Developmentally distinct *in vivo* effects of FSH on proliferation and apoptosis during testis maturation

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Abstract

The critical influence of follicle stimulating hormone (FSH) on male fertility relates both to its impact on Sertoli cell proliferation in perinatal life and to its influence on the synthesis of Sertoli cell-derived products essential for germ cell survival and function in the developing adult testis. The nature and timing of this shift of germ cells to their reliance on specific Sertoli cell-derived products are not defined. Based on existing data, it is apparent that the dominant function of FSH shifts between 9 and 18 day postpartum (dpp) during the first wave of spermatogenesis from driving Sertoli cell proliferation to support germ cells. To enable comprehensive analysis of the impact of acute *in vivo* FSH suppression on Sertoli and germ cell development, FSH was selectively suppressed in Sprague–Dawley rats by passive immunisation for 2 days and/or 4 days prior to testis collection at 3, 9 and 18 dpp. The 3 dpp samples displayed no measurable changes, while 4 days of FSH suppression decreased Sertoli cell proliferation and numbers in 9 dpp, but not 18 dpp, animals. In contrast,

germ cell numbers were unaffected at 9 dpp but decreased at 18 dpp following FSH suppression, with a corresponding increase in germ cell apoptosis measured at 18 dpp. Sixty transcripts were measured as changed at 18 dpp in response to 4 days of FSH suppression, as assessed using Affymetrix microarrays. Some of these are known as Sertoli cell-derived FSH-responsive genes (e.g. StAR, cathepsin L, insulin-like growth factor binding protein-3), while others encode proteins involved in cell cycle and survival regulation (e.g. cyclin D1, scavenger receptor class B 1). These data demonstrate that FSH differentially affects Sertoli and germ cells in an age-dependent manner *in vivo,* promoting Sertoli cell mitosis at day 9, and supporting germ cell viability at day 18. This model has enabled identification of candidate genes that contribute to the FSH-mediated pathway by which Sertoli cells support germ cells.

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Introduction

Sperm production in the adult male requires establishment of the Sertoli cell population and initiation of the first spermatogenic wave, events that occur during foetal and early postnatal life. In the rat, Sertoli cells proliferate until around 15 days after birth, setting the full complement of Sertoli cells. Surrounded by Sertoli cells, the gonocytes multiply in the foetal testis and then enter a period of quiescence lasting a week. They resume mitosis at 3 days after birth and migrate to the basement membrane at the seminiferous cord perimeter. Once they have contacted the basement membrane they are called spermatogonia. Spermatogonia subsequently pass through three developmental phases involving mitosis, meiosis and spermiogenesis to form spermatozoa, with the germ cells completing

the first wave of spermatogenesis at 43 days after birth in the rat (de Rooij 1998).

The mechanisms by which the Sertoli and germ cell populations are regulated during the first spermatogenic wave are under investigation and appear to be influenced by a complex network of interacting signals. It is known that pituitary-derived follicle stimulating hormone (FSH) exerts its effects on the developing germ cells indirectly, as only the Sertoli cells bear FSH receptors. A key role of FSH in setting the size of the Sertoli cell population in early postnatal life has been attributed to its stimulatory effect on Sertoli cell division (Orth 1984, Orth *et al.* 1988, Boitani *et al.* 1995). The response of the Sertoli cell to FSH changes during the first wave of spermatogenesis in rodents, and this influences the proliferation of Sertoli cells (Boitani *et al.* 1995, Meehan *et al.* 2000,

Buzzard *et al.* 2003). Synthesis of several Sertoli cell products also changes during development, however whether some of these are regulated by FSH *in vivo* remains to be determined (Munsie *et al.* 1997, Yan *et al.* 2001, Fragale *et al.* 2001, Migrenne *et al.* 2003, Pellegrini *et al.* 2003). FSH–regulated apoptosis during the first spermatogenic wave has not been examined, however in adult rats, germ cells are lost through apoptosis (Meachem *et al.* 1999), rather than through reduced proliferation when FSH is manipulated (McLachlan *et al.* 1995). However the regulation of adult spermatogenesis cannot be assumed to be mediated in the same manner as is the first spermatogenic wave, as shifts in the expression patterns of key regulatory genes (i.e., Bcl-2 family members and stem cell factor (SCF) have been observed as Sertoli cells mature and eventually mature germ cell populations emerge (Munsie *et al.* 1997, Huang *et al.* 1992, Meehan *et al.* 2001).

A large number of FSH responsive genes have been identified, however in most cases, their specific contribution to Sertoli cell and germ cell viability and function is undefined. The interaction of SCF with its receptor, c-kit, is essential for germ cell progression and survival in first wave and adult spermatogenesis (Yoshinaga *et al.* 1991, Packer *et al.* 1995), and expression of SCF in Sertoli cells has been shown to be regulated by FSH *in vitro* and *in vivo* (Rossi *et al.* 1993, McLean *et al.* 2002). Expression of the transcription factor DMRT1 in Sertoli cells can be elevated by FSH *in vivo* (Chen & Heckert 2001) and a role for DMRT1 in early postnatal testicular development relating to the termination of Sertoli cell proliferation has been deduced from the phenotype of *DMRT -/-* mice (Raymond *et al.* 2000). Some genes encoding apoptotic regulators in the Bcl-2 family of proteins have also been shown to be responsive to FSH, including *Bcl-w* and *Bok* (Yan *et al.* 2000, Suominen *et al.* 2001). A recent study using microarray analyses has defined 100–300 known transcripts that are regulated by FSH in cultures of Sertoli cells from 20 days post partum (dpp) rats (McLean *et al.* 2002). It is now timely to identify the FSH regulated genes involved in testicular development and spermatogenesis using an *in vivo* approach.

To more precisely understand the dynamic mechanisms by which FSH acts in immature rats, we set out to identify the cells that respond to *in vivo* changes in FSH levels during the first wave of spermatogenesis and to examine the functional changes in these cells. Administration of an antibody to FSH was performed to selectively suppress FSH for two or four days by immunoneutralisation. This acute FSH suppression differentially affected Sertoli and germ cell numbers during development as assessed by the, unbiased, optical disector stereological technique, corresponding with selective changes in proliferation and apoptosis in these cell types. Rat genomic microarrays enabled identification of candidate genes regulated by FSH from 14 to 18 dpp. The value of this model for

interrogating the changing roles of FSH in the first wave of spermatogenesis is discussed.

Material and Methods

Animals

Male outbred Sprague–Dawley rat pups with mothers were obtained from the Monash University Central Animal House (Clayton, Australia). They were maintained at 20 °C in a fixed 12 h light:12 h darkness cycle with free access to food and water in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (1997, National Health and Medical Research Council, Australia). This study was approved by the Monash Medical Centre Animal Ethics Committee.

Passive immunisation against FSH

The ability of the polyclonal ovine antisera raised against rat FSH (FSHAb) to neutralise rat FSH *in vitro* and *in vivo* has been previously described (Meachem *et al.* 1998). The level of neutralisation of serum FSH achieved in adult rats was greater than 90% (reaching the limit of assay detection). Rat pups were immunised with the FSHAb or with normal sheep immunoglobulin (ConAb) at a daily dose of 10 mg/kg, a 5-fold higher dose than that previously administered to adult rats. Each animal received s.c. injections 2 days prior to death at 3 days dpp, and 2 and 4 days prior to death at 9 and 18 dpp. Ten rats were injected for each data point.

Tissue collection and preparation

One h prior to death, each rat received 5-bromodeoxyurdine (BrdU; 50 mg/kg, s.c. Sigma) to enable proliferation analysis. Rats were killed 24 h after the final injection of the antibody, at 3, 9 or 18 dpp, by decapitation. Trunk blood was collected and allowed to clot overnight at 4 °C prior to serum collection for hormone assays. Due to the small amounts of serum collected for day 3 and 9 dpp rats, these samples were pooled in groups of three and two animals, respectively. The testes were then excised and weighed. The right testis of each animal was snap frozen on dry ice and stored at − 75 °C for RNA preparation, while left testis was immersion-fixed with Bouin's solution for less than 5 h and sliced into 2-mm thick slabs orthogonal to the long axis of the testis. The testes from 9 and 18 dpp rats were divided into two, or four slices, respectively, and half of each processed into hydroxyethylmethacrylate resin (Technovit 7100; Kulzer and Co. GmBH, Friedrichsdorf, Germany) according to the manufacturer's instructions, while the other half was used for routine embedding into paraffin. The whole left testis from 3 dpp rats was embedded in resin and paraffin in every second

animal. Thick (25 μm) resin sections were serially cut (Supercut Microtome, Reichert Jung 2050, Nossloch, Germany), stained with the Periodic acid-Schiff's reaction reagents and counterstained with Mayer's Haematoxylin for the determination of cell number. Thin (5μm) paraffin sections were placed on Superfrost Plus slides for analysis of proliferation and apoptosis.

Cell number estimates using the optical disector

The optical disector stereological method (Wreford 1995) was used to determine the total number of cell nuclei per testis. All measurements were performed using a 100 × objective on an Olympus BX-50 microscope (Olympus, Tokyo, Japan). A microcator (D 8225; Heideinhain, Traunreut, Germany) that monitored scanned depth was attached to the microscope stage. The images were captured by a JVC TK-C1381 video camera coupled to a Pentium PC computer using a Screen Machine II fast multimedia video adapter (FAST, Hamburg, Germany). The software package, CASTGRID V1·60 (Olympus, Denmark, Germany), was used to generate an unbiased counting frame superimposed on video image. Fields were selected by a systematic uniform random sampling scheme as previously described (McLachlan *et al.* 1994, Wreford 1995) with the use of a motorized stage (Multi-control 2000; ITK, Lahnau, Germany). The final screen magnification was 2708-fold.

Sertoli cells were identified by their irregularly shaped nuclei, which were often positioned towards to the basement membrane and contained multiple nucleoli. Gonocytes were relatively large circular to ovoid cells with large circular nuclei, centrally located within the epithelium. Type A and B spermatogonia/preleptotene spermatocytes, leptotene/zygotene spermatocytes and pachytene spermatocytes were identified according to the characteristics described (Russell *et al.* 1990). Type B spermatogonia and preleptotene spermatocytes were not readily distinguishable at these stages of development and were counted as one. At least 300 Sertoli cell nuclei in total per testis were counted using the unbiased counting frame ($175–430\ \mu m^2$) and at least 80 gonocytes, spermatogonia (type A, B spermatogonia and preletotene spermatocytes) or spermatocytes (leptotene, zygotene and pachytene spermatocytes) were counted employing a larger frame ($430–1923\ \mu m^2$). The frame size was selected based on cell frequency at different time points; less abundant cell types were counted in a larger counting frame. No correction for shrinkage was required (Meachem *et al.* 1996). Slides were masked prior to each type of quantitation (cell number, proliferation and apoptosis) to facilitate unbiased counting.

Proliferation analysis

BrdU incorporation into testicular cells at 3, 9 and 18 dpp was detected by immunohistochemistry as previously described (Schlatt *et al.* 1999), with minor modifications as described below (see Fig 1A). In brief, slides bearing paraffin-embedded tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate buffer (0·1 M, pH 6; 90–95 °C for 10 min then room temperature for 20 min). Sections were treated successively with trypsin (0·25%, 1·5 min) and 3% hydrogen peroxide in methanol (20 min) and washed in PBS. All subsequent procedures were performed at room temperature, washes were in PBS, and incubations were in a humid chamber. Sections were treated with first with Superblock (40 min; DAKO, Carpenteria, CA, USA) and then with the monoclonal antibody to BrdU (clone BU-33, 4μl/ml in PBS, 1 h; Sigma). Following washes, biotinylated rabbit anti-mouse IgG was added (1:300 in PBS, 30 min; Zymed, California, USA). Sections were washed and incubated first in ABC complex (40 min; Vectastain Elite, Vector Laboratories, Burlingame, CA) and then with Tyramide Signal Amplification Biotin reagent (30 min; PerkinElmer Life Sciences, Inc, Boston, MA, USA) according to the manufacturers' instructions. After washing, diaminobenzidine (DAB) was added to reveal sites of antibody binding with a dark brown reaction product (2–3 min; DAKO Liquid DAB Substrate Chromogen System), and sections were counterstained with Mayer's Haematoxylin (3 min; Sigma), blued in Scott's tap water (1 min), and finally dehydrated and mounted in Depex (BDH Laboratory Suppliers, Poole, Dorset, UK) under glass coverslips. Cell types with anti-BrDU nuclear staining were identified on the basis of their location within the cord and the size and shape of cell nuclei, as described above.

The percentage of BrdU labelled cells was assessed using an unbiased counting frame of $430\ \mu m^2$. To determine the proliferation index for each cell type, the total number of BrdU-labelled cells was divided by the total number of labelled and unlabelled cells.

Apoptosis analysis

Tissue sections (5 μm) were deparaffinized and rehydrated prior to the detection of DNA fragmentation (see Fig 1B). Apoptotic cells were detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labelling (TUNEL) method (Meachem *et al.* 1999). Apoptotic cells were visualised using the chromogen DAB and processed as described for BrDU detection. On control sections, the TdT enzyme was omitted. TUNEL-positive cell types were identified based on their location within the cord, their size, and the shape of the cell nucleus.

The percentage of cells with TUNEL labelling was assessed using an unbiased counting frame of $430\ \mu m^2$ and the apoptotic index calculated by dividing the number of TUNEL labelled cells by the total number of labelled and unlabelled cells.
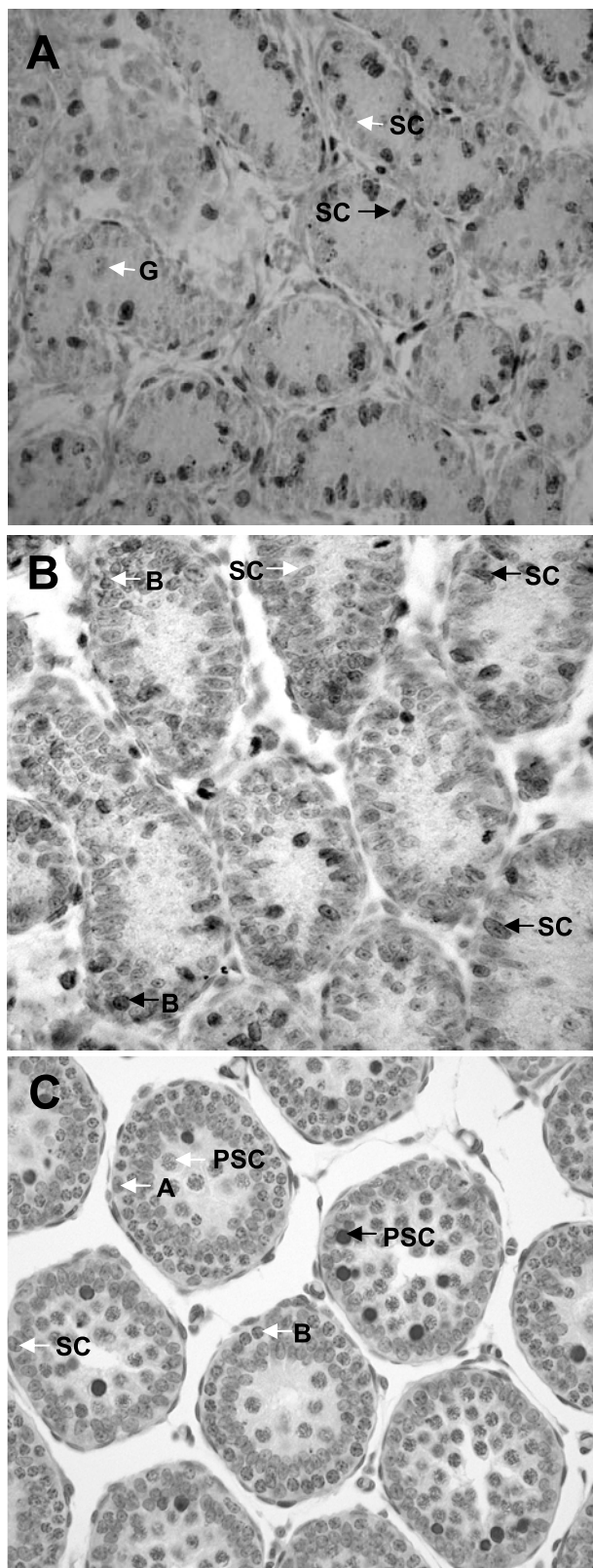
Serum androgen levels

Serum androgen levels were measured by RIA using iodinated histamine-testosterone in combination with an acidic buffer (pH 5·1) to disrupt binding between testosterone and binding proteins in unextracted serum samples (O'Donnell *et al.* 1994). Serum samples (5–25µl) were assayed in duplicate across two assays. Within assay variation for both assays was 3%, and between assay variation was 9%. Assay sensitivity was 0·6 ng/ml.

Serum inhibin levels

Immunoreactive inhibin was measured by heterologous RIA as described previously (Robertson *et al.* 1988). Results are expressed in terms of an in-house rat ovarian extract calibrated against human recombinant (hr)-inhibin. Iodinated hr-inhibin was used as tracer. The antiserum used was rabbit antiserum (#1989) which is directed towards the α-subunit thereby measuring both inhibin A and B, and cross-reacts 288% with pro-·C, the prosequence of the inhibin alpha subunit (Robertson *et al.* 1989). Goat anti-rabbit IgG (GAR#12; Monash Institute of Reproduction & Development, Monash University, Melbourne, Australia) was used as second antibody. The assay buffer used was 0·01 M PBS containing 0·5% BSA (Sigma). Mouse serum pools diluted in a dose-dependent manner and were parallel to the standard curve (data not shown). The samples were run in a single assay in 20µl duplicates. The within-assay variation was 7·2%. Assay sensitivity was 0·17 ng/ml.

Oligonucleotide microarray hybridisation

To identify genes regulated by FSH at the 18 day time point, total RNA was isolated from total testes using acid phenol extraction (Chomczynski & Sacchi 1987). RNA from two individual animals treated from 14 dpp-18 dpp with either FSHAb or ConAb (4 samples in total) was used. The gene expression profile of FSHAb- and ConAb-treated testis were individually determined using Affymetrix RG_U34A rat chips (Affymetrix Inc., Santa Clara, CA, USA) as previously described (McLean *et al.* 2002). The RNA from the FSHAb- and the

Figure 1 Representative photomicrographs of cross sections of the testis from rats receiving normal sheep immunoglobulin for up to 4 days. Panel A. BrdU incorporation in the 3 dpp rat testis detected by immunohistochemistry. White arrows: nucleus of unlabelled Sertoli cell (SC) and gonocyte (G). Black arrow: nucleus of a labelled Sertoli cell. Panel B. BrdU incorporation in the 9 dpp rat testis. White arrows: nucleus of unlabelled Sertoli cell (SC) and type B spermatogonia Black arrows: nucleus of labelled Sertoli cell (SC) and type B spermatogonia. Panel C. DNA fragmentation indicative of cellular apoptosis detected by TUNEL in 18 dpp rat testis. White arrows: nucleus of unlabelled Sertoli cell (SC), type B spermatogonia (B) and pachytene spermatocyte (PSC). Black arrows: nucleus of labelled pachytene spermatocytes (PSC).

ConAb-treated testis was extracted and purified before biotin labelling and hybridisation to the microarray gene chip. Two replicates of each sample were hybridised to separate chips giving a total of four chips in the analysis. Each gene set on the Affymetrix chip is composed of 16 pairs of 24-mer oligonucleotides, with 8799 genes on each RG_U34A chip. Included in each set is one anti-sense strand specific for the gene and one anti-sense strand with single point mutations is used as a comparative negative control. The labelled RNA was visualized on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA, USA). An initial two way comparison was performed using the Affymetrix Suite software. In both experiments the FSHAb- and ConAb-treated chips were compared against each other.

Bioinformatics and microarray statistics

The bioinformatics and statistical analysis performed is as previously described (Eisen *et al.* 1998, Chaudhary *et al.* 2005, Kezele *et al.* 2002). Microarray output was examined visually for excessive background noise and physical anomalies. The default Microarray Suite (MAS, Silicon Genetics, Redwood City, CA, USA) statistical values were used for all analyses. An absolute analysis using MAS was performed to assess the relative abundance of the transcripts based on signal and detection (present, absent, or marginal) for the 16 different oligonucleotides per gene and comparison for analysis. The absolute analysis from MAS was imported into GeneSpring 5·1 software (Silicon Genetics, Redwood City, CA, USA). The data were normalized within GeneSpring using the default/recommended normalisation methods. These include setting of signal values below 0·01 to 0·01, total chip normalisation to the 50th percentile, and normalisation of each gene to the median. These normalisations allowed for the comparison of data based on relative abundance in any sample set rather than compared with a specific control value. Transcripts expressed differentially at a statistically significant level were determined using a one-way ANOVA parametric test with variances not assumed equal, and a *P*-value cutoff of 0·05. This was applied to all samples and considered all transcripts represented on the arrays. Two independent samples for each treatment group were analyzed was performed and allowed a 2×2 factorial comparison in the experiment. Subsequently, expression restrictions were applied to identify the transcripts expressed in a significant manner. These restrictions were designed so that the remaining transcripts met the following requirements in addition to being expressed in a significant manner: 1) each transcript have a signal value of at least 100 in the average of both samples, from at least one of the treatments tested and 2) had an average fold change of 1·5 or greater in signal intensity between treatments. Transcripts that passed these restrictions were considered for further analysis. Previous studies have

shown that microarray data correlates well with real-time quantitative PCR and Northern analysis (Eisen *et al.* 1998, Chaudhary *et al.* 2005, Kezele *et al.* 2002, Sadate-Ngatchou *et al.* 2004) . Therefore, microarray data does not need to be confirmed as previously suggested (Shima *et al.* 2004). However, two selected genes were used in a real-time quantitative PCR procedure as previously described (McChlery & Clarke 2003) to help confirm the microarray procedure. The microarray chip data can be accessed at www.skinner.wsu.edu.

Reverse transcription (RT) and real-time PCR analysis

Real time PCR was used to measure the relative levels of two candidate FSH-regulated genes. Total RNA collected from two individual day 18 rats treated with either ConAb or with FSH Ab for 4 days was treated to remove residual genomic DNA (Ambion DNA-free Treatment Kit; Ambion, Austin TX, USA). RNA (500 ng) was converted to cDNA in a final volume of 20 µl using Superscript II according to the manufacturer's protocol (Invitrogen). For each sample, the absence of contaminating genomic DNA in cDNA samples was confirmed using reactions in which the RT enzyme was omitted. Quantitative RT-PCR analysis was performed using the Roche LightCycler (Roche, Mannheim, Germany) and the FastStart DNA Master SYBR-green 1 system (Roche). Oligonucleotide primer sequences for IGFBP-3, Smad3, and beta-actin were obtained either from published sources or Frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi;version 3·0 (Table 1). For PCR analysis, sample cDNA was diluted 1:10- to 1:50-fold, and PCR reaction conditions, including Mg^{2+} concentration, primer concentration, anneal time and extension time were optimized for each primer pair as summarized in Table 1. For all PCR analyses, standard curves were generated using dilutions of an immature rat testicular cDNA preparation of arbitrary units (i.e. 17 dpp). PCR of all standards and samples were performed using duplicate reactions for approximately 40–45 cycles, after which a melting curve analysis was performed to monitor PCR product purity (see Table 1). In initial experiments, PCR product identities were verified by agarose gel electrophoresis and DNA sequencing (data not shown).

Statistics

A two-sample *t*-test was used to determine differences between FSHAb and ConAb treated samples with the assumption that data were normally distributed for all histological and hormone data. If data did not show normal distribution, then a Mann–Whitney test was carried out using Sigmastat for Windows version 2·0 (Jandel Corporation, CA, USA). Data are expressed as mean ± S.E.M., $n=7$ rats per group. Statistical analysis of microarray data is described in Bioinformatics and microarray statistics.

Table 1 Primer-specific LightCycler conditions used for PCR amplification of rat IGF-BP3, Smad 3 and beta-actin

Name [Accession No.]	Primer sequence	Product size (bp)	Primer concentration (pmol)	Mg^{2+} concentration (mM)	Annealing temperature (°C)	Extension time (Sec)	Data temperature (°C)*	PCR product Tm (°C)**
Rat IGF-BP3 [NM_012588]	sense 5'-AATGCTGGGAGTGTGGAAAG-3' antisense 5'-GCGGTATCTACTGGCTCTGC-3'	145	30	3·5	58	18	90	90
Rat Smad3 [U66479]	sense 5'-AAGGGCGAGCAGAACGGG-3' antisense 5'-GGGATGGAATGGCTGTAGTC-3'	425	35	3·0	57	19	92	92
Rat β-actin [NM_031144]	sense 5'-CCGTAAAGACCTCTATGCCAACA-3' antisense 5'-GCTAGGAGCCAGGGCAGTAATC-3'	103	50	2·5	67	5	80	86

*Temperature at which the fluorescence of the PCR product was quantified during LightCycler analysis; **PCR products have characteristic melting points (Tm). The Tm for a DNA product is defined as the temperature at which half of the DNA helical structure is lost, and is determined by melting temperature analysis on the LightCycler.

Results

Testicular weights and serum hormone measurements

Testicular weights were not significantly different following 2 days of FSH suppression in 3 dpp rats compared with their corresponding controls (Table 2), however testicular weights were reduced in 9 and 18 dpp animals after 2 (to 84% of control) and 4 days (to 78% of control) of FSH suppression. FSH suppression did not affect serum androgen levels at any time point compared with controls (Table 2). Serum inhibin levels following 2 days of FSH suppression in 3, 9 and 18 dpp rats were significantly reduced to 74%, 71% and 69%, and after 4 days 73% and 53% in 9 and 18 dpp rats compared with control values, respectively (Table 2). This reduction in circulating inhibin levels is presumed to reflect the successful neutralisation of FSH bioactivity through administration of the antibody raised against FSH, as FSH normally stimulates production of inhibin from Sertoli cells.

Cell populations decreased in FSH withdrawn rats

At 3 dpp there were no differences in gonocyte ($0·098 \pm 0·010$ vs $0·091 \pm 0·054$ million/testis) or Sertoli cell numbers ($3·50 \pm 2·4$ vs $3·50 \pm 2·3$ million/testis) following 2 days of FSHAb treatment compared with ConAb treatment (Fig 2A), correlating no change in testis weight. At 9 dpp, there was a significant reduction (to 63% of control; $P<0·05$) in the number of Sertoli cells following 4 days of FSH suppression ($16·8 \pm 1·7$ vs $23·0 \pm 1·7$ million/testis), while spermatogonial numbers were unchanged ($0·9 \pm 0·08$ vs $1·1 \pm 0·4$ million/testis) (Fig 2B). At 18 dpp no difference in the number of Sertoli cells were observed in response to 4 days of FSHAb treatment ($40·2 \pm 3·20$ vs $44·2 \pm 4·18$ million/testis). A reduction in spermatogonia to 75% ($16·9 \pm 1·8$ vs $22·2 \pm 1·0$ million/testis, $P<0·05$) of control group values was observed following 4 days of FSH suppression. Spermatocyte number tended to be reduced to 80% ($12·4 \pm 1·1$ vs $15·5 \pm 1·0$ million/testis, $P=0·061$) of control group values was observed following 4 days of FSH suppression, although this did not achieve significance (Fig 2C).

FSH suppression decreases Sertoli cell proliferation in day 9 rats

At 3 dpp, no differences in Sertoli cell proliferation were observed between FSHAb- and ConAb-treated rats ($23·4\% \pm 3·2$ vs $27·2\% \pm 1·8$); no BrdU labelled gonocytes were observed in these samples (Refer Fig 1). At 9 dpp, there was a significant reduction (to 66% of control, $P<0·01$) in the proliferation rate of Sertoli cells following FSH suppression ($14·3\% \pm 0·6$ vs $21·6\% \pm 1·2$) (Fig 2A), while no change was observed for spermatogonia ($23·2\% \pm 2·4$ vs $21·1\% \pm 1·6$, $P=0·11$) (Fig 3A). At

Table 2 The testicular weights (mg), serum testosterone (ng/ml) and inhibin (ng/ml) levels at 3 dpp, 9 dpp and 18 dpp following 2 and 4 days of FSH suppression. Rats were treated with normal sheep immunoglobulin (ConAb) and with antibody raised against rat FSH (FSHAb) at 3 dpp, 9 dpp and 18 dpp following 2 and 4 days of treatment. The data are expressed as mean ± s.e.m., with $n=10$ rats/group.

	Testicular weight (mg)	Serum testosterone (ng/ml)	Serum inhibin (ng/ml)
Treatment			
3 days post partum			
+ConAb 2d	5·1 ± 0·2	0·64 ± 0·08	3·57 ± 0·21
+FSHAb 2d	5·1 ± 0·1	0·63 ± 0·11	2·65 ± 0·06**
9 days post partum			
+ConAb 2d	17·9 ± 0·9	1·36 ± 0·3	4·53 ± 0·3
+FSHAb 2d	15·2 ± 0·5*	1·18 ± 0·1	3·23 ± 0·2*
+ConAb 4d	18·7 ± 0·4	0·89 ± 0·2	4·17 ± 0·24
+FSHAb 4d	14·6 ± 0·5**	0·70 ± 0·1	3·03 ± 0·19*
18 days post partum			
+ConAb 2d	75·4 ± 3·0	0·92 ± 0·3	3·51 ± 0·4
+FSHAb 2d	63·2 ± 3·4*	0·92 ± 0·2	2·43 ± 0·2*
+ConAb 4d	74·1 ± 1·2	1·80 ± 0·5	5·75 ± 0·31
+FSHAb 4d	57·9 ± 2·1**	1·36 ± 0·2	3·09 ± 0·20**

Asterisks denote significant differences between rats receiving FSHAb and their corresponding controls. *$P<0·01$ and **$P<0·001$.

18 dpp, in response to FSH suppression, there was no change in Sertoli cell ($6·8\% ± 2·3$ vs $9·5\% ± 1·0$, $P=0·30$), spermatogonial ($19·6\% ± 1·9$ vs $23·3\% ± 1·0$, $P=0·11$) or spermatocyte proliferation ($11·9\% ± 1·4$ vs $10·8\% ± 1·7$, $P=0·64$) (Fig 3B) compared with their corresponding controls. These data indicate that the reduction in testis weight due to FSH suppression at 9 dpp, but not at 18 dpp, reflects the lower number of Sertoli cells in these samples. The incidence of apoptosis was next investigated as an alternative basis for the reduced testis weights in the 18 dpp samples (Fig 3B).

Germ cell apoptosis is affected by FSH suppression in 18 dpp rats

No apoptosis was observed in Sertoli cells and gonocytes at 3 dpp (Refer Fig 1). No differences in Sertoli cell ($0·6\% ± 0·2$ vs $0·9\% ± 0·2$, $P=0·36$) or spermatogonial apoptosis ($6·6\% ± 1·1$ vs $4·6\% ± 0·9$, $P=0·20$) were observed following 4 days of FSH suppression at 9 dpp compared with controls (Fig 4A). However, at 18 dpp, there was a significant elevation of spermatogonial apoptosis (250% of control) in response to FSH suppression ($15·5\% ± 1·6$ vs $6·2\% ± 1·0$, $P<0·001$); spermatocyte apoptosis increased to 136% of control levels ($16·1\% ± 2·2$ vs $11·8\% ± 1·4$, $P=0·13$), although this did not achieve significance (Fig 4B). No change in Sertoli cell apoptosis was observed at any time point (Fig 4). These data indicate that a shift occurs between 9 and 18 dpp within the germ cell population towards reliance for support cues on FSH-derived products. The nature of this support system

and the effects of its removal were examined using a genomic microarray to identify genes regulated by altered levels of FSH at 18 dpp.

Microarray analysis

Thirty genes were identified in the 18 dpp samples as up-regulated and 30 as down-regulated (Table 3) following FSH suppression by treatment with FSHAb for 4 days. These 60 genes of interest met the selection criteria outlined previously; all give at least one signal intensity greater than 100, are flagged as changing, and have fold changes of 1·5 or greater.

Amongst these genes, 12 out of 60 (20%) have previously been reported to be regulated by FSH, predominantly through *in vitro* studies. Many of the genes on this list have not yet been identified as being expressed in the testis (26/60), and some of those known to be in the testis have not previously been assessed for regulation by FSH (14/34). This latter category of candidate *in vivo* FSH target genes have been reported to be expressed in Sertoli cells (4/14), germ cells (8/14) and/or non-Sertoli somatic cells (6/14); clearly the non-Sertoli cells expressed genes that would be regulated indirectly, through FSH regulation of Sertoli cell biosynthesis or other functions.

RT-PCR analysis

IGFBP-3 mRNA expression levels (measured in two individual animals) in FSHAb-treated rats were significantly elevated approximately 2·5-fold above the levels
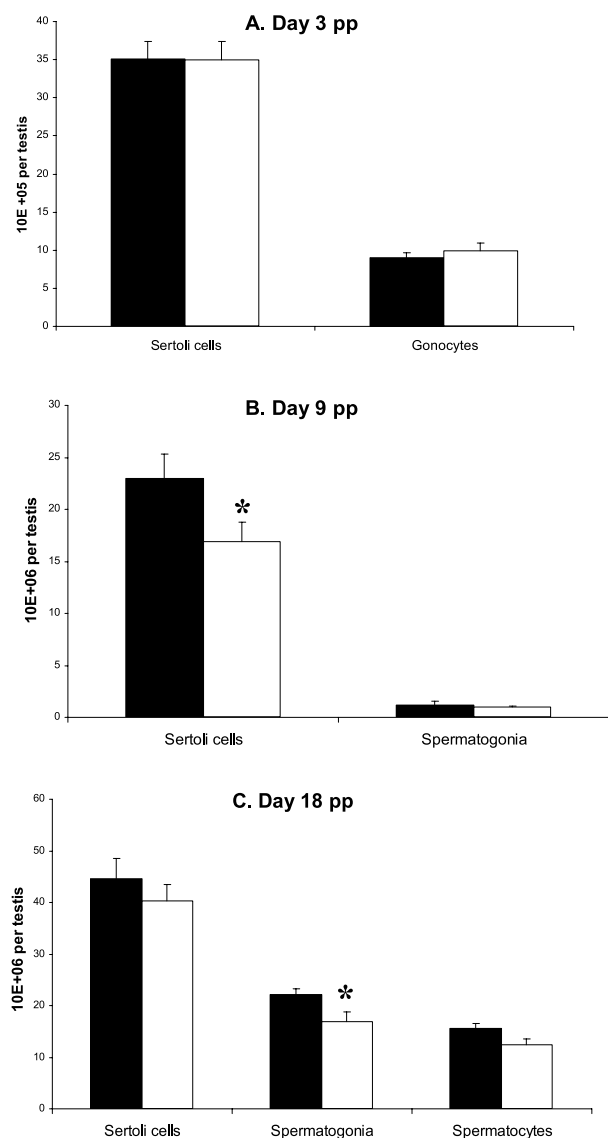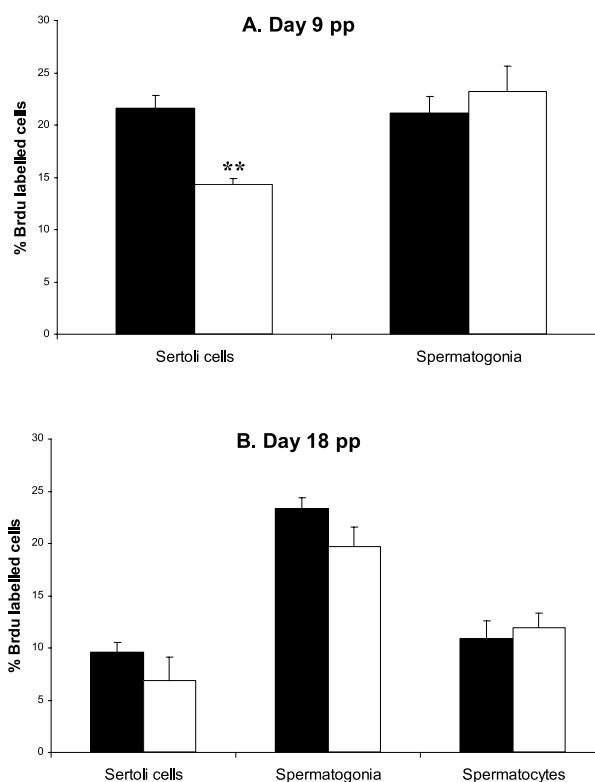
Figure 2 The number of Sertoli cells, spermatogonia and spermatocytes at 3 dpp (A), 9 dpp (B) and 18 dpp (C) following 2 and 4 days of FSH suppression. Cell numbers in rats treated with normal sheep immunoglobulin (black bars) and rats treated with antibody raised against rat FSH (white bars) at 3 dpp, 9 dpp and 18 dpp. Sertoli cell, spermatogonial and spermatocyte numbers are expressed as mean ± S.E.M., where $n=7$ rats/group. Asterisk identify significant differences between treated and control groups at $P<0.05$.
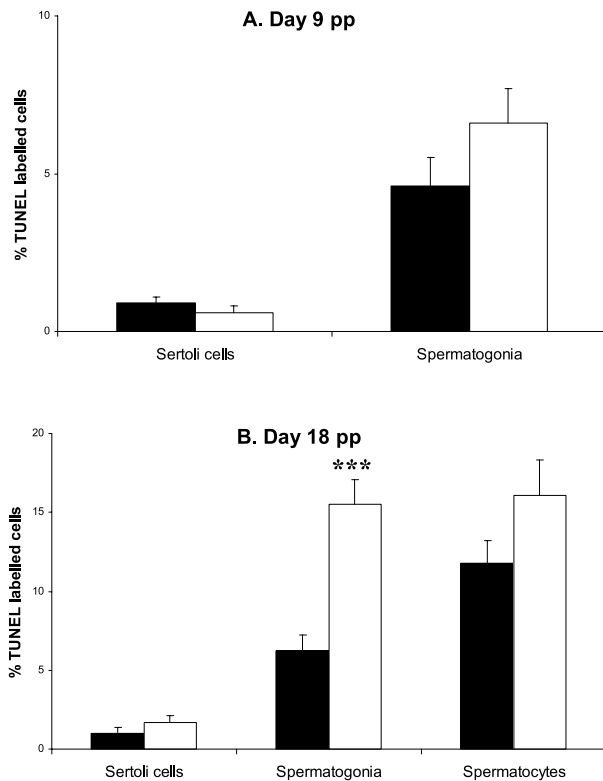

Figure 3 Proliferation of specific cell populations of the seminiferous epithelium at 9 dpp (A) and 18 dpp (B) following 4 days of FSH suppression. Percentage of cells incorporating BrdU in rats treated with normal sheep immunoglobulin (black bars) and rats treated with antibody raised against rat FSH (white bars) at 9 dpp and 18 dpp. The percentages of labelled versus unlabelled total cells, Sertoli cells, spermatogonia and spermatocytes are expressed as mean ± S.E.M., where $n=7$ rats/group. Asterisks denote significant differences between treated and control groups at $P<0.01$.

measured in two individual ConAb-treated rats. In contrast, Smad 3 mRNA expression was significantly reduced by approximately 6 -fold following FSHAb treatment compared with ConAb-treated rats (Fig 5).

Discussion

These data illustrate that the influence of FSH on Sertoli and germ cells are dynamic and cell-type specific during the first spermatogenic wave in the rat. We developed this model to enable a co-ordinated documentation of specific cellular changes, in association with the identification of testicular genes, for which expression is affected by variance in bioactive FSH levels. The results are in accord with previous observations that FSH and activin regulate Sertoli and germ cell proliferation in an age-dependent manner *in vitro* (Boitani *et al.* 1995, Fragale *et al.* 2001). Our findings expand on earlier analyses by measuring proliferation and apoptosis in the same samples and establishing a method for identification of FSH target genes *in vivo*. More specifically, this study demonstrated for the first time *in vivo* that acute FSH suppression reduces both the Sertoli and germ cell populations, first by inhibiting Sertoli cell proliferation from 5 to 9 dpp, and later by inducing germ cell apoptosis between 14 and 18 dpp (see Fig 6).

Acute suppression of FSH for 4 days resulted in decreased testis weights in 9 and 18 dpp rats. This decrease

Figure 4 Apoptosis in specific cell populations of the seminiferous epithelium at 9 dpp (A) and 18 dpp (B) following 4 days of FSH suppression. The percentages of TUNEL labelled cells in rats treated with normal sheep immunoglobulin (black bars) and rats treated with antibody raised against rat FSH (white bars) at 9 dpp and 18 dpp. The percentages of labelled versus unlabelled total cells, Sertoli cells, spermatogonia and spermatocytes are expressed as mean ± s.e.m., where $n=7$ rats/group. Asterisks denote significant differences between treated and control groups at $P<0.001$.

in testicular weight was attributed, at least in part, to the decreased Sertoli cell population at 9 dpp and a decreased germ cell population in 18 dpp rats as assessed by the optical disector stereological technique. The Sertoli cell population decrease resulted from inhibition of proliferation and not from elevated apoptosis. In contrast, the reduction in spermatogonial and spermatocyte cell number at 18 dpp resulted from elevated apoptosis of germ cells.

Sertoli cell development

Our findings are in accord with other studies which show that FSH plays a central role in regulating the size of the Sertoli cell population (Meachem *et al.* 1996) by supporting Sertoli cell proliferation during early postnatal life (Orth 1984, Orth *et al.* 1988, Boitani *et al.* 1995). Whether FSH plays a role in terminating Sertoli cell proliferation around 15 days after birth is beyond the scope of this study, however data from other *in vitro* and *in vivo*

studies indicate a role for FSH in this process (Baker & O'Shaughnessy 2001, Buzzard *et al.* 2002). Survival of Sertoli cells in the postnatal rat does not appear to be influenced by FSH levels, as evidenced by the fact that FSH suppression did not induce Sertoli cell apoptosis, though the possibility remains that there are residual levels of serum FSH in this model which are sufficient to support Sertoli cell survival. We predict that a greater impact on testicular physiology could be apparent if complete FSH withdrawal were achieved.

Several other factors have been demonstrated to regulate Sertoli cell development in the time interval under investigation here, including thyroid hormone (Cooke & Meisami 1991, Van Haaster *et al.* 1992), activin (Boitani *et al.* 1995, Fragale *et al.* 2001, Buzzard *et al.* 2003), glial cell line-derived neurotrophic factor (GDNF) (Hu *et al.* 1999), interleukin-1 (Petersen *et al.* 2002), and transforming growth factor (TGF) and epidermal growth factor (Petersen *et al.* 2001). Interactions between these factors and FSH-mediated regulation of Sertoli cell function is not well understood, though two previously identified FSH regulated target genes, cyclin D2 (Buemer *et al.* 2000) and DMRT-1 (Raymond *et al.* 2000) are potential mediators.

In this study, the FSHAb dose administered was five times higher than that previously shown to neutralise greater than 90% of serum FSH in adult rats (Meachem *et al.* 1998, 1999). This dose was selected because serum FSH levels are 3–5 higher during postnatal life than in adulthood, as measured using RIA (Kirby *et al.* 1992). In our previous studies with this antibody, neutralisation of bioactive material was determined using an FSH *in vitro* bioassay, based on the induction of aromatase activity in immature rat Sertoli cells and assay of free FSH from rat serum using HPLC and RIA (Meachem *et al.* 1998, 1999). The significant reduction in serum inhibin levels following FSHAb administration in the present study are a positive indicator of the efficacy of FSH immunoneutralisation. Whether FSH regulates Sertoli cell development in the 3 dpp testis remains unclear. Our data suggest that Sertoli cell proliferation and apoptosis are not governed by FSH between 1 and 3 dpp in the rat. Consistent with this, Buzzard *et al.* (2003) reports that proliferation of purified Sertoli cells from rats younger than 6 dpp is not affected by FSH. On the other hand, Boitani *et al.* (1995) and Meehan *et al.* (2000) reported that Sertoli cell proliferation in 3 dpp rat testis fragment cultures was enhanced in the presence of FSH. Differences between our new data and that reported in earlier studies may reflect differences between *in vitro* and *in vivo* models, with the latter being uniquely employed in the present study. It is possible that removal of FSH for 2 days *in vivo* may be insufficient to observe a subtle but functionally important change in Sertoli cell proliferation at this age, due to this relatively small population of Sertoli cells present at this time (<4 million Sertoli cells/ 3 dpp testis). It may be necessary to extend the period of FSH suppression or to

Table 3 Genes up and down regulated by FSH in day 18 postpartum rat testes. Day 15 rats were treated with normal sheep immunoglobulin or with an antibody raised against rat FSH for 4 days. Results are based on two microarray hybridizations of target synthesized from RNA from two independent rat testes from each treatment group. The Affymetrix microarray data set was analyzed by pairwise comparison using Gene Sifter. Sample sets with a signal at ≥100 detected in at least one position on the array and a fold change of 1·5 or greater are reported. References for those genes previously reported to be in the rat testis and/or regulated by FSH are in parentheses.

	Accession number	Fold change	Average signal FSHAb	+/- s.e.m.	Testis expression previously reported	FSH regulation previously reported
Genes up-regulated by FSH suppression						
Insulin-like growth factor binding protein 3	AI009405; M31837	6·47; 3·12	17·45	5·85; 1·99	Yes; Sertoli cells (Smith et al. 1990, Rappaport & Smith 1995, Khan et al. 2002)	Yes
Testin	U16858	3·97	30·65	0·90	Yes; Sertoli cells (Grima et al. 1998, Lui et al. 2003)	Yes
HNF-3/forkhead homolog-1	L13201	3·14	36·65	0·39	Yes; (Kaestner et al. 1998, Migrenne et al. 2003, Wolfrum et al. 2003)	No
2·4 kb repeat DNA right terminal region	X05472	2·62	53·0	1·99	No	No
Endothelin 1	M64711	2·46	36·15	0·46	Yes; Sertoli cells (Fantoni et al. 1993, Tripiciano et al. 1999)	Yes
cDNA clone rx01264 3'	AI639097	2·44	121·55	0·25	No	No
ATP-binding cassette, sub-family C sulfonylurea receptor 2B mRNA (SUR2B);	AF019628	2·33	20·75	1·01	Yes; Leydig cells (Schinkel & Jonker 2003, O'Shaughnessy et al. 2002)	No
Cyclin D1	AI231257	2·04	8·50	1·60	Yes; gonocytes & spermatogonia (Wolgemuth et al. 1998, Buemer et al. 2000)	No
cDNA, 3' end /clone=RKIBA73	AA892986	1·98	6·05	0·90	No	No
Lipase A, lysosomal acid	S81497	1·98; 1·84	55·95	0·63; 0·63	Yes; Guinea pig (Kabbaj et al. 2001)	No
SH3 domain binding protein CR16	U31159	1·93	10·8	0·63	No	No
G-protein-coupled receptor 105	U76206	1·81	8·85	0·56	No	No
Fibrinogen, beta polypeptide	M35602	1·80	7·85	0·52	No	No
Cyclic Protein-2=cathepsin L proenzyme	S85184	1·74; 1·67	58·15	0·12; 0·12	Yes; Sertoli cells (Penttila et al. 1995, Wright et al. 2003)	Yes
Mast cell protease 7	U67910	1·74	20·6	0·85	No	No
UDP glycosyltransferase 1 family, polypeptide A7	AF039212	1·71	38·5	0·18	No	No
N-myc downstream-regulated gene 2	AA799560	1·70	185·55	0·10	No	No
Rat retinol-binding protein (RBP)	M10934	1·69	38·50	0·37	Yes; Sertoli cells (Eskild et al. 2000)	Yes
Crystallin, alpha B	M55534	1·67	43·90	0·84	Yes; spermatocytes (Kato et al. 1991)	No
Similar to microsomal glutathione S-transferase 3 (LOC289197)	AA892234	1·66	358·95	0·25	No	No
Ribonuclease 7 precursor; eosinophil cationic protein	D88586	1·63	30·7	0·27	No	No
Mitogen activated protein kinase kinase kinase 1	U48596	1·58	321·3	0·55	No	No
Cadherin 2; N-Cadherin	AF097593	1·57	65·15	0·30	Yes; Sertoli cells (Lampa et al. 1999)	Yes
Rat nuclear receptor co-regulator 1	M81766	1·56	75·85	0·09	Yes; Sertoli cells (Dufour & Kim 1999, Kastner et al. 1996)	No
Reelin	AA893471	1·55	39·25	0·44	Yes; (Ikeda & Terashima 1999, Andersen et al. 2003)	No
Vdup1; upregulated by 1,25-dihydroxyvitamin D-3	AI014169	1·55	490·7	0·20	No	No
PC4; interferon-related developmental regulator 1	AI014163	1·54	78·05	0·37	Yes; germ cells (Mbikay et al. 1997)	No
N-acetylglucosaminyltransferase I	D16302	1·51	132·45	0·09	Yes; (Nozaki et al. 2003)	No
mRNA for MHC class II antigen RT1.B-1 beta-chain	M36151	1·51	50·90	2·25	Yes; germ cells (Mori et al. 2000)	No
Rattus norvegicus transcribed sequences	AA891962	1·51	11·6	0·16	No	No

Table 3 *Continued*

	Accession number	Fold change	Average signal FSHAb	+/- s.e.m.	Testis expression previously reported	FSH regulation previously reported
Genes up-regulated by FSH suppression						
Steroidogenic acute regulatory protein	AB001349; AB006007	4·34; 4·21	59·85; 81·05	6·81; 1·36	Yes; Sertoli cells (Gregory & De Phillip 1998, McLean et al. 2002, Manna et al. 2003)	Yes
Aminolevulinic acid synthase 2	D86297	3·00	188·20	0·64	No	No
Phosphodiesterase 4B	AA799729	2·55; 1·80	144·5	0·51; 0·42	Yes; Sertoli cells (Swinnen et al. 1991)	Yes
MAD homolog 3 (Drosophila)	U66479	2·39	40·65	0·12	Yes; Sertoli, Leydig cells & spermatocytes (Xu et al. 2003)	No
Melanoma inhibitory activity; cartilage derived retinoic acid sensitive protein	U67884	2·27	357·45	1·12	No	No
Syndecan 1	S61865	2·24	82·10	0·48	Yes; Sertoli cells (Brucato et al. 2002)	Not tested
75 kDa glucose regulated protein	S78556	2·08	534·40	0·23	No	No
Gap junction membrane channel protein alpha 1 (gja-1); connexin43	AI029183	2·06	50·10	0·38	Yes; Sertoli, Leydig & germ cells (Sommersberg et al. 2000, Bravo-Moreno et al. 2002)	Yes
Neuronal activity-regulated pentraxin	S82649	2·05	253·4	1·51	No	No
Protein C inhibitor	AB013128	2·03	676·65	0·28	Yes; Leydig & germ cells (Uhrin et al. 2000, Odet et al. 2003)	No
Organic anion transporter protein 3 (OAT3); solute carrier family 21 (fatty acid transporter), member 7	AF041105	1·92	27·30	0·45	Yes	No
Amyloid beta (A4) precursor-like protein 2	X77934	1·87	431·55	0·20	Yes; (von der Kammer et al. 1994)	No
R. norvegicus cDNA clone rx05024 3'	AI639115	1·82	52·90	0·44	No	No
R. norvegicus similar to Esophagus cancer-related gene-2 protein precursor (ECRG-2) (LOC301016), mRNA	AA799734	1·82	336·75	0·23	No	No
Adducin 3, gamma	U35775	1·79	127·8	0·00	No	No
Monocarboxylate transporter (MCT1); solute Carrier family 16, member 1	D63834	1·77	45·8	0·20	Yes; germ cells (Jackson et al. 1997, Boussouar et al. 2003, Goddard et al. 2003)	No
Scavenger receptor class B, member 1	AA874843	1·77	345·00	0·35	Yes; Sertoli & Leydig cells (Reaven et al. 2000, Kawasaki et al. 2002)	No
Lysyl oxidase	AI234060	1·76	44·3	0·16	Yes; (Asuncion et al. 2001, Maki et al. 2001)	No
Transcribed sequence with moderate similarity to protein ref: NP_061921·1 (*Homo sapiens*) hypothetical protein FLJ20752 (*H. sapiens*)	AA799531	1·67	174·65	0·42	Yes; (Ericcson et al. 1993)	No
Proteolipid protein	AI070277	1·67	169·75	0·20	Yes; (Li et al. 2002)	No
IP63 protein	X99330	1·66	80·35	0·08	No	No
Fumarate hydratase 1	AI171734	1·63	61·45	0·18	No, but it is ubiquitous	No
cAMP responsive element modulator	U04835	1·63	36·85	0·74	Yes; (Walker et al. 1994, West et al. 1994, Monaco et al. 1995)	Yes
Lamin B1	U72353	1·63	41·55	0·59	Yes; Sertoli, Leydig, peritubular & germ cells (Machiels et al. 1997)	No
Cyclin D2	AA899106	1·60	279·55	0·45	Yes; Sertoli cells & spermatogonia (Wolgemuth et al. 1998, Buemer et al. 2000, Strohmann et al. 2004)	Yes
Androgen Binding Protein (ABP); sex hormone binding globulin	M31179	1·59	873·95	0·11	Yes; Sertoli cells (Skinner et al. 1989, Danzo 1995, Larriba et al. 1995, Joseph et al. 1997)	Yes
Superoxide dismutase 3	X68041	1·57	147·15	0·21	No	No
Chondroitin sulfate proteoglycan	AA818894	1·56	17·85	0·34	Yes; Leydig cells (Ungefroren et al. 1995)	No
Nicotinamide nucleotide transhydrogenase	AA891872	1·53	405·45	0·30	Yes; (Arkblad et al. 2002)	No
Transcribed sequence with moderate similarity to protein sp: P00722 (*E. coli*) BGAL_ECOLI Beta-galactosidase (Lactase)	AA892522	1·52	600·8	0·39	No	No
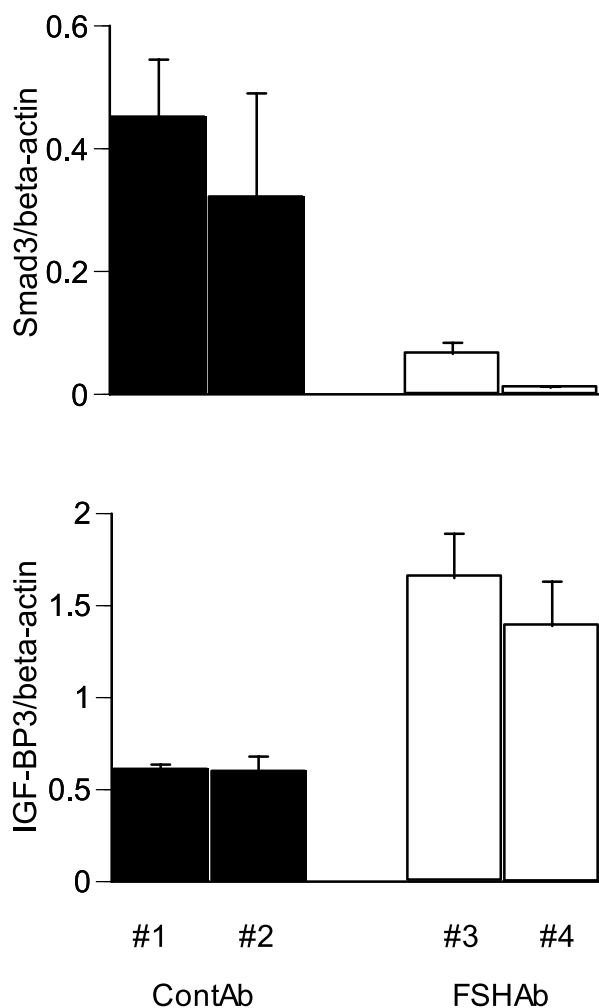
Figure 5 Expression levels of rat Smad 3 and IGFBP-3 by real time PCR. Two rats treated with normal sheep immunoglobulin (black bars) and treated with antibody raised against rat FSH (white bars) were tested to validate data obtained from the microarray analysis. Each sample was measured over three separate experiments in terms of ng of mRNA normalised to beta actin. Data are mean ± S.D.

increase the dose of neutralising antibody if in fact residual levels of FSH exist. The Sertoli cell numbers measured in control antibody-treated groups in this study are in accord with our previous study (Meachem *et al.* 1996) and that of others (Simorangkir *et al.* 1997), reporting numbers quantified by the optical disector stereological technique, in rats of similar ages.
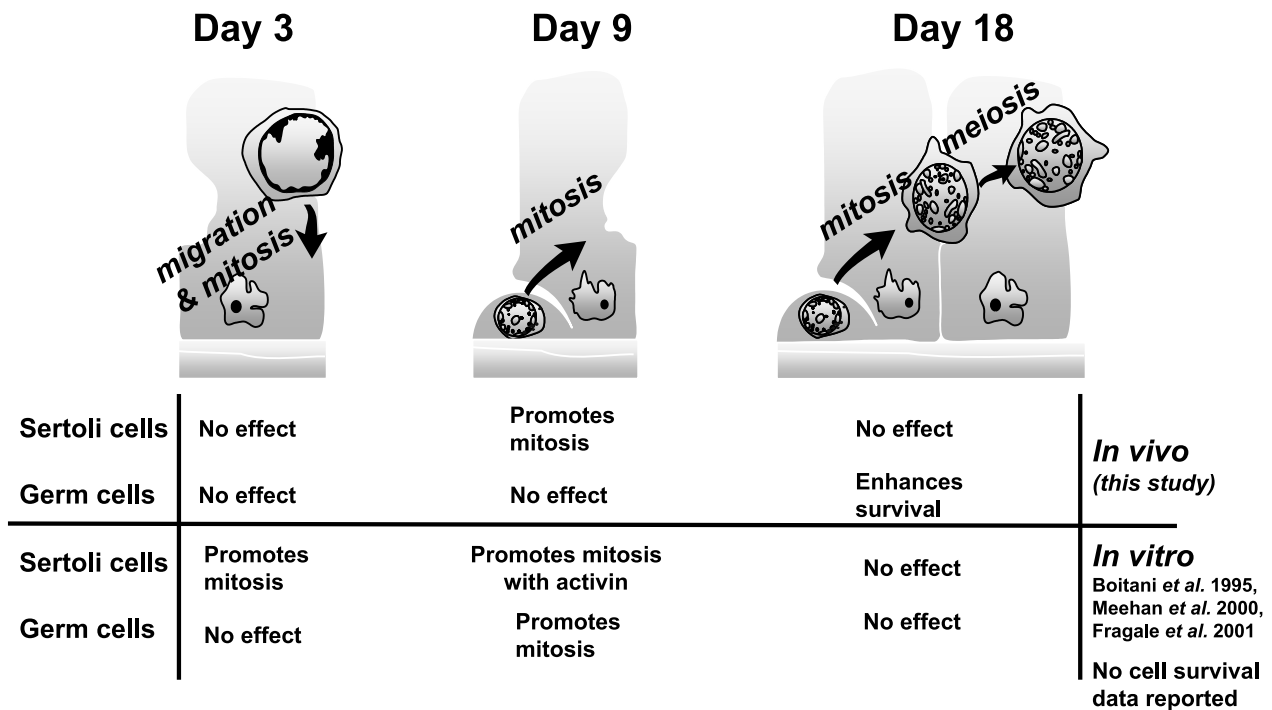
Germ cell development

These data provide no evidence that FSH supports the maturation of gonocytes into type A spermatogonia. Studies of congenitally deficient hypogonadal mice (hpg) lacking both FSH and LH show that gonocyte number is

not altered in these newborn mice compared with wild-type controls, however gonocyte number is significantly reduced at 5 dpp, suggesting that gonocyte survival may be in part regulated by gonadotrophins. Whether this is an effect specific to FSH is yet to be elucidated (Baker & O'Shaughnessy 2001). Several other recent studies highlight the impact of locally produced members of the TGF↳ superfamily on germ cell development, including bone morphogenetic protein 4 (BMP4) (Pellegrini *et al.* 2003), activin (Boitani *et al.* 1995, Meehan *et al.* 2000) and GDNF (Meng *et al.* 2000, Yomogida *et al.* 2003), and production of both inhibin (an activin antagonist) and GDNF is regulated by FSH (Tadokoro *et al.* 2002). Meehan *et al.* (2000) provides *in vitro* evidence, using 1 and 3 day cultures of 3 dpp rat testis fragments, that FSH plus follistatin mediates gonocyte maturation into type A spermatogonia, but that FSH alone does not support gonocytes, as assessed on a per Sertoli cell basis. Meehan's study reported that activin independently elevated gonocyte number 4-fold above that of DMEM treated fragments and that follistatin prevented this activin-induced rise, giving rise to the concept that balance between activin and follistatin can influence germ cell maturation at the onset of spermatogenesis. Another facet of first wave initiation, gonocyte migration, relies at least in part on the interaction of c-kit and its FSH-regulated ligand, SCF (Orth *et al.* 2000), and it is apparent that the population of migratory gonocytes have stem cell activity, while those lacking pseudopods are undergoing apoptosis (Orwig *et al.* 2002). Thus influence of FSH at this age may reflect a complex set of interacting signals.

Several lines of evidence indicate that FSH enables spermatogonial survival in the adult rat, (Shetty *et al.* 1996, Meachem *et al.* 1999) without directly enhancing germ cell proliferation *in vivo* (McLachlan *et al.* 1995). In immature rats rendered FSH- and LH-replete by hypophysectomy, administration of FSH reduced the number of degenerating cells (Russell *et al.* 1987) and prevented germ cell apoptosis (Tapanainen *et al.* 1993). Evidence from culture of adult rat seminiferous tubules (Henriksen *et al.* 1996) and of immature rat testis fragments (Boitani *et al.* 1993) indicates that FSH also enhances proliferation of differentiated spermatogonia *in vitro*. The significant reduction in the spermatogonial population after 4 days of FSH suppression in the current study provides evidence that FSH influences survival of germ cell populations from 14–18 dpp. The reduction in germ cell number reduction appears attributable to a 2·5-fold higher apoptotic index compared with control levels, not to a change in proliferation rate. We also observed that spermatogonial numbers were not influenced by FSH at 9 days after birth, and this finding is inconsistent with *in vitro* data (Boitani *et al.* 1995). Low levels of circulating FSH may be present in the animals examined in this study; this may be sufficient to support spermatogonial development, as small increases in FSH can significantly impact the recovery of

Figure 6 Dynamic effects of FSH on Sertoli and germ cell development during first wave in rats assessed through *in vivo* and *in vitro* analyses. Time points listed illustrate progressive maturation of germ cells and Sertoli cells in the first wave of spermatogenesis in rat. At 3 dpp, Sertoli cells are proliferating and gonocytes are re-entering the cell cycle and migrating to the basement membrane of the seminiferous cord to become undifferentiated and stem cell spermatogonia. At 9 dpp, proliferating Sertoli cells and differentiating spermatogonia are observed. By 18 dpp, Sertoli cell proliferation has ceased, and the cords have transformed into seminiferous tubules as tight junctions form between Sertoli cells and vectorial secretion from this epithelium commences. Germ cells that have progressed to the pachytene stage of meiosis are predominant in the seminiferous tubules. In this study, suppression of FSH by passive immunisation for 2 and 4 days revealed the age-dependent differential *in vivo* affects of FSH on Sertoli and germ cells: promotion of Sertoli cell mitosis at 9 dpp and increasing germ cell viability at 18 dpp. These data are in agreement with previous *in vitro* observations that FSH regulates Sertoli cell proliferation in an age-dependent manner, interacting with activin to promote Sertoli proliferation and germ cell development at discrete ages. This comparison also highlights differences that may specifically reflect the use of *in vitro* versus *in vivo* models.

spermatogonial number in adult rats (Meachem *et al.* 1998). In the present study, spermatogonial number was assessed using a sensitive, unbiased optical disector method appropriate for counting these cells. Effects of lowered FSH levels may have been masked due to factors associated with counting a small population of cells. Spermatogonial and spermatocyte numbers in control antibody-treated rats in this study were in accord with our (Meachem *et al.* 1996) and that of others (Simorangkir *et al.* 1997) reporting data that were assessed in rats of similar ages by the using the optical disector technique.

Age dependent changes in cell survival resulting from FSH suppression may be due to developmentally regulated changes in the cellular synthesis of apoptotic regulators, such that documented for members of the Bcl-2 family during the first wave of mouse spermatogenesis (Meehan *et al.* 2001). The *Bcl-w* (Yan *et al.* 2000) and *Bok* (Suominen *et al.* 2001) mRNAs were shown to be up- and down-regulated respectively by FSH *in vitro*, and the *bcl-w* knockout mouse has elevated germ cell apoptosis following

14 dpp, indicating the potential influence of Bcl-2 family members on the cellular responses in this model.

Identification of genes regulated by FSH in vivo

Interrogation of a rat gene microarray with RNA from 18 dpp rats in this study identified 60 genes that were regulated by 4 days of FSH suppression *in vivo*. Twenty percent of these differentially expressed genes have been previously identified as FSH-regulated, predominantly through *in vitro* methods, a finding which indicates the validity of this model and experimental approach. For example, in an analysis of 20 dpp rat Sertoli cells using the same microarray platform, FSH-responsive mRNAs were identified at 2, 4, 8 and 24 h of culture (McLean *et al.* 2002). In accord with the present analysis, that *in vitro* analysis identified the genes encoding steroidogenic acute regulatory (StAR) protein and endothelin as down- and up- regulated, respectively, in the absence of FSH. FSH has now been identified through both *in vitro*

(McLean *et al.* 2002) and *in vivo* studies to be one of the wide variety of transcriptional regulators of StAR mRNA synthesis (Gregory & De Phillip 1998, Manna *et al.* 2003). Cyclin D2 and androgen binding protein (ABP) are additional Sertoli cell proteins up-regulated by FSH that are required for normal testicular function (Skinner *et al.* 1989, Danzo 1995, Larriba *et al.* 1995, Sicinski *et al.* 1996, Joseph *et al.* 1997), while protein C inhibitor, also required for spermatogenesis (Uhrin *et al.* 2000, Odet *et al.* 2003), appears to be synthesized only in germ cells and Leydig cells (Odet *et al.* 2003). *In vivo* confirmation of the negative regulation by FSH of genes encoding insulin-like growth factor binding protein-3 (IGFBP-3) (Smith *et al.* 1990, Rappaport & Smith 1995, Khan *et al.* 2002), HNF-3/ forkhead (Wolfrum *et al.* 2003), transferring (Kaestner *et al.* 1998), endothelin-1 (Fantoni *et al.* 1993, Tripiciano *et al.* 1999) and cathepsin-L (Penttila *et al.* 1995, Wright *et al.* 2003) is presented. Members of the HNF-3/ forkhead transcription factor family are part of a signal transduction cascade from Akt/protein kinase B that regulates transcription of apoptosis-related genes (Wolfrum *et al.* 2003). HNF-gamma is known to regulate transferrin (Kaestner *et al.* 1998), a gene that is itself regulated by FSH (Migrenne *et al.* 2003); we hypothesize that HNF-3 represents an intermediate for FSH regulation of several genes.

The high concordance of the present data with previous reports provides validation of the methodological approach used to identify *in vivo* targets of FSH regulation, though some differences may be noted. In this study, FSH suppression led to elevated testicular N-cadherin mRNA, but in a previous 2 day *in vitro* analysis, FSH alone did not affect N-cadherin expression; FSH in combination with testosterone stimulated it (Lampa *et al.* 1999). This most likely reflects a specific difference between *in vitro* and *in vivo* models regarding hormone responses; alternatively, the duration of suppression or addition may underpin the difference between these two results.

In addition, novel candidates for FSH regulation have been uncovered, some of which may be indirectly regulated by altered levels of available FSH or represent bona fide novel FSH-regulated genes. Targets not previously identified in the testis include aminolevulinic acid synthase 2 and melanoma inhibitory activity.

Several categories of encoded gene products within the array of candidate FSH target genes may aid identification of the pathways and processes that underpin the reduction in germ cell survival that was measured after 4 days of treatment with the FSHAb. Most striking are the genes relating to cell cycle regulation and apoptosis (e.g. lamin, cyclin D1, cyclin D2, Scavenger Receptor B1). Products of other FSH target genes comprise or influence the architecture of the seminiferous epithelium (e.g. cadherin, cathepsin L), while others are known to function in signal transduction (e.g. HNF3, MAPKKK, Smad 3, rat retinol-binding protein) and to participate in regulation of hor-

monal inputs (StAR and androgen binding protein). However, because the microarray analysis presented in this study addresses a single time point, early and acutely affected FSH gene targets may be undetected. The cellular responses identified in this study (i.e. germ cell apoptosis) would have been initiated before the time point of analysis, so future studies will address this in order to more fully comprehend the pathways through which FSH levels influence Sertoli cell function and germ cell survival.

The limit for microarray signal changes considered to be significant was a 1·5-fold difference between the control and treatment groups. The highest difference detected was 6·57-fold, with several others showing around a 4-fold difference. While this level of change is low in comparison to systems where a single cell type is tested, these data do demonstrate that this approach can identify FSH-regulated genes in a complex cell mixture (i.e. total testis). This is, in fact, comparable to the situation that might be required for analysis of clinical samples, where the small sample size would preclude the use of cell separation methods prior to analysis. The fact that up- and down-regulation of genes is measurable following 4 days of treatment indicates that in conditions where FSH suppression has been long term, FSH target gene expression interrogation may be a useful tool for identifying the basis of pathological change. It is important to note that some alterations in gene expression may be in part attributed to the reduction in testicular weight as a consequence of cell loss. However, we believe the disproportionate change in gene expression (1·5–6 fold) compared with the 22% reduction in testis weight following FSH suppression indicates that the changes in gene expression are primarily due to specific FSH effects.

In conclusion, the model used in this study has successfully identified distinct developmental responses to FSH in Sertoli and germ cells in the postnatal rat and identified candidate genes that may underpin these responses at 18 dpp. These data illustrate the switch in Sertoli cell function during the first spermatogenic wave, as they cease proliferation and establish a niche for support of germ cell development. In addition, the emerging dependence of germ cells on Sertoli cell-derived products can be explored through identification of FSH-target genes and the pathways in which they function.

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