**	$\mathbf{6.98 \times 10^{-4}}$	***DAZ1/DAZ3/DAZ2/DAZ4, DMC1, RUVBL1, SYCP1***
DNA recombination	4	6.78	7.34×10^{-4}	*MSH5, DMC1, RUVBL1, SYCP1*
Meiotic recombination	**3**	**5.08**	$\mathbf{8.87 \times 10^{-4}}$	***MSH5, DMC1, SYCP1***
M phase	5	8.47	.001109199	*MSH5, DMC1, SYCP1, MAD2L1, MDC1, BUB1B*
Cell cycle	8	13.56	.001228391	*MSH5, DMC1, SYCP1, RB1CC1, MAD2L1, MDC1, RAD9B, BUB1B*
Meiosis I	**3**	**5.08**	**.001420763**	***MSH5, DMC1, SYCP***
Sexual reproduction	**5**	**8.47**	**.001557728**	***DAZ1/DAZ3/DAZ2/DAZ4, DMC1,RUVBL1, SYCP1***
DNA repair	5	8.47	.002119799	*MSH5, TOP2A, CRY1, MAD2L1, RAD9B*
Response to DNA damage stimulus	5	8.47	.003055314	*MSH5, TOP2A, CRY1, MAD2L1, RAD9B*
Response to endogenous stimulus	5	8.47	.003832754	*MSH5, TOP2A, CRY1, MAD2L1, RAD9B*
Reproduction	**5**	**8.47**	**.003832754**	***DAZ1/DAZ3/DAZ2/DAZ4, DMC1, RUVBL1, SYCP1***
Primary metabolism	27	45.76	.005542536	*GINS2, DMC1, H2AFV, RB1CC1, BUB1B, UBE2F, MSH5, TOP2A, DNMT1, CRY1, TEX14, SAP30, MDC1, PTBP2, HNRPA2B1, TRIP13, SLBP, RUVBL1, TSP50, EIF1B, DNMT1, SNRPB, SMURF1, CSTF3, RAD9B, DMRTC2, DPEP3*
Meiosis	**3**	**5.08**	**.005605975**	***MSH5, DMC1, SYCP1***
M phase of meiotic cell cycle	**3**	**5.08**	**.005605975**	***MSH5, DMC1, SYCP1***
Meiotic cell cycle	**3**	**5.08**	**.005835985**	***MSH5, DMC1, SYCP1***
Cellular metabolism	27	45.76	.009990932	*GINS2, DMC1, H2AFV, RB1CC1, BUB1B, UBE2F, MSH5, TOP2A, SYCP1, CRY1, TEX14, SAP30, MDC1, PTBP2, HNRPA2B1, TRIP13, SLBP, RUVBL1, TSP50, EIF1B, DNMT1, SNRPB, SMURF1, CSTF3, RAD9B, DMRTC2, DPEP3*
RNA processing	5	8.47	.010524072	*HNRPA2B1, SNRPB, SLBP, CSTF3, PTBP2*
Metabolism	28	47.46	.012775914	*GINS2, DMC1, H2AFV, RB1CC1, BUB1B, UBE2F, MSH5, TOP2A, SYCP1, CRY1t, TEX14, SAP30, MDC1, PTBP2, HNRPA2B1, TRIP13, SLBP, RUVBL1, TSP50, EIF1B, DNMT1, SNRPB, LOC150763, SMURF1, CSTF3, RAD9B, DMRTC2, DPEP3*
mRNA processing	4	6.78	.012824716	*SNRPB, SLBP, CSTF3, PTBP2*
mRNA metabolism	4	6.78	.01788918	*SNRPB, SLBP, CSTF3, PTBP2*
RNA metabolism	5	8.47	.022531644	*HNRPA2B1, SNRPB, SLBP, CSTF3, PTBP2*
RNA 3′-end processing	2	3.39	.025443074	*SLBP, CSTF3*
mRNA 3′-end processing	2	3.39	.025443074	*SLBP, CSTF3*
Cellular physiological process	32	54.24	.029135935	*NUP107, GINS2, DMC1, H2AFV, RB1CC1, RANBP5, MAD2L1, BUB1B,UBE2F, MSH5, TOP2A, TOP2A, SYCP1, CRY1, TEX14, SAP30, MDC1, PTBP2, SETX, HNRPA2B1, SLBP, TRIP13, RUVBL1, TSP50, EIF1B, SNRPB, DNMT1, SMURF1, CSTF3, RAD9B, DMRTC2, ACTR3, DPEP3*
Mitotic checkpoint	2	3.39	.032272232	*MAD2L1, MAD2L1, BUB1B*

[a] Major relevant pathways and genes are marked in bold.
[b] Out of a total number of 59 separate genes in the cluster.

Table 7. *Enrichment of gene ontologies in expanded gene lists: DAVID pathway analysis of cluster 2*[a]

Gene Ontology Term	No. of Genes	%[a]	Enrichment *P*	Gene Symbol
Steroid biosynthesis	9	25.71	1.33×10^{-12}	*HSD17B6, HMGCR, DHCR24, SC4MOL, CYP11A1, STAR, TM7SF2, CYP17A1, DHCR7*
Steroid metabolism	10	28.57	1.22×10^{-11}	*HSD17B6, HMGCR, DHCR24,SC4MOL, CYP11A1, LDLR, STAR, TM7SF2, CYP17A1, DHCR7*
Sterol metabolism	7	20.00	5.17×10^{-9}	*HMGCR, DHCR24, SC4MOL, CYP11A1, LDLR, TM7SF2, DHCR7*
Lipid biosynthesis	9	25.71	7.56×10^{-9}	*HSD17B6, HMGCR, DHCR24, SC4MOL, CYP11A1, STAR, TM7SF2, CYP17A1, DHCR7*
Lipid metabolism	12	34.29	1.19×10^{-8}	*HSD17B6, HMGCR, DHCR24, SC4MOL, CYP11A1, LDLR, PTGES, STAR, TM7SF2, CYP17A1, AZGP1, DHCR7*
Cellular lipid metabolism	11	31.43	1.70×10^{-8}	*HSD17B6, HMGCR, DHCR24, SC4MOL, CYP11A1, LDLR, PTGES, STAR, TM7SF2, CYP17A1, DHCR7*
Cholesterol metabolism	6	17.14	1.34×10^{-7}	*HMGCR, DHCR24, CYP11A1, LDLR, TM7SF2, DHCR7*
Sterol biosynthesis	5	14.29	1.94×10^{-7}	*HMGCR, DHCR24, SC4MOL, TM7SF2, DHCR7*
Cholesterol biosynthesis	4	11.43	9.84×10^{-6}	*HMGCR, DHCR24, TM7SF2, DHCR7*
Alcohol metabolism	7	20.00	1.78×10^{-5}	*HMGCR, DHCR24, SC4MOL, CYP11A1, LDLR, TM7SF2, DHCR7*
Hormone biosynthesis	4	11.43	2.08×10^{-5}	*HSD17B6, CYP11A1, STAR, CYP17A1*
Biosynthesis	12	34.29	6.25E-05	*HSD17B6, HMGCR, DHCR24, SC4MOL, CYP11A1, PRPS2, LDLR, SERP1, STAR, TM7SF2, CYP17A1, DHCR7*
Hormone metabolism	4	11.43	1.14×10^{-4}	*HSD17B6, CYP11A1, STAR, CYP17A1*
C21-steroid hormone biosynthesis	3	8.57	1.97×10^{-4}	*CYP11A1, STAR, CYP17A1*
C21-steroid hormone metabolism	3	8.57	2.37×10^{-4}	*CYP11A1, STAR, CYP17A1*
Androgen biosynthesis	2	5.71	.005804242	*HSD17B6, CYP11A1*
Androgen metabolism	2	5.71	.01157595	*HSD17B6, 2CYP11A1*
Cellular biosynthesis	8	22.86	.012738723	*HSD17B6, HMGCR, CYP11A1, PRPS2, LDLR, PGRMC1, STAR, CYP17A1*
Metabolism	23	65.71	.032542447	*CYB5A, GSTA3, GSTA1, HMGCR, SC4MOL, LDLR, PRPS2, ATP13A3, CYP17A1, AZGP1, UBE2F, TIGD3, HSD17B6, PAPSS2, DHCR24, CYP11A1, SLC6A6, PTGES, STAR,PGRMC1, TM7SF2, DIRAS3, DHCR7*

[a] Out of a total number of 35 separate genes in the cluster.

Further genes known to be expressed by Leydig cells and showing significant down-regulation during gonadotropin suppression were *CHGA* and *PROK1*. Expression of *CHGA* correlates with LH levels (Ortega et al, 2004). However, in the bovine testis, *CHGA* expression is also high in spermatogonia (Payan-Carreira et al, 2006) with a reducing gradient of expression with maturation up to the round spermatid stage. Elongating and elongated spermatids do not express *CHGA*. Thus, reduced expression of *CHGA* in the treated men might also reflect suppression of spermatogenesis. *PROK1*, a prokineticin, encodes EG-VEGF, which is expressed in Leydig cells and is thought to be involved in the integrity and proliferation of blood vessels in the testis (Samson et al, 2004). Knock-out in mice of 1 of its receptors, *Pkr2*, causes severe atrophy of the reproductive system (Matsumoto et al, 2006), an effect partly due to low concentrations of gonadotropins associated with lack of GnRH but also thought to have a direct testicular component. In the ovary, expression of *PROK1* positively correlates with granulosa cell steroidogenesis (Kisliouk et al, 2003).

PGRMC1 (*HPR6*) encodes a component of the putative membrane progesterone receptor and is able to activate cytochrome P450 enzymes (Crudden et al, 2005). A reduction in its expression might therefore contribute to reduced expression of such enzymes. The array data suggested suppression in the CT but not CTD groups, but qRT-PCR analysis demonstrated similar suppression in both groups.

Although small changes in expression could simply be due to loss of a proportion of some germ cell types, alteration in the dynamics of Sertoli cell–germ cell adherens junctions was suggested by changes in expression of *RHOB*, a member of the *RAS* gene family (Lui et al, 2003). The breakdown of adherens junctions is necessary for germ cells to detach from Sertoli cells at spermiation, suggesting the possibility that reduction in expression of *RHOB* and its associated regulatory transcription factor *TAF4B* (Geles et al, 2006) could

Table 8. *Summary of genes identified*

Gene Symbol	Full Gene Name	Gene Ontology Function	Cellular Localization[a]
CYP17A1	Cytochrome P450c17-alpha-hydroxylase/ 17,20-lyase	C21-steroid hormone biosynthesis	Leydig cells
CYP11A1	Cytochrome P450c11 side-chain cleavage	C21-steroid hormone biosynthesis	Leydig cells
HSD17B6	Hydroxysteroid (17-beta) dehydrogenase 6 homolog	Androgen biosynthesis	Leydig cells
STAR	Steroidogenic acute regulatory protein	Steroid biosynthesis	Leydig and Sertoli cells
DHCR24	24-Dehydrocholesterol reductase	Cholesterol biosynthesis, MAP kinase phosphatise activity	Spermatogonia, Sertoli and Leydig cells
DPEP3	Dipeptidase 3	Membrane dipeptidase activity	Spermatocytes, Leydig cells
GSTA2	Glutathione S-transferase A2	Glutathione transferase activity	Spermatogonia
PRPS2	Phosphoribosyl pyrophosphate synthetase 2	Kinase activity; nucleoside, nucleotide, and nucleic acid biosynthesis; and metabolism	Sertoli cells, spermatogonia, Leydig cells
PAPSS	3′-Phosphoadenosine 5′-phosphosulfate synthase	Adenylylsulfate kinase activity; ATP binding; nucleobase, nucleoside, nucleotide, and nucleic acid metabolism; nucleotidyltransferase activity	Germ cells, Leydig cells
HMGCS2	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2 (mitochondrial)	Cholesterol biosynthesis	Spermatogonia
CT45-1, 3	Cancer/testis antigen CT45-1/3	Unknown	Testis
INSL3	Insulinlike 3 (Leydig cell)	Cell-cell signaling, hormone activity, insulin receptor binding, spermatogenesis	Leydig cells
SC4MOL	Sterol-C4-methyl oxidaselike	Steroid metabolism, sterol biosynthesis, C-4 methylsterol oxidase activity	Sertoli cells, spermatogonia
CYB5	Cytochrome b_5	Cytochrome *c* oxidase activity, fatty acid metabolism, transport	Spermatogonia, Leydig cells
APOE	Apolipoprotein E	Cholesterol homeostasis, induction of apoptosis, lipid transport, lipoprotein metabolism, antioxidant activity	Sertoli cells, spermatogonia
TAF4B	TAF4b RNA polymerase II, TATA box binding protein (TBP)–associated factor, 105 kd	Transcription factor activity	Spermatogonia, Sertoli and Leydig cells
SYCP1	Synaptonemal complex protein 1	Synaptonemal complex formation, DNA binding, meiotic recombination, spermatogenesis	Spermatogonia, spermatocytes
SYCP3	Synaptonemal complex protein 3	Synaptonemal complex formation, DNA binding, meiotic recombination, spermatogenesis	Spermatocytes
DMC1	DMC1 dosage suppressor of mck1 homolog	ATP binding, damaged DNA binding, DNA repair, meiotic recombination, spermatogenesis	Germ cells, Leydig cells
DAZL	Deleted in azoospermialike	Cell differentiation, nucleotide binding, regulation of translation, RNA binding, spermatogenesis	Spermatogonia, spermatocytes
MSH5	mutS Homolog 5		
TEX11	Testis expressed 11	Protein binding	Spermatogonia, Leydig cells
TEX14	Testis expressed 14	Nucleotide binding, protein amino acid phosphorylation, protein kinase activity, transferase activity	Germ cells, Leydig cells
TEX101	Testis expressed 101	Receptor activity	Spermatocytes, Leydig cells
HORMAD1	HORMA domain containing 1	DNA-binding HORMA	Spermatocytes, Leydig cells
HORMAD2	HORMA domain containing 2	DNA-binding HORMA	Germ cells, Leydig cells
CHGA	Chromogranin A	Calcium ion binding	Germ cells
PROK1	Prokineticin 1, (EG-VEGF)	Growth factor activity	Germ cells
PGRMC1	Progesterone receptor membrane component 1	Receptor activity, steroid binding	Spermatogonia, Leydig cells
RHOB	*ras* Homolog gene family, member B	Cell adhesion, cell differentiation, nucleotide binding, protein binding, protein transport, transformed cell apoptosis	Spermatogonia, Sertoli and Leydig cells

Table 8. *Continued*

Gene Symbol	Full Gene Name	Gene Ontology Function	Cellular Localization[a]
WISP2	WNT1-inducible signaling pathway protein 2	Cell adhesion, cell-cell signaling, insulinlike growth factor binding, protein binding, regulation of cell growth, signal transduction	Leydig cells
CST9L	Cystatin 9-like (mouse)	Cysteine protease inhibitor activity	Germ cells
FLJ35767	FLJ35767 protein (similar to mouse Tex19)	Unknown	

[a] Cellular localization based on published data on human and/or mouse homologs as indicated in the text or in searches of the GermOnline database of the transcriptome of male gametogenesis (Chalmel et al, 2007), available at http://www.germonline.org/index.html.

block this process as a mechanism for the failure of spermiation observed during male contraceptive regimens (Matthiesson and McLachlan, 2006). *RhoB* expression at adherens junctions is increased by 1-(2, 4-dichlorobenzyl)-indazole-3-carbohydrazide (adjudin), which interferes with adhesion of spermatids and spermatocytes to Sertoli cells, and hence their maturation, and is a promising approach to reversible male contraception (Lui et al, 2003; Mruk et al, 2006). The *WNT1* pathway is also involved in the regulation of adherens junctions (Wechezak and Coan, 2003), and *WISP2*, a component of the *WNT1* signaling pathway (Banerjee et al, 2003), was reduced in both treatment groups. A further potential gene associated with regulation of adherens junctions and the only gene identified as increased in both treatment groups is *CST9L*, a cysteine protease inhibitor (Siu and Cheng, 2004).

Of the remaining genes in which levels were observed to fall in the treated groups, *PAPSS* and *PRPS2* are genes involved in nucleotide metabolism, and *GSTA2* and *DPEP3* have a function in glutathione metabolism. Both of these biological processes were found to be statistically overrepresented in the testis when higher level gene ontology and KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis was performed on the expressed gene set ($P = .03$ and .05, respectively), indicating their importance in the testis.

Very few genes were differentially suppressed between the 2 treatment groups. Some were only significantly different from controls in one group (eg, CT group: *APOE*, *HSD17B6*, *STAR*, *PRPS2*, *GSTA2*; CTD group: *SYCP1*, *DPEP3*), but qPCR analysis showed significantly different expression in CT compared with CTD in only 1 gene, *FLJ35767*. This is an expressed sequence tag (EST) isolated from a human testis library in which the predicted coding sequence shows similarity to 2 mouse proteins, Tex19 and a hypothetical protein. *Tex19* is expressed in spermatogonia (Wang et al, 2001). The finding that *FLJ35767* is down-regulated further by desogestrel than by a GnRH antagonist and testosterone alone suggests a progestogenic effect, as we have previously reported for *SRD5A1* (Walton et al, 2006). This provides further evidence for direct progestogenic effects on the testis. However, it is striking that only 1 such gene was identified in this study, indicating that it might be of limited biological significance. The absence of any significant differences between suppressors and nonsuppressors (other than for *INSL3*) within the treatment groups was disappointing. Given that the data were so tight between individuals, it seems likely that an array approach will require more refined tissue sampling to be of value in this key issue.

In this study, we identify the 10% of genes most highly expressed in the human testis. Only a small subset of genes was observed to show changes in expression after 4 weeks of gonadotropin suppression. Functional grouping indicates that genes associated with steroidogenesis are most markedly regulated by short-term gonadotropin suppression, with other Leydig cell genes also highly regulated, most notably *INSL3*. *INSL3* might be a candidate for future contraceptive targeting. Within the tubule compartment, genes involved in early meiosis showed reduced expression, with regulation of a number of genes that might be involved with adherens junctions also identified. Such changes could underlie the observation that disruption of spermiation is an early component of the response to gonadotropin withdrawal. Future studies examining histological expression of these genes should help define whether they are down-regulated in individual cells or whether certain early germ cell stages expressing them are lost because of a lack of gonadotropin support.

Acknowledgments

We are grateful to Ann Kerr for her assistance with patient recruitment, to the staff of the vasectomy clinic at the Edinburgh Well Woman clinic for their organizational skills, and to Mr Ian Wallace, FRCS, for performing the testicular biopsies without complication.

References

Amory JK, Page ST, Anawalt BD, Coviello AD, Matsumoto AM, Bremner WJ. Elevated end-of-treatment serum INSL3 is associated with failure to completely suppress spermatogenesis in men receiving male hormonal contraception. *J Androl*. 2007;28:548–554.

Anand-Ivell RJ, Relan V, Balvers M, Coiffec-Dorval I, Fritsch M, Bathgate RA, Ivell R. Expression of the insulin-like peptide 3 (INSL3) hormone-receptor (LGR8) system in the testis. *Biol Reprod*. 2006;74:945–953.

Anderson RA, Baird DT. Male contraception. *Endocr Rev*. 2002;23: 735–762.

Aquila S, Middea E, Catalano S, Marsic S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S, Ando S. Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Hum Reprod*. 2007;22:2594–2605.

Banerjee S, Saxena N, Sengupta K, Tawfik O, Mayo MS, Banerjee SK. WISP-2 gene in human breast cancer: estrogen and progesterone inducible expression and regulation of tumor cell proliferation. *Neoplasia*. 2003;5:63–73.

Bay K, Hartung S, Ivell R, Schumacher M, Jurgensen D, Jorgensen N, Holm M, Skakkebaek NE, Andersson AM. Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis. *J Clin Endocrinol Metab*. 2005;90:3410–3418.

Bay K, Matthiesson KL, McLachlan RI, Andersson AM. The effects of gonadotropin suppression and selective replacement on insulin-like factor 3 secretion in normal adult men. *J Clin Endocrinol Metab*. 2006;91:1108–1111.

Benjamini Y, Yekutieli D. Quantitative trait Loci analysis using the false discovery rate. *Genetics*. 2005;171:783–790.

Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett*. 2004; 573:83–92.

Chalmel F, Rolland AD, Niederhauser-Wiederkehr C, Chung SSW, Demougin P, Gattiker A, Moore J, Patard J-J, Wolgemuth DJ, Jégou B, Primig M. The conserved transcriptome in human and rodent male gametogenesis. *Proc Natl Acad Sci U S A*. 2007;104: 8346–8351.

Collins LL, Lee HJ, Chen YT, Chang M, Hsu HY, Yeh S, Chang C. The androgen receptor in spermatogenesis. *Cytogenet Genome Res*. 2003;103:299–301.

Critchley HO, Robertson KA, Forster T, Henderson TA, Williams AR, Ghazal P. Gene expression profiling of mid to late secretory phase endometrial biopsies from women with menstrual complaint. *Am J Obstet Gynecol*. 2006;195:406, e1–16.

Crudden G, Loesel R, Craven RJ. Overexpression of the cytochrome p450 activator hpr6 (heme-1 domain protein/human progesterone receptor) in tumors. *Tumour Biol*. 2005;26:142–146.

Dharia S, Slane A, Jian M, Conner M, Conley AJ, Parker CRJr. Colocalization of P450c17 and cytochrome b5 in androgen-synthesizing tissues of the human. *Biol Reprod*. 2004;71: 83–88.

El-Hefnawy T, Manna PR, Luconi M, Baldi E, Slotte JP, Huhtaniemi I. Progesterone action in a murine Leydig tumor cell line (mLTC-1), possibly through a nonclassical receptor type. *Endocrinology*. 2000;141:247–255.

Freeman TC, Goldovsky L, Brosch M, van Dongen S, Mazière P, Grocock RJ, Freilich S, Thornton J, Enright AJ. Construction, visualisation and clustering of transcription networks from microarray expression data. *PLoS Comput Biol*. 2007;3(10):e20.

Geles KG, Freiman RN, Liu WL, Zheng S, Voronina E, Tjian R. Cell-type–selective induction of c-jun by TAF4b directs ovarian-specific transcription networks. *Proc Natl Acad Sci U S A*. 2006;103: 2594–2599.

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 2004; 5:R80.

Goldovsky L, Cases I, Enright AJ, Ouzounis CA. BioLayout (Java): versatile network visualisation of structural and functional relationships. *Appl Bioinformatics*. 2005;4:71–74.

Habib GM, Shi ZZ, Cuevas AA, Lieberman MW. Identification of two additional members of the membrane-bound dipeptidase family. *FASEB J*. 2003;17:1313–1315.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4:249–264.

Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA, Hsueh AJ. Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc Natl Acad Sci U S A*. 2004;101:7323–7328.

Kisliouk T, Levy N, Hurwitz A, Meidan R. Presence and regulation of endocrine gland vascular endothelial growth factor/prokineticin-1 and its receptors in ovarian cells. *J Clin Endocrinol Metab*. 2003;88:3700–3707.

Levi O, Lutjohann D, Devir A, von Bergmann K, Hartmann T, Michaelson DM. Regulation of hippocampal cholesterol metabolism by apoE and environmental stimulation. *J Neurochem*. 2005;95:987–997.

Li L, Kaplan J. Characterization of yeast methyl sterol oxidase (ERG25) and identification of a human homologue. *J Biol Chem*. 1996;271:16927–16933.

Lui WY, Lee WM, Cheng CY. Sertoli–germ cell adherens junction dynamics in the testis are regulated by RhoB GTPase via the ROCK/LIMK signaling pathway. *Biol Reprod*. 2003;68:2189–2206.

Martin LJ, Tremblay JJ. The human 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase type 2 promoter is a novel target for the immediate early orphan nuclear receptor Nur77 in steroidogenic cells. *Endocrinology*. 2005;146:861–869.

Matsumoto AM, Karpas AE, Paulsen CA, Bremner WJ. Reinitiation of sperm production in gonadotropin-suppressed normal men by administration of follicle-stimulating hormone. *J Clin Invest*. 1983; 72:1005–1015.

Matsumoto AM, Paulsen CA, Bremner WJ. Stimulation of sperm production by human luteinizing hormone in gonadotropin-suppressed normal men. *J Clin Endocrinol Metab*. 1984;59:882–887.

Matsumoto S, Yamazaki C, Masumoto KH, Nagano M, Naito M, Soga T, Hiyama H, Matsumoto M, Takasaki J, Kamohara M, Matsuo A, Ishii H, Kobori M, Katoh M, Matsushime H, Furuichi K, Shigeyoshi Y. Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proc Natl Acad Sci U S A*. 2006;103:4140–4145.

Matthiesson KL, McLachlan RI. Male hormonal contraception: concept proven, product in sight? *Hum Reprod Update*. 2006;12: 463–482.

McLachlan RI, O'Donnell L, Stanton PG, Balourdos G, Frydenberg M, de Kretser DM, Robertson DM. Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones, and germ cell populations in normal young men. *J Clin Endocrinol Metab*. 2002;87:546–556.

McLachlan RI, Robertson DM, Pruysers E, Ugoni A, Matsumoto AM, Anawalt BD, Bremner WJ, Meriggiola C. Relationship between serum gonadotropins and spermatogenic suppression in men undergoing steroidal contraceptive treatment. *J Clin Endocrinol Metab*. 2004;89:142–149.

Mruk DD, Wong CH, Silvestrini B, Cheng CY. A male contraceptive targeting germ cell adhesion. *Nat Med*. 2006;12:1323–1328.

Nieschlag E, Zitzmann M, Kamischke A. Use of progestins in male contraception. *Steroids*. 2003;68:965–972.

Ortega HH, Lorente JA, Mira GA, Baravalle C, Salvetti NR. Constant light exposure causes dissociation in gonadotrophin secretion and

inhibits partially neuroendocrine differentiation of Leydig cells in adult rats. *Reprod Domest Anim*. 2004;39:417–423.

Payan-Carreira R, Rodrigues P, Carvalho PR. Chromogranin-A expression in the bovine testis. *Anim Reprod Sci*. 2006;96:146–153.

Radich JP, Mao M, Stepaniants S, Biery M, Castle J, Ward T, Schimmack G, Kobayashi S, Carleton M, Lampe J, Linsley PS. Individual-specific variation of gene expression in peripheral blood leukocytes. *Genomics*. 2004;83:980–988.

Robert NM, Martin LJ, Tremblay JJ. The orphan nuclear receptor NR4A1 regulates insulin-like 3 gene transcription in Leydig cells. *Biol Reprod*. 2006;74:322–330.

Samson M, Peale FV, Jr., Frantz G, Rioux-Leclercq N, Rajpert-De Meyts E, Ferrara N. Human endocrine gland-derived vascular endothelial growth factor: expression early in development and in Leydig cell tumors suggests roles in normal and pathological testis angiogenesis. *J Clin Endocrinol Metab*. 2004;89:4078–4088.

Shah C, Modi D, Sachdeva G, Gadkar S, Puri C. Coexistence of intracellular and membrane-bound progesterone receptors in human testis. *J Clin Endocrinol Metab*. 2005;90:474–483.

Siu MK, Cheng CY. Interactions of proteases, protease inhibitors, and the beta1 integrin/laminin gamma3 protein complex in the regulation of ectoplasmic specialization dynamics in the rat testis. *Biol Reprod*. 2004;70:945–964.

Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol*. 2004;3:article 3.

Themmen APN, Huhtaniemi IT. Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocr Rev*. 2000;21:551–583.

Walton MJ, Bayne RA, Wallace I, Baird DT, Anderson RA. Direct effect of progestogen on gene expression in the testis during gonadotropin withdrawal and early suppression of spermatogenesis. *J Clin Endocrinol Metab*. 2006;91:2526–2533.

Wang PJ, McCarrey JR, Yang F, Page DC. An abundance of X-linked genes expressed in spermatogonia. *Nat Genet*. 2001;27:422–426.

Wechezak AR, Coan DE. Subcellular distribution of Wnt-1 at adherens junctions and actin-rich densities in endothelial cells. *Exp Cell Res*. 2003;288:335–343.

Whitney AR, Diehn M, Popper SJ, Alizadeh AA, Boldrick JC, Relman DA, Brown PO. Individuality and variation in gene expression patterns in human blood. *Proc Natl Acad Sci U S A*. 2003;100:1896–1901.

Zhang P, Mellon SH. Multiple orphan nuclear receptors converge to regulate rat P450c17 gene transcription: novel mechanisms for orphan nuclear receptor action. *Mol Endocrinol*. 1997;11:891–904.