Pathways involved in testicular germ cell apoptosis in immature rats after FSH suppression

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Abstract

FSH is a key regulator of testis function, required for the establishment of full complements of Sertoli and germ cells during postnatal testis development and for the maintenance of spermatogenesis in the adult. FSH plays an important role in germ cell survival rather than proliferation, in the window between 14 and 18 days of testicular development, which coincides with the cessation of Sertoli cell proliferation and the onset of germ cell meiosis during the first wave of spermatogenesis. This study aimed to identify the pathway(s) of apoptosis regulated by changes in FSH levels in 14- to 18-day-old rats, using a model of *in vivo* FSH suppression by passive immunoneutralization with a rat anti-FSH antibody. Apoptotic pathways were identified by immunohistochemistry using pathway-specific proteins as markers of the intrinsic (activated caspase 9) and extrinsic (activated caspase 8) pathways, followed by quantification of cell numbers using stereological techniques. In addition, RT-PCR was used to

assess the expression of pathway-specific genes. We previously reported a 2.5-fold increase in spermatogonial apoptosis in these samples after 4 days of FSH suppression, and now show that this increase correlates with a 9.8-fold ($P < 0.001$) increase in the frequency of caspase 9-positive spermatogonia in the absence of caspase 8 immunoreactivity. By contrast, spermatocytes exhibited both increased caspase 9 (7.5-fold; $P < 0.001$) and caspase 8 (5.7 fold; $P < 0.001$) immunoreactivities after 4 days of FSH suppression. No significant change in the transcription levels of candidate genes required for either pathway was detected. This study demonstrates that, in the seminiferous tubules, FSH suppression induces spermatogonial apoptosis predominantly via the intrinsic pathway, while spermatocyte apoptosis occurs via both the intrinsic and extrinsic pathways.

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Introduction

Sperm output in adulthood relies on the establishment of the Sertoli cell population and initiation of germ cell development during foetal and early postnatal life. In rats, Sertoli cells proliferate from the time of gender specification at embryonic day 11.5 until around 15 days after birth (15 dpp), and this complement of Sertoli cells is sustained throughout adulthood (Orth *et al.* 1988). The first wave of spermatogenesis is initiated when gonocytes differentiate first into spermatogonia at 3 dpp; these first become spermatocytes at ~9 dpp, form haploid spermatids by 18 dpp and then first form spermatozoa at 43 dpp (de Rooij 1998).

Both Sertoli and germ cell populations are regulated during the first wave of spermatogenesis by endocrine signals including follicle-stimulating hormone (FSH) and luteinizing hormone (LH)/testosterone (T). A key role for FSH in regulating the size of the Sertoli cell population in early postnatal life has been attributed to its stimulatory effect on

Sertoli cell division (Orth 1984, Boitani *et al.* 1995, Orth *et al.* 1998, Meachem *et al.* 2005), with no evidence for an effect on survival (Meachem *et al.* 2005). We have previously demonstrated the absence of significant changes in apoptosis and proliferation rates of germ cells in 3 dpp and 9 dpp rats following FSH suppression by immunoneutralization of the circulating hormone (Meachem *et al.* 2005). However, at 18 dpp, we documented a 2.5-fold increase in spermatogonial apoptosis (control versus FSH-suppressed groups: $6.2 \pm 1.0\%$ vs $15.5 \pm 1.6\%$; $P < 0.001$), suggesting that FSH acts as a germ cell survival factor rather than as a proliferative factor (Meachem *et al.* 2005). The apoptotic pathway(s) by which this germ cell apoptosis is (are) executed in response to FSH suppression is unknown.

Two pathways of apoptosis are described as active in the testis: the intrinsic and extrinsic pathways (reviewed in Sinha-Hikim *et al.* 2003a). The intrinsic pathway (or mitochondrial pathway) involves translocation of BAX from the cytosol to the mitochondria where it causes cytochrome C release into

the cytosol. Cytochrome C then binds to the apoptotic protease activating factor-1, resulting in the activation of the initiator caspase 9 and subsequent activation of executioner caspases 3, 6 and 7, leading to apoptosis (Adams & Cory 1998, Green 2000, Hengartner 2000). The BCL2 protein family members, such as BCL2L2 (formerly BCL-W), have been shown to be involved in this pathway by the formation of dimers with BAX (Adams & Cory 1998). The extrinsic pathway (or death receptor pathway) involves Fas ligand stimulation of FAS protein on target cells, which then activates the initiator caspase 8, and subsequently activates executioner caspases, effecting apoptosis (Nagata & Golstein 1995, Lee *et al.* 1997).

In adult rats, the intrinsic pathway has recently been shown to be involved in spermatogonial apoptosis following selective FSH suppression (Ruwanpura *et al.* 2007), while both the intrinsic and extrinsic pathways are involved in spermatocyte and spermatid apoptosis following selective T withdrawal (Nandi *et al.* 1999, Woolveridge *et al.* 1999). During the first wave of spermatogenesis, the extrinsic pathway of apoptosis functions during programmed germ cell apoptosis (Moreno *et al.* 2006, Codelia *et al.* 2007, Lizama *et al.* 2007). In immature (8–13 dpp) *hpg* mice lacking gonadotropins, spermatogonial and spermatocyte apoptosis occurs via both apoptotic pathways (Chausiaux *et al.* 2008). However, in immature rats, the regulation of each of these apoptotic pathways by hormones (FSH and/or LH/T) remains unknown.

To understand the mechanisms of FSH action on germ cell apoptosis in the immature rat *in vivo*, we have used the experimental model of FSH suppression by passive immunoneutralization with an anti-FSH antibody administered for 4 days to 14 dpp normal rats (Meachem *et al.* 2005). We hypothesized that acute FSH suppression would result in accelerated germ cell apoptosis via activation of the intrinsic and/or extrinsic pathways. In this study, we aimed to determine the pathway(s) involved in germ cell apoptosis by employing antibody detection systems directed to the activated caspase forms (aCaspase 9: intrinsic pathway, aCaspase 8: extrinsic pathway) in combination with germ cell enumeration by stereology. In addition, we quantified the expression levels of candidate genes from both apoptotic pathways.

Materials and Methods

Animals

Testicular tissues were obtained from male 18 dpp outbred Sprague–Dawley rats after 4 days of acute FSH suppression starting on 14 dpp (Meachem *et al.* 2005). All investigations conformed to the NHMRC/CSIRO/AAC Code of Practice for the Care and Use of Animals for Experimental Purposes and were approved by the Monash University Standing Committee on Ethics in Animal Experimentation.

Experimental design

Passive immunization against FSH Rats at 14 dpp ($n=5$ per group) were immunized either with a purified in-house polyclonal ovine immunoglobulin fraction derived from antisera raised against rat FSH (FSHAb) or with normal sheep immunoglobulin (ConAb; Meachem *et al.* 1998, 2005). Each animal received a daily dose of FSHAb (10 mg/kg in saline) by s.c. injections for 4 days, a fivefold higher dose of FSHAb (Meachem *et al.* 2005) capable of neutralizing >90% of serum FSH levels in adult rats within 24 h (Meachem *et al.* 1998). FSHAb treatment does not alter the serum testosterone in this immature rat model (Meachem *et al.* 2005). Rats were killed 24 h after their final injection at 18 dpp.

Positive control tissues The heat-treated adult rat testis (courtesy of Dr Amiya Sinha Hikim) was used as the positive control for immunohistochemical identification of intrinsic pathway activation (Sinha-Hikim *et al.* 2003b).

Tissue collection and preparation

Testes were excised and the right testis of each rat snap frozen in liquid nitrogen and stored at -80°C for total RNA preparation. The left testes were immersion fixed with Bouin's solution for 5 h, weighed and then sampled (Meachem *et al.* 2005). The sampled tissues were then embedded in paraffin for immunohistochemistry. Prior to immunostaining, 5 μm tissue sections were prepared, deparaffinized and hydrated through a series of progressively decreasing ethanol concentrations and rinsed with PBS (10 mM, pH 7.4).

Assessment of apoptotic pathways

The activation of apoptotic pathways has been assessed with a previously validated immunohistochemistry procedure employing antibodies against the activated forms of pathway-specific caspases (Ruwanpura *et al.* 2007). In brief, aCaspase 9 antibody (0.76 $\mu\text{g}/\text{ml}$ in PBS; this rabbit polyclonal antibody detects p17 and p37 of aCaspase protein; Cell Signaling Technology, Danvers, MA, USA) was used to identify the intrinsic pathway activation, and aCaspase 8 antibody (2.4 $\mu\text{g}/\text{ml}$ in PBS; this mouse monoclonal antibody detects only the N-terminal region of the p18 subunit; Novocastra Laboratories, Newcastle, UK) was used for the extrinsic pathway detection. On negative control sections, the primary aCaspase 9 and 8 antibodies were substituted with the same concentration of rabbit and mouse IgG antibodies (Biosciences, Franklin Lakes, NJ, USA) respectively.

Quantification of labelled cells

Stereological techniques were applied to determine the percentages of aCaspase 9- or 8-labelled cells as described previously (Ruwanpura *et al.* 2007, 2008). Upon activation,

caspases translocate from the cytoplasm to the nucleus; therefore intracellular localization of activated caspase varies during apoptotic pathway activation. The aCaspase-labelled cells were identified by brown nuclear, cytoplasmic and whole cell staining. Germ cell types were identified by their location within the seminiferous tubules, in conjunction with their nuclear size and shape. Cells within the seminiferous epithelium were classified as spermatogonia, spermatocytes or Sertoli cells (Russell *et al.* 1990). The percentages of labelled cells was assessed using an unbiased counting frame of 405 μm^2 per field superimposed on a video image by CASTGRID V1.60 software package (Olympus, Denmark, Germany), wherein 50–200 cell nuclei for each cell group were counted per rat. All slides were masked prior to the analysis. The extent of caspase activation was calculated by dividing the number of labelled cells by the total number of labelled and unlabelled cells in each group.

Immunofluorescence and confocal studies

To determine the prevalence of cells exhibiting the marker of each apoptotic pathway, the co-localization of TUNEL-labelled cells with aCaspase 9 or 8 proteins was detected by confocal microscopy using immunofluorescent dual labelling (Ruwanpura *et al.* 2007, 2008). *In situ* detection of cells with DNA fragmentation was performed on tissue sections using an Apoptag fluorescein *in situ* apoptosis detection kit (Chemicon International, Temecula, MA, USA). For staining of aCaspases, antibodies to aCaspase 9 (Cell Signaling Technology) or aCaspase 8 (2.4 $\mu\text{g}/\text{ml}$ in PBS; this rabbit monoclonal antibody detects only the cleaved product p18, 41, 43 of active caspase 8 protein; Cell Signaling Technology) with secondary antibody goat anti-rabbit Alexa 546 secondary antibody (Molecular Probes, Eugene, OR, USA) were used. For negative control sections, TdT was omitted and the lack of secondary antibody cross-reactivity was verified by the substitution of the equivalent concentration of an isotype control antibody.

The proportions of TUNEL-labelled germ cells that were either aCaspase 9- or 8-positive were quantified by counting all the labelled and dual labelled cells, as described previously (Ruwanpura *et al.* 2007, 2008). TUNEL-labelled cells were observed with varying aCaspase staining intensity and were therefore designated as aCaspase-low or aCaspase-high. Only aCaspase-high TUNEL cells were included in the quantification for dual labelling.

Total RNA extraction and reverse transcription (RT)

Real-time PCR was used to measure the relative levels of five candidate apoptotic pathway-specific genes. Total RNA was extracted from testes treated with ConAb and FSHAb ($n=3$ per group), using a total RNA extraction kit (Qiagen). Any contaminating residual genomic DNA was removed using a DNase-free kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA (500 ng) was reverse

transcribed to cDNA in a final volume of 20 μl using Superscript III according to the manufacturer's protocol (Invitrogen). The absence of contaminating genomic DNA in total RNA samples was confirmed using reactions in which RT enzyme was omitted.

Real-time PCR analysis

Quantitative real-time PCR analysis was performed using the Roche LightCycler and the FastStart DNA Master SYBR Green I system (Roche). Oligonucleotide primer sequences specific for rat β -actin (*Actb*), *Bax* and *Bcl2l2* were designed using Primer3 (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), and *Casp9* (caspase 9 gene), *Casp8* (caspase 8 gene) and *Fas* were obtained from published sources (Table 1). For PCR analyses, sample cDNA was diluted 1:4- to 1:80-fold. PCR conditions, including primer concentrations, Mg^{2+} concentration, annealing temperature and time, and extension time were optimized for each primer pair (Table 1). For all PCR analyses, standard curves were produced using dilutions of an immature 18 dpp ConAb-treated rat testicular cDNA preparation assigned an arbitrary unitage. PCR of standards was performed using duplicate reactions, and samples were measured in triplicate for ~ 38 –40 cycles, after which a melting curve analysis was performed to monitor PCR product purity (Table 1). Amplification of a single PCR product was confirmed by agarose gel electrophoresis and DNA sequencing (data not shown). To compare the expression levels of the apoptotic pathway-specific genes in groups, we normalized the data collected in our RNA analysis with the housekeeping gene, *Actb*.

Statistical analysis

All statistical analyses were performed using SigmaStat for Windows version 3.1 (Jandel Corporation, San Rafael, CA, USA). Data showing a normal distribution were analyzed using a *t*-test, and if data did not show normal distribution, Mann–Whitney test was performed. Data are expressed as mean $\pm$ S.E.M. (for all histological data) or S.D. (for all gene expression levels), with $n=3$ –5 rats per group as indicated.

Results

FSH suppression increases intrinsic pathway activity

In immature rats, 4 days of FSH suppression increased the proportion of TUNEL-labelled germ cells that were also positive for aCaspase 9 (by 1.3-fold) to $60.5 \pm 2.3\%$ ($P=0.03$), compared with $53.5 \pm 1.6\%$ in ConAb-treated samples (Fig. 1A), while no differences were seen in the proportion of aCaspase 8-positive, TUNEL-labelled germ cells between groups (Fig. 1C, D, F and G).

Table 1 Primer-specific LightCycler conditions used for PCR amplification of candidate genes

Name (accession)	Primer sequence	Product size (bp)	Primer conc (pmol)	Mg ²⁺ conc (mM)	Anneal temp (°C)	Extension time (s)	Data temp (°C) ^a	PCR product melt temp (°C) ^b
Rat Bax (AF235993)	F CCAGCATCGACGAGAGG R CGGAGGAAGTCCAGTGCC	342	40	2.0	60	16	84	89.2
Rat Bcl2/2 (AF096291)	F GAGTTTGAGACCCGCTCC R GTCTTCACTGATGCCAGTT	362	40	3.0	60	16	82	91.1
Casp9 (NM031632)	F AGCCAGATGCTGCCATAC R CAGGAGACAAACCTGGGAA	132	40	3.0	66	10	82	88
Huang et al. (2005)	F CTGCGAAGATCGACGATTA R CATGTCCTGCATTTTGATGG	123	40	4.0	66	10	82	84.2
Casp8 (AF288372)	F GCAATGCTTCTCTGTGACCACT R GCTGTGTGCTCGATCTCATCG	351	40	3.5	67	15	82	85.4
Huang et al. (2006)	F CCGTAAAGACCTCTATGCCAACA R GCTAGGAGCCAGGGCAGTAATC	103	50	2.5	67	15	72	86
Rat Fas (D26112)								
Tolba et al. (2006)								
Rat Actb (NM031144)								

^aRefers to the temperature at which the fluorescence of the PCR product was quantified during LightCycler analysis.
^bAs determined by melting temperature analysis on the LightCycler.

FSH suppression leads to germ cell apoptosis via the intrinsic apoptotic pathway

FSHAb treatment compared with ConAb treatment for 4 days resulted in a 9.8-fold increase in aCaspase 9-labelled spermatogonia ($0.6 \pm 0.4\%$ vs $5.6 \pm 0.3\%$; $P < 0.001$) and a 7.5-fold increase in aCaspase 9-labelled spermatocytes ($1.6 \pm 0.4\%$ vs $11.7 \pm 1.1\%$; $P < 0.001$; Fig. 2A–C). No aCaspase 9-labelled Sertoli cells were observed in any samples (data not shown).

FSH suppression leads to spermatocyte apoptosis via the extrinsic apoptotic pathway

In immature rats treated with ConAb and FSHAb for 4 days, no aCaspase 8-labelled spermatogonia were observed. However, FSHAb treatment for 4 days resulted in a 5.7-fold increase in aCaspase 8-labelled spermatocytes ($2.1 \pm 0.8\%$ vs $11.8 \pm 1.1\%$; $P < 0.001$), compared with ConAb treatment (Fig. 3A–C). A few aCaspase 8-labelled Sertoli cells were observed in both ConAb- and FSHAb-treated rats (data not shown).

FSH suppression has no effect on intrinsic and extrinsic pathway-specific candidate gene expression levels

Changes in the expression of three intrinsic pathway-specific genes, *Casp9*, *Bcl2/2* and *Bax*, and two extrinsic pathway-specific genes, *Casp8* and *Fas*, were examined by real-time PCR analysis of whole testis RNA. After 4 days of FSH suppression, consistent trends indicating reductions in *Casp9* (1.9-fold), *Bcl2/2* (1.4-fold), *Bax* (1.4-fold), *Casp8* (1.7-fold) and *Fas* (1.3-fold) mRNA levels were seen compared with the ConAb treatment group; however, these did not achieve significance (Fig. 4).

Discussion

We demonstrate here that suppression of FSH affects the activity of both intrinsic and extrinsic apoptotic pathways in the immature rat testis in a cell-selective manner. We previously reported a 2.5-fold increase in spermatogonial apoptosis (control versus FSH-suppressed groups: $6.2 \pm 1.0\%$ vs $15.5 \pm 1.6\%$; $P < 0.001$) occurs following acute FSH suppression for 4 days in 18 dpp old rats (Meachem et al. 2005) and now show that this occurs principally via the intrinsic, rather than the extrinsic pathway. In addition, we show that FSH actions are important in spermatocyte survival through the regulation of both apoptotic pathways. No significant change in mRNA expression levels of pathway-specific genes was documented. These data indicate that the mechanism by which FSH influences germ cell survival differs between germ cell subtypes during the first wave of spermatogenesis.

Figure 1 The proportion of TUNEL-labelled germ cells with aCaspase 9 or 8 reactivity (156/298 vs 130/213:100/238 vs 123/283 respectively) in 18 dpp rats that had received ConAb (white bars) or FSHAb (black bars) for 4 days (A). Data are mean $\pm$ S.E.M. ($n=5$ per group). Asterisks denote significant differences between FSH-suppressed rats and the corresponding ConAb-treated rats ($*P<0.05$). Photomicrographs of rat testis cross sections illustrate the presence of aCaspase 9 (B–D) and 8 (E–F), both shown in red in rat germ cells with caspase activity. TUNEL-positive cells are shown in green. Dual labelling of caspases with TUNEL appears in yellow (see arrows). (B) shows a cross section of heated adult rat testis (intrinsic pathway quality control; see also Ruwanpura *et al.* 2007). (C) and (E) illustrate cells containing aCaspase 9 and 8 respectively in testes of rats that received ConAb for 4 days. (D) and (F) indicate aCaspase 9 and 8 respectively in testes of rats receiving FSHAb for 4 days. Inserts are negative controls incubated with an isotype-matched irrelevant antibody.

Figure 2 The percentage of aCaspase 9-labelled spermatogonia and spermatocytes (A), in 18 dpp rats that received ConAb (white bars) or FSHAb (black bars) for 4 days. Data are means $\pm$ S.E.M. ($n=5$ per group). Asterisks denote significant differences between FSH-suppressed rats in comparison with corresponding ConAb-treated samples (** $P<0.001$). Photomicrographs represent the staining of aCaspase 9 in testis cross sections for ConAb (B) and FSHAb (C) groups. Inserts illustrate the absence of a signal in negative controls.

This is the first demonstration that reduced FSH levels lead to decreased spermatogonial survival selectively via the intrinsic apoptotic pathway during the first wave of spermatogenesis. BAX, a component of the intrinsic pathway, is understood to function to control apoptosis in germ cells. In mice lacking *Bax*, spermatogonial numbers are elevated as the consequence of the failure of programmed apoptosis during the first wave of spermatogenesis (Knudson *et al.* 1995, Koji 2001). BAX protein also appears to be localized to spermatogonia at this time (Rodriguez *et al.* 1997). Similar to the findings presented here, we have previously shown that FSH withdrawal primarily affects intrinsic pathway activity in spermatogonia of adult rats (Ruwanpura *et al.* 2007). Data from gonadotropin-suppressed men demonstrated that gonadotropins regulate spermatogonial survival via only the intrinsic pathway, even though specific effects of FSH on spermatocyte survival on these men are unknown (Ruwanpura *et al.* 2008). However, the mechanism by which FSH regulates the intrinsic apoptotic pathway and spermatogonial development remains elusive. Based on

knowledge from other systems, the actions of FSH-regulated factors could provoke changes in mitochondrial permeability, allowing factors such as cytochrome C and BAX to be transported through the mitochondria, leading to apoptosis (Erkkila *et al.* 1999). The absence of various extrinsic pathway components in spermatogonia, such as FAS (Nandi *et al.* 1999), provides a simple explanation of why spermatogonial apoptosis via this pathway was not seen in mammals (D'Alessio *et al.* 2001, Chausiaux *et al.* 2008, Ruwanpura *et al.* 2007, 2008).

Our data suggest that FSH regulates both intrinsic and extrinsic apoptotic pathways during the meiotic phase of the first wave of spermatogenesis. This is in agreement with the observations of the phase when meiosis begins in immature (13 dpp) *hpg* mice. Chausiaux *et al.* (2008) reported increases in cleaved caspase 8 and 9 protein levels coinciding with increased spermatocyte apoptosis. During the first wave of spermatogenesis, physiological spermatocyte apoptosis correlates with BAX redistribution, cytochrome C redistribution and the activation of caspase 3 and FAS up-regulation (Lizama *et al.* 2007).

Figure 3 The percentage of aCaspase 8-labelled spermatogonia and spermatocytes (A) in ConAb (white bars) or FSHAb (black bars) samples. Data are means $\pm$ S.E.M. ($n=5$ per group). Asterisks denote significant differences between treatment groups (** $P<0.001$). Photomicrographs demonstrate aCaspase 8 signals in ConAb (B) and FSHAb (C) samples respectively. Inserts illustrate negative control signals.

Circulating FSH levels also affect spermatocyte and spermatid apoptosis via both pathways in FSH-suppressed adult rats and gonadotropin-suppressed men (Ruwanpura *et al.* 2007, 2008). It is of note that while previously we measured a 1.5-fold increase in spermatocyte apoptosis (control versus FSH-suppressed groups: $11.8 \pm 1.4\%$ vs $16.1 \pm 2.2\%$; $P=0.13$) in FSH-suppressed immature rats (Meachem *et al.* 2005), we now report a six- to eightfold increase in caspase activity within this cell population. This difference most likely reflects the fact that initiator caspase activation is an upstream event in the apoptosis pathway, while TUNEL marks the latter phase of the pathway and such cells are rapidly lost.

Interestingly, in the subset of cells positive for TUNEL reactivity, FSH suppression only showed significant increases in the intrinsic pathways, but not in the extrinsic pathways (i.e. using dual labelling immunofluorescence). However, the significant increase in the proportion of aCaspase 8-labelled spermatocytes indicates that the approach employed was sufficient to detect extrinsic pathway activity in the germ cells (i.e. using single labelling immunohistochemistry). The apparent lack of change in caspase 8 activity recorded here

for all apoptotic germ cells may be further refined using molecular markers to distinguish the subpopulations of these cells, and the samples in the present study will be suited for such an analysis when such markers that are compatible with the staining procedures used here become available. There may be another possible explanation for the no change in caspase 8 activity. It may be due to crosstalk between the apoptotic pathways. In many cell types, caspase 8 directly activates the executioner caspase, while in some cells FAS triggering induces the intrinsic pathway via a cleavage of the BCL2 protein family member, BID. BID can then induce BAX-mediated release of cytochrome C from mitochondria, further committing the cell to apoptosis via the intrinsic pathway (Scaffidi *et al.* 1999, Said *et al.* 2004). This type of crosstalk has been seen for programmed spermatocyte apoptosis during the first wave of spermatogenesis in normal rats (Moreno *et al.* 2006, Lizama *et al.* 2007). However, we have not undertaken such analyses in this study. To our knowledge, there is no evidence for this crosstalk in other germ cell death models such as hormone deprivation, heat or genotoxic stress.

Figure 4 The mRNA levels of *Casp9*, *Bcl2l2*, *Bax*, *Casp8* and *Fas* in ConAb (white bars) or FSHAb (black bars) samples are presented following normalization to the *Actb* value for each sample. Data are mean $\pm$ s.d. ($n=3$ per group). No significant differences were recorded between treatment groups.

Interestingly, despite detection of both the intrinsic and extrinsic pathway-activated executioner proteins in germ cells, we observed no significant change in the transcript levels of *Bax*, *Bcl2l2* and *Fas* following FSH suppression. However, the transcript levels may not necessarily reflect active protein concentration, due to the potential for post-translational regulation of protein activities. As active protein levels were not quantified in the present study, we cannot address these possibilities. In immature *hpg* mice, decreased *Bax* transcription was proposed to be due to androgen deficiency (Chausiaux *et al.* 2008), since *Bax* activity is androgen dependent in prostate cancer (Lin *et al.* 2006). Even though caspase activation is often thought of principally at the protein level controlling proteolytic cascades, some studies have reported increases in caspase mRNA due to apoptotic stimuli (Huang *et al.* 2005, Chausiaux *et al.* 2008). FSH suppression had no effect on the transcription of the initiator caspase genes in this study. On the other hand, in the *hpg* mouse testis, *Casp9* and *Casp8* expression levels were significantly increased, as assessed by microarray analysis (Chausiaux *et al.* 2008), although this was probably due to the deficiency in testosterone rather than lack of FSH.

In conclusion, this study reveals that FSH suppression regulates spermatogonial apoptosis via the intrinsic apoptotic pathway, while spermatocyte apoptosis occurs via both the intrinsic and extrinsic apoptotic pathways during the first wave

of spermatogenesis. In this study, FSH has its effect at the protein levels for caspase 8 and caspase 9, not at the transcript levels of various pathway-related genes. Understanding the basic mechanisms in which hormones regulate germ cell progression in the immature testis is an important step towards the understanding how normal testicular function is established.

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