Effects of FSH on testicular mRNA transcript levels in the hypogonadal mouse

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Abstract

FSH acts through the Sertoli cell to ensure normal testicular development and function. To identify transcriptional mechanisms through which FSH acts in the testis, we have treated gonadotrophin-deficient hypogonadal (*hpg*) mice with recombinant FSH and measured changes in testicular transcript levels using microarrays and real-time PCR 12, 24 and 72 h after the start of treatment. Approximately 400 transcripts were significantly altered at each time point by FSH treatment. At 12 h, there was a clear increase in the levels of a number of known Sertoli cell transcripts (e.g. *Fabp5*, *Lgals1*, *Tesc*, *Scara5*, *Aqp5*). Additionally, levels of Leydig cell transcripts were also markedly increased (e.g. *Ren1*, *Cyp17a1*, *Akr1b7*, *Star*, *Nr4a1*). This was associated with a small but significant rise in testosterone at 24 and 72 h. At 24 h, androgen-dependent Sertoli cell transcripts were up-regulated (e.g. *Rhox5*, *Drd4*, *Spinlw1*, *Tubb3* and *Tsx*) and this trend continued up to 72 h. By contrast with the somatic cells, only five germ cell transcripts (*Dkkl1*, *Hdc*, *Pou5f1*, *Zfp541* and *1700021K02Rik*) were altered by FSH within the time-course of the experiment. Analysis of canonical pathways showed that FSH induced a general decline in transcripts related to formation and regulation of tight junctions. Results show that FSH acts directly and indirectly to induce rapid changes in Sertoli cell and Leydig cell transcript levels in the *hpg* mouse but that effects on germ cell development must occur over a longer time-span.

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Introduction

Postnatal testicular growth, spermatogenesis and fertility are dependent upon the pituitary gonadotrophins FSH and LH. LH acts directly on Leydig cells to stimulate androgen production, while androgens and FSH stimulate spermatogenesis through direct action on the Sertoli cells (McLachlan *et al.* 2002). The role of gonadotrophins is clearly seen in the hypogonadal (*hpg*) mouse that lacks GnRH (Mason *et al.* 1986) and, consequently, has undetectable circulating levels of LH and FSH (Cattanach *et al.* 1977). The gonads of the *hpg* mouse remain in a pre-pubertal state throughout life, with spermatogenesis blocked at early meiosis (Cattanach *et al.* 1977, Myers *et al.* 2005) although treatment with exogenous gonadotrophins or androgens will increase testicular growth and restore germ cell development (Charlton *et al.* 1983, Singh & Handelsman 1996*a*,*b*, Haywood *et al.* 2003). In recent years, generation of mice lacking individual hormones or hormone receptors has allowed us to investigate more clearly the roles played by LH, FSH and androgen in the regulation of testicular function. In particular, study of mice lacking androgen receptors (AR) in the Sertoli cells (SCARKO (De Gendt *et al.* 2004)) has shown that androgens are essential for spermatocyte progression through meiosis. By contrast, mice lacking FSH (FSHβKO (Kumar *et al.* 1997)) or the FSH receptor (FSHRKO; Dierich *et al.* 1998, Abel *et al.* 2000) are fertile with all stages of spermatogenesis present. Nevertheless, in FSHRKO and FSHβKO mice there is a reduction in sperm number and quality (Krishnamurthy *et al.* 2001, Wreford *et al.* 2001) suggesting that FSH action optimises spermatogenesis. In addition, comparison of SCARKO mice with mice lacking both FSHR and AR on the Sertoli cells has shown that FSH acts to increase Sertoli cell number, total germ cell number and the number of germ cells associated with each Sertoli cell (Abel *et al.* 2008). This is achieved by an increase in the number of spermatogonia and enhanced entry of these cells into meiosis (Abel *et al.* 2008).

Previous studies have identified a number of Sertoli cell products or mRNA transcripts that are FSH-sensitive including, for example, inhibin, AR, transferrin, doublesex and mab-3 related transcription factor1 (DMRT), androgen-binding protein and inducible cAMP early repressor (Morris *et al.* 1988, Verhoeven & Cailleau 1988, Skinner *et al.* 1989, Monaco *et al.* 1995, Chen & Heckert 2001). In addition, an earlier study has

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used arrays to examine the short-term effects (up to 24 h) of a single injection of FSH on testicular gene expression *in vivo* (Sadate-Ngatchou *et al.* 2004). From these studies, we can now identify a number of transcripts acutely regulated by FSH but we continue to lack a clear understanding of how FSH acts to regulate testicular development and function over the longer term. To address this issue, we have carried out a comprehensive review of the effects of more prolonged FSH treatment (multiple injections up to 72 h) on transcript levels in the testis of the *hpg* mouse.

Materials and methods

Animals and treatments

hpg mice from the original colony first identified at the MRC Laboratories, Harwell, Oxford (Cattanach *et al.* 1977) were bred at Oxford. The *hpg* mutation was identified by PCR analysis of tail DNA as previously reported (Lang 1995). All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and with the approval of a local ethical review committee.

Male *hpg* mice, 10 weeks of age and in group sizes of 3–4, were injected subcutaneously with 8 IU recombinant human FSH (rhFSH) (Serono Ltd) in 0·2 ml PBS (PBS, pH 7·4, Sigma Aldrich) at the start of the experiment and every 12 h thereafter for 12, 24 or 72 h. This dose of recombinant hormone had previously been shown to induce a significant increase in testis weight in *hpg* mice when given for 1 week (Abel and Charlton unpublished). Mice were killed 1 h after the last injection, testes removed, snap frozen in liquid nitrogen and stored at −70 °C.

Testicular histology

Three *hpg* mice treated as above were killed at each time point. The testes were weighed and one testis from each animal was fixed in 1% glutaraldehyde, 4% paraformaldehyde, in phosphate buffer, 0·1 M, pH 7·2 for 24 h at 4 °C, and embedded in araldite. Semi-thin, 1 μm sections were cut and stained with toluidine blue.

DNA microarray

Three or four animals from FSH-treated or control *hpg* groups were killed at each time point and the RNA from testes of individual animals extracted on RNeasy columns (Qiagen). RNA was quantified using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE, USA) and RNA quality was checked using the Agilent bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). Samples of total RNA (8 μg) from individual animals were reverse transcribed and then *in vitro* transcribed and hybridised to mouse MOE430A arrays (Affymetrix, Santa Clara, CA, USA) ($n=3$ or 4 for each group) according to the GeneChip expression technical manual (Affymetrix) as previously reported (Baban & Davies 2008). All the experiments were designed and information compiled in compliance with MIAME guide lines. Gene transcript levels were determined from data image files using algorithms in Gene Chip Operating Software (GCOS1.2, Affymetrix).

The array data were generated in two batches. In the first experiment control, 12 and 72 h FSH groups were extracted and hybridised to the arrays and in a subsequent experiment control and 24 h FSH groups were processed in the same way. Each treatment group was analysed against its own control. Differentially expressed genes were identified using the Welch *t*-test, variance not assumed equal, $P<0{\cdot}05$. Analysis of canonical pathways was carried out using Ingenuity Pathways Analysis (www.ingenuity.com).

The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession GSE8924.

Real-time PCR

Total RNA was extracted from individual testes of control or FSH-treated *hpg* mice using Trizol (Life technologies) and residual genomic DNA was removed by DNAse treatment (DNA-free, Ambion Inc., Austin, TX, USA, supplied by AMS Biotechnology, Abingdon, UK). RNA (1 μg) was reverse transcribed using random hexamers (Ambion) and Moloney murine leukaemia virus reverse transcriptase (Life Technologies) as previously described (Hirst *et al.* 2004).

Quantitative real-time PCR was used to confirm changes in selected mRNA transcripts identified from the microarray analysis or to examine other transcripts of potential interest. The real-time PCR used either the Taqman (*Inha*, *Inhba*, *Inhbb* and *Hdc*) or the SYBR green (all other transcripts) method in a 96-well plate format. For Taqman, Universal Taqman master mix, and optimised primer and probe sets were purchased from Applied Biosystems (Warrington, UK) and used according to the manufacturer's recommendations in a 25 μl volume. For SYBR green, each reaction contained 5 μl 2×SYBR mastermix (Stratagene, Amsterdam, Netherlands), primer (100 nM) and template in a total volume of 10 μl. The thermal profile used for amplification was 95 °C for 8 min followed by 40 cycles of 95 °C for 25 s, 63 °C for 25 s and 72 °C for 30 s. At the end of the amplification phase a melting curve analysis was carried out on the products formed. All primers were designed by Primer Express 2.0 (Applied Biosystems) using parameters previously described (Czechowski *et al.* 2004).

No-RT controls for each sample were screened to check for the presence of residual genomic DNA. The primers and probes used for real-time SYBR PCR are shown in Supplementary Table 1, see supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol42/issue4/. Different animals were used to provide RNA for real-time PCR and microarray studies.

Hormone assay

In a separate study adult *hpg* mice were treated with rhFSH as above and intratesticular levels of testosterone measured by RIA following ethanol extraction as previously described (O'Shaughnessy & Sheffield 1990). The limit of detection of the assay was 25 fmol/testis.

Statistical analysis

With the exception of the array studies described above, the effects of FSH treatment were analysed initially by single-factor ANOVA followed by *post hoc* analysis using Fisher's test.

Results

Testicular weight and histology after rhFSH treatment

There was a significant increase in testis weight within 12 h of the start of FSH treatment and weight continued to increase up to 24 h (Fig. 1A). This weight increase was accompanied by an apparent increase in tubular diameter with clear formation of a tubular lumen (Fig. 1B). On the semi-thin light micrographs, there was also an apparent increase in vacuolation of the Sertoli cell cytoplasm by 24 h which became more marked by 72 h (Fig. 1B). This was confirmed on electron micrographs with several small vacuoles apparent within the cytoplasm at 24 h and larger vacuoles present at 72 h (Fig. 1C). There was no clear advancement of spermatogenesis within the timescale of the experiment.

Hormone levels

Intratesticular testosterone levels were undetectable in control *hpg* mice (<25 fmol/testis (<12 fmol/mg tissue), $n=8$) and increased to low but consistently detectable levels 24 h after the start of treatment with FSH (65·0±12·4 fmol/testis (19·1±3·6 fmol/mg), $n=4$) and remained detectable up to 72 h (76·2±45·0 fmol/testis (19·5±11·5 fmol/mg), $n=4$).

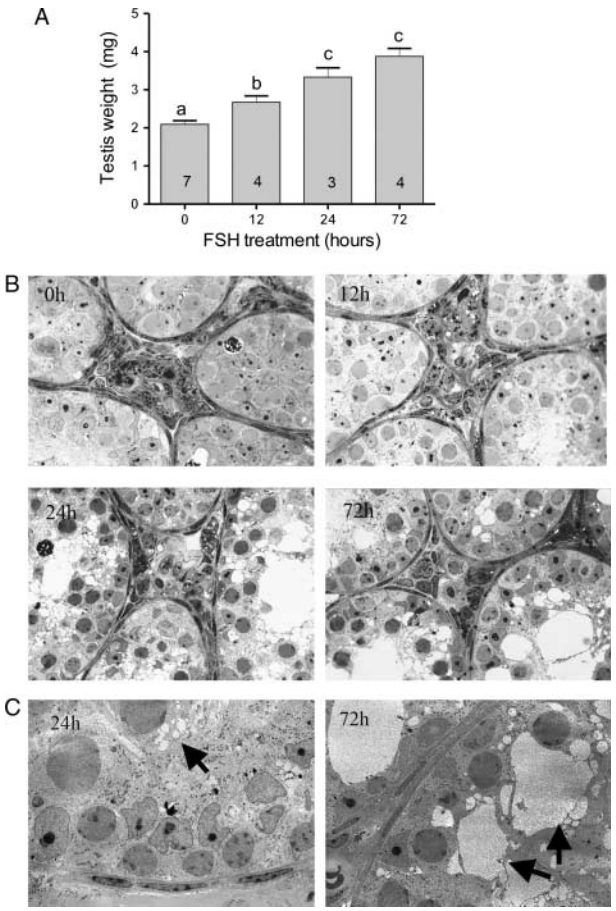

Figure 1 Effect of rhFSH on testis weight and morphology in *hpg* mice. A) Testis weights of control adult *hpg* mice and mice treated every 12 h with FSH (*n* for each group is shown in the histogram). B) Semi-thin sections of testes from control adult *hpg* mice and mice treated with FSH for 12, 24 and 72 h. Note the appearance of vacuoles within the cytoplasm of the Sertoli cell at 24 and 72 h post-treatment. C) Electron micrographs at 24 and 72 h, arrows indicate the progression from multiple small vacuoles to fewer large vacuoles.

Microarray data

Analysis of the array data showed that there were 182, 164 and 203 transcripts significantly (>2-fold) increased in the *hpg* testis 12, 24 and 72 h after the start of FSH treatment and 162, 411 and 215 significantly decreased at the same times. Transcripts with the highest fold changes in expression at each time during treatment are listed in Table 1 and the complete list of significantly altered transcripts (>2-fold) is shown in Supplementary Table 2, see supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol42/issue4/. At 12 h after the start of FSH treatment, there was a clear increase in the levels of a number of transcripts known to be expressed in the Sertoli cells (e.g. *Fabp5*, *Lgals1*, *Tesc* and *Scara5*;

Table 1 Effects of FSH treatment on testicular transcript levels – highest-regulated transcripts from microarray studies[a]

Fold change	Gene symbol	Gene title	Fold change	Gene symbol	Gene title
Transcripts up-regulated 12 h after start of treatment			*Transcripts down-regulated 12 h after start of treatment*		
20·7	Ren1	Renin 1 structural	7·27	Myh8	Myosin, heavy polypeptide 8
18·2	Dmkn	Dermokine	6·65	Rin2	Ras and Rab interactor 2
13·3	Aqp5	Aquaporin 5	5·03	Pdgfc	Platelet-derived growth factor C
12·1	Wif1	Wnt inhibitory factor 1	4·55		Transcribed locus[b]
11·0	Fabp5	Fatty acid binding protein 5, epidermal	4·53	Bcan	Brevican
6·6	Tubb3	Tubulin, β 3	4·46	Rgs11	Regulator of G-protein signalling 11
6·5	Cyp17a1	Cytochrome P450, family 17a 1	4·45	Tmem37	Transmembrane protein 37
6·3	Akr1b7	Aldo-keto reductase family 1 B7	4·43	Fhod3	Formin homology 2 domain containing 3
6·0	Lgals1	Lectin, galactose binding, soluble 1	4·38	Derl3	Der1-like domain family, member 3
5·9	Col4a1	Procollagen, type IV, α1	4·01	Ddit4l	DNA-damage-inducible transcript 4-like
5·7	Pappa	Pregnancy-associated plasma protein A	3·98	BC013672	cDNA sequence BC013672
5·2	Star	Steroidogenic acute regulatory protein	3·97	Scin	Scinderin
4·6	Ldlr	Low density lipoprotein receptor	3·96	Ddit4l	DNA-damage-inducible transcript 4-like
4·6	Tesc	Tescalcin	3·89	Cabc1	Chaperone, ABC1 complex-like
4·6	Rps6ka2	Ribosomal protein S6 kinase 2	3·86	Krt20	Keratin 20
4·6	Scara5	Scavenger receptor class A 5	3·74	Tmem140	Transmembrane protein 140
4·4	Hgsnat	Heparan N-acetyltransferase	3·71	Dbp	D site albumin promoter binding protein
4·4	Hs3st1	Heparan sulphate 3-*O*-sulphotransferase 1	3·71	Rnasel	Ribonuclease L
4·2	Syne1	Synaptic nuclear envelope 1	3·70	Spsb1	splA receptor domain and SOCS box 1
4·1	Slc38a5	Solute carrier family 38, member 5	3·67	Tnni3	Troponin I, cardiac
4·0	Gpd1	Glycerol-3-phosphate dehydrogenase 1	3·65	Cdo1	Cysteine dioxygenase 1, cytosolic
3·9	Dos	Downstream of Stk11	3·57	Stard8	START domain containing 8
3·8	Svs5	Seminal vesicle secretory protein 5	3·55	Slc40a1	Solute carrier family 40, member 1
3·8	Bhmt	Betaine-homocysteine methyltransferase	3·52	Hdac5	Histone deacetylase 5
3·7	D9Ertd280e	Chr 9, ERATO Doi 280	3·32	Dbp	D site albumin promoter binding protein
3·7	Tnfrsf12a	Tumour necrosis factor receptor 12a	3·23	Chdh	Choline dehydrogenase
3·7	1200016-E24Rik	RIKEN cDNA 1200016E24	3·23	8030411-F24Rik	RIKEN cDNA 8030411F24 gene
3·7	Nr4a1	Nuclear receptor subfamily 4, group A1	3·23	Per3	Period homolog 3 (*Drosophila*)
3·6	Dkk3	Dickkopf homolog 3	3·22	Ctnna2	Catenin, α 2
3·6	Cyp51	Cytochrome P450, family 51	3·19	Trim47	Tripartite motif protein 47
Transcripts up-regulated 24 h after start of treatment			*Transcripts down-regulated 24 h after start of treatment*		
28·8	Lin7c	Lin-7 homolog C (*C. elegans*)	9·9	Ddit4l	DNA-damage-inducible transcript 4-like
18·0	Cyp17a1	Cytochrome P450, family 17a1	9·9	Rin2	Ras and Rab interactor 2
13·6	Cyp11a1	Cytochrome P450, family 11a1	9·1	Slc40a1	Solute carrier family 40, member 1
11·0	Fabp5	Fatty acid binding protein 5, epidermal	8·0	Rgs11	Regulator of G-protein signalling 11
10·1	Rhox5	Reproductive homeobox 5	7·6	Igfbp3	Insulin-like growth factor binding protein 3
9·4	Star	Steroidogenic acute regulatory protein	7·4	Myh6	Myosin, heavy polypeptide 6 α
8·7	Slc38a5	Solute carrier family 38, member 5	7·2	Apbb2	Amyloid precursor protein-binding B2
7·9	Aqp5	Aquaporin 5	6·7	Rassf5	Ras association domain family 5
7·4	Tubb3	Tubulin, β 3	6·3	Tmem37	Transmembrane protein 37
7·0	Drd4	Dopamine receptor 4	6·3	Chdh	Choline dehydrogenase
6·3	Tesc	Tescalcin	6·2	Itga9	Integrin α 9
5·5	Lgals1	Lectin, galactose binding, soluble 1	6·2	Thbd	Thrombomodulin
5·0	Spinlw1	Eppin	5·9	Fcgr2b	Fc receptor, IgG, low affinity IIb
4·6	Osr1	Odd-skipped related 1	5·8	Trim47	Tripartite motif protein 47
4·5	Fads2	Fatty acid desaturase 2	5·7	Spsb1	splA receptor domain and SOCS box 1
4·2	Pappa	Pregnancy-associated plasma protein A	5·6	Ddit4l	DNA-damage-inducible transcript 4-like
4·2	Scara5	Scavenger receptor class A5	5·5	Vnn1	Vanin 1
4·2	Pscdbp	Pleckstrin homology binding protein	5·5	Fcgr2b	Fc receptor, IgG, low affinity IIb
4·1	Plac8	Placenta-specific 8	5·4	Ptprd	Protein tyrosine phosphatase, receptor D
4·0	Gpt2	Glutamic pyruvate transaminase 2	5·4	Nkx3-1	NK-3 transcription factor, locus 1
4·0	Rps6ka2	Ribosomal protein S6 kinase 2	5·3	Fcgr2b	Fc receptor, IgG, low affinity IIb
4·0	Gpd1	Glycerol-3-phosphate dehydrogenase 1	5·1	Ctgf	Connective tissue growth factor
4·0	Hdc	Histidine decarboxylase	5·0	H19	H19 fetal liver mRNA
3·8	Igf1	Insulin-like growth factor 1	5·0	Pdgfc	Platelet-derived growth factor C

(continued)

Table 1 *Continued*

Fold change	Gene symbol	Gene title	Fold change	Gene symbol	Gene title
3·7	Fah	Fumarylacetoacetate hydrolase	4·9	Hsd17b11	Hydroxysteroid (17β) dehydrogenase 11
3·7	Mpzl2	Myelin protein zero-like 2	4·9	Cabc1	Chaperone, ABC1 complex-like
3·6	Insl3	Insulin-like 3	4·9	9630031F1-2Rik	RIKEN cDNA 9630031F12 gene
3·4	Wif1	Wnt inhibitory factor 1	4·8	Ptch1	Patched homolog 1
3·3	Inha	Inhibin α	4·7	Smoc2	SPARC related modular calcium binding 2
3·2	Col18a1	Procollagen, type XVIII, α 1	4·7	Fhod3	Formin homology 2 domain containing 3
Transcripts up-regulated 72 h after start of treatment			*Transcripts down-regulated 72 h after start of treatment*		
35·0	Drd4	Dopamine receptor 4	13·4	Igfbp3	Insulin-like growth factor binding protein 3
21·1	Slc38a5	Solute carrier family 38a5	8·2	Rgs11	Regulator of G-protein signalling 11
14·9	Rhox5	Reproductive homeobox 5	6·6	Rin2	Ras and Rab interactor 2
13·9	Fabp5	Fatty acid binding protein 5, epidermal	6·5	Clca1	Chloride channel calcium activated 1
8·4	Spinlw1	Eppin	6·0	Slc40a1	Solute carrier family 40 1
7·6	Klk1b24	Kallikrein 1-related peptidase b24	5·6	Spsb1	splA receptor domain and SOCS box 1
6·6	Tubb3	Tubulin, β 3	4·9	Ifitm1	Interferon induced transmembrane 1
5·9	Hdc	Histidine decarboxylase	4·7	Myh8	Myosin, heavy polypeptide 8
5·5	Fah	Fumarylacetoacetate hydrolase	4·6	Fhod3	Formin homology 2 domain containing 3
5·5	Tsx	Testis specific X-linked gene	4·4	Bcan	Brevican
5·4	Zcchc18	Zinc finger, CCHC domain 18	4·4	Tmem140	Transmembrane protein 140
4·6	Sct	Secretin	4·4	BC013672	cDNA sequence BC013672
4·6	Gpd1	Glycerol-3-phosphate dehydrogenase 1	4·3	Tmem37	Transmembrane protein 37
4·5	Myh1	Myosin, heavy polypeptide 1	4·0	Erbb3	v-erb-b2 homolog 3
4·5	Fabp4	Fatty acid binding protein 4, adipocyte	3·9	Dst	Dystonin
4·5	St8sia2	ST8 sialyltransferase 2	3·9	Xist	Inactive X specific transcripts
4·5	Tgfb1	Transforming growth factor, β 1	3·8	Arhgdig	Rho GDP dissociation inhibitor γ
4·3	Pscdbp	Pleckstrin homology binding protein	3·7	Edn1	Endothelin 1
4·2	Igf1	Insulin-like growth factor 1	3·7	6330403-K07Rik	RIKEN cDNA 6330403K07 gene
4·1	Scara5	Scavenger receptor class A, member 5	3·7	Jun	Jun oncogene
4·1	Sept6	Septin 6	3·6	Pla2g5	Phospholipase A2, group V
4·0	D17H6-S56E-5	Chr 17, human D6S56E 5	3·6	Apbb2	Amyloid β precursor protein-binding B2
4·0	Pappa	Pregnancy-associated plasma protein A	3·6	H19	H19 fetal liver mRNA
4·0	Klk1	Kallikrein 1	3·6	Hspb1	Heat shock protein 1
3·9	Dmkn	Dermokine	3·6	Fcgr2b	Fc receptor, IgG, low affinity IIb
3·8	Tpd52l1	Tumour protein D52-like 1	3·5		Transcribed locus[c]
3·8	Inha	Inhibin α	3·5	Adi1	Acireductone dioxygenase 1
3·8	Slc25a5	Solute carrier family 25, member 5	3·4	H2-T23	Histocompatibility 2, T region locus 23
3·7	Car3	Carbonic anhydrase 3	3·4	Scin	Scinderin
3·7	Pde4b	Phosphodiesterase 4B, cAMP specific	3·3	Rnasel	Ribonuclease L

[a]If a transcript is represented more than once on the array only the highest fold change is shown in this table. Supplementary Table 2 shows the complete significant dataset.
[b]Net affy number 1454967_at.
[c]Net affy number 1436092_at.

Kingma *et al.* 1998, Perera *et al.* 2001, Dettin *et al.* 2003, Jiang *et al.* 2006) and, perhaps surprisingly, in the Leydig cells (e.g. *Ren1*, *Cyp17a1*, *Akr1b7*, *Star*, *Ldlr* and *Nr4a1*; Deschepper *et al.* 1986, Le Goascogne *et al.* 1991, Song *et al.* 2001, Baron *et al.* 2003; Table 1). By 24 h after the start of FSH treatment, androgen-dependent Sertoli cell transcripts appeared in the list of up-regulated transcripts (e.g. *Rhox5*, *Drd4*, *Spinlw1* and *Tubb3*; Lindsey & Wilkinson 1996, Cunningham *et al.* 1998, Denolet *et al.* 2006, O'Shaughnessy *et al.* 2007) and this trend became more marked by 72 h. By contrast with the somatic cells, very few germ cell genes appear on the lists of significantly regulated transcripts. Only *Hdc* (increased 4·0- and 5·9-fold at 24 and 72 h respectively; Safina *et al.* 2002) and *1700021K02Rik* (*Spatial*) (increased 3·1-fold at 72 h) (Irla *et al.* 2003) were significantly altered by FSH within the time-span of these studies (Table 1 and Supplementary Table 2).

Few of the transcripts down-regulated following FSH treatment have been localised in the testis with the

exception of *Igfbp3* which has been shown to be of Sertoli cell origin (Smith *et al.* 1990). In order to identify more of the differentially expressed transcripts on the arrays, which may be of a Sertoli cell origin, up- and down-regulated transcripts were compared with those identified as being of likely Sertoli cell origin by Chalmel *et al.* (2007) using a cell isolation, GeneChip and clustering approach (Supplementary Table 2A and B). A degree of caution is required as some known Sertoli cell transcripts (e.g. *Rhox5*) are missing from the list generated by Chalmel *et al.* (2007) probably due to Chip sensitivity or the subsequent filtering process. Nevertheless, of the transcripts up-regulated at 12 h, 44% matched to the data from Chalmel *et al.* (2007). Interestingly, this number declined to 35% at 24 h and 27% at 72 h after the start of FSH treatment (Supplementary Table 2A). The number of down-regulated transcripts that matched the Sertoli cell list was 27, 21 and 28% at 12, 24 and 72 h respectively (Supplementary Table 2B).

Real-time PCR

Leydig cell genes

To confirm results from the array studies, real-time PCR was used to measure the effect of FSH treatment on testicular expression of selected transcripts which are known to be expressed exclusively in the Leydig cells (O'Shaughnessy *et al.* 2002). Eight mRNA species were tested which had shown an increase in transcript levels on the arrays after FSH (*Star*, *Cyp17a1*, *Hsd17b3*, *Akr1b7*, *Lhr*, *Cyp11a1*, *Insl3* and *Ren1*) (Fig. 2A). Results from the real-time PCR studies confirmed that seven of these transcripts are regulated by FSH in the *hpg* testis, although no change in *Lhr* was seen. Two other Leydig cell mRNA species (*Hsd3b6* and *Sult1e1*) that had not shown any response to FSH on the arrays were also tested by real-time PCR (Fig. 2A). Levels of *Hsd3b6* did not show a response to FSH but there was a significant, if variable, increase in *Sult1e1* after 72 h.

Androgen-dependent genes

The array studies showed clearly that a number of androgen-dependent Sertoli cell transcripts were altered after FSH treatment. Real-time PCR was used to confirm changes in selected transcripts (*Rhox5*, *Tsx*, *Drd4*, *Spinlw1* (*Eppin*) and *Igfbp3*) shown previously to be androgen-regulated (Lindsey & Wilkinson 1996, Denolet *et al.* 2006, O'Shaughnessy *et al.* 2007; Fig. 2B). In agreement with results from the array studies four transcripts (*Rhox5*, *Tsx*, *Drd4* and *Spinlw1*) showed increased expression 24–72 h after FSH treatment while one transcript (*Igfbp3*) showed a significant decrease in expression (Fig. 2B).

Sertoli cell genes

In addition to androgen-dependent Sertoli cell genes described above, 16 other Sertoli cell transcripts were measured by real-time PCR following FSH treatment of adult *hpg* mice (Fig. 3). Of these transcripts, seven had shown significantly increased expression on the arrays (*Tesc*, *Lgals1*, *Aqp5*, *Dhh*, *Pappa*, *Wnt4* and *Shbg*), six had not shown any significant change (*Trf*, *Wt1*, *Amh*, *Spata2*, *Tjp1* and *Gdnf*), two had shown a significant decrease (*Fshr* and *Rgs11*) after FSH treatment and one transcript (*Defb19*) was not on the array. Results from real-time studies confirmed increased transcript levels for six out of the seven mRNA species identified on the array (the exception was *Shbg*) and for the one transcript (*Defb19*) not on the array (Fig. 3A). Both transcripts decreased on the arrays after FSH treatment also showed a significant decrease by real-time PCR (Fig. 3A). Interestingly, however, the real-time PCR data showed there was a significant increase in levels of three out of the six transcripts that were not significantly changed on the arrays (*Trf*, *Wt1* and *Amh*; Fig. 3A). Two of these transcripts (*Trf* and *Amh*) had shown a greater than twofold increase on the arrays but had not reached significance. Differences between results from real-time PCR and arrays may be a matter of sensitivity and variability of the two techniques or may be due to the choice of primers, away from the 3′ region targeted by these arrays.

The inhibin subunits are expressed in a number of cell types in the testis (Barakat *et al.* 2008) although it might be expected that initial responsiveness to FSH would be predominantly localised in the Sertoli cells. On the arrays, both *Inha* and *Inhbb* were significantly increased by FSH (Table 1 and Supplementary Table 2) while there was no effect on *Inhba*. Results from the real-time PCR studies reflected the same pattern of results (Fig. 3B).

Germ cell genes

The number of known germ cell transcripts on the array affected by FSH, within the time-span of the study, was not high. This was, therefore, investigated further using real-time PCR. Expression of nine germ cell transcripts known to be expressed predominantly in spermatogonia (*Stra8*, *Pou5f1*, *Dkkl1* and *Spo11*), spermatocytes (*Mybl1*, *Zfp541*) or spermatids (*1700021K02Rik*, *Hdc* and *Tp1*) was measured following FSH treatment (Fig. 4). Expression levels of *Stra8*, *Spo11*, *Mybl1* and *Tp1* were unaffected by treatment but there was a transient increase in *Pou5f1* at 12 h while *Dkkl1* and *Zfp541* were significantly increased at 72 h. Expression of *Hdc* and *1700021K02Rik* (shown previously to be increased on the array) was increased at all times after treatment.

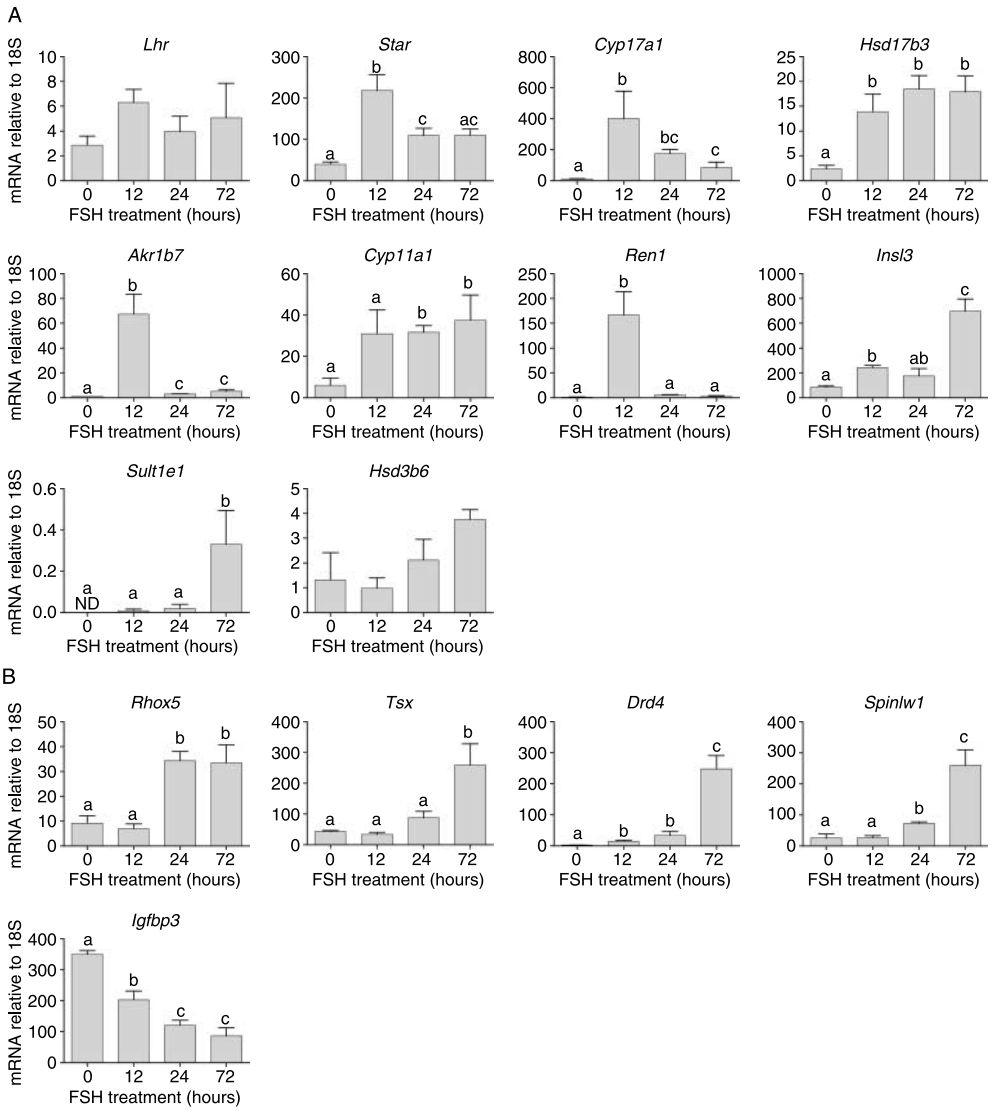

Figure 2 Real-time PCR measurements of mRNA transcript levels in testes from adult *hpg* mice treated for 0 (control), 12, 24 or 72 h with FSH. Data show results from Leydig cell-specific transcripts (A) and from Sertoli cell-specific, androgen-dependent transcripts (B). The mean ± S.E.M. of three or four animals per group is shown. Groups with different letter superscripts are significantly different.

Other transcripts

Results from the array studies identified a number of transcripts regulated by FSH but without known function and/or known expression pattern in the testis. Levels of three of these transcripts (*Wif1*, *Dmkn*, *Dkk3*) were measured in *hpg* testes after FSH treatment (Fig. 5). In all cases, FSH caused a significant increase in transcript levels confirming the results of the array study.

Canonical pathway analysis

Analysis of canonical pathways showed that components of the cholesterol biosynthetic pathway were significantly increased at 12 h but not at other times (Supplementary Table 3A, see supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.endocrinology-journals.org/content/vol42/issue4/) and that there was a general decline in transcripts encoding factors involved in formation and regulation of tight junctions (Supplementary Table 3B).

Discussion

FSH is essential for optimum fertility in the adult male but uncertainty remains about how it acts to regulate Sertoli cell activity and spermatogenesis. The *hpg* mouse

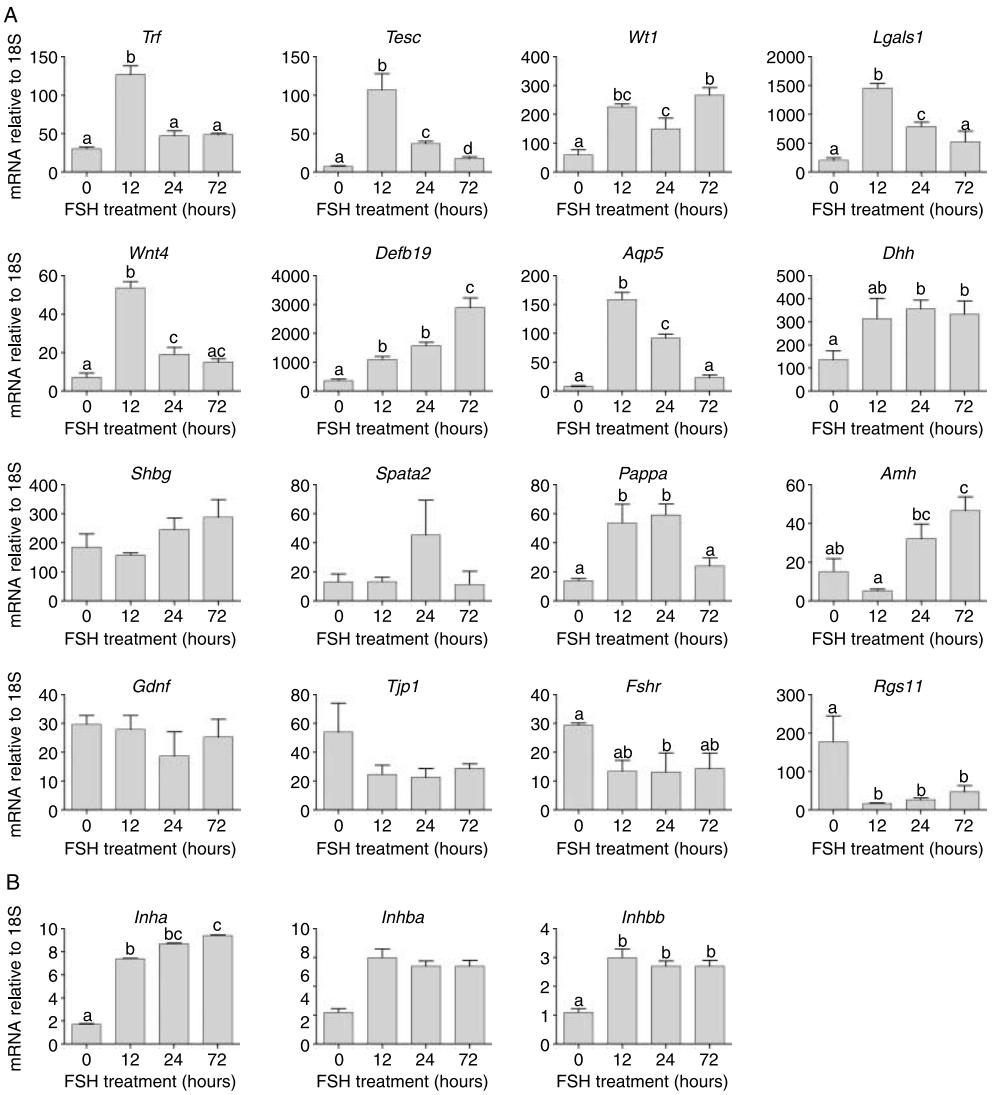

Figure 3 Real-time PCR measurements of Sertoli cell-specific mRNA transcript levels in testes from adult *hpg* mice treated for 0 (control), 12, 24 or 72 h with FSH. Results show the mean ± S.E.M. of three or four animals per group. Groups with different letter superscripts are significantly different; where no superscripts are shown there was no difference between groups.

is an excellent model system with which to test the effects of FSH since the Sertoli cells have not been exposed to the hormone but express FSHR and are sensitive to FSH action. This study is an extension of earlier work by Sadate-Ngatchou *et al.* (2004) using a longer treatment period, different array chips with a larger characterised gene set (MOE430A chips (14 000 characterised genes) versus MG U74Av2 chips (6000 characterised genes, 6500 ESTs)), a significantly larger animal cohort and recombinant FSH. In addition, the purpose of this study was to follow changes in testicular transcript levels in the *hpg* in response to maintained levels of FSH rather than the acute response to a single administration. Together, the two studies complement each other and serve to identify transcripts regulated by FSH over the short and medium term. Interestingly, at 12 h after the start of FSH administration, when both studies can be directly compared with a degree of caution, there were only 44 differentially transcripts common to both studies, 25 up-regulated (e.g. *Cyp17*, *Ren1*, *Fos*, *Hdc*, *Col4a1*) and 19 down-regulated (e.g. *Rgs11*, *Cdo1*, *Erbb3*, *Ptk2b*, *Vnn1*). This low number of transcripts in common may be due to a combination of the chips used, the age of the animals, the number of animals used and the treatment regime.

In this study, the total number of transcripts altered at each time point did not vary markedly across the treatment period but only 39 transcripts were

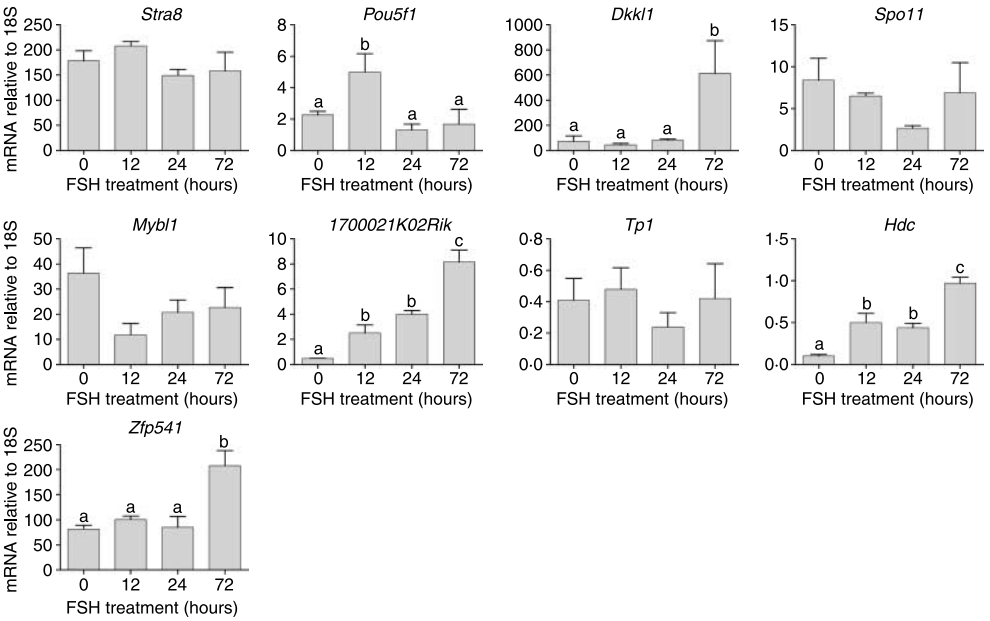

Figure 4 Real-time PCR measurements of germ cell-specific mRNA transcript levels in testes from adult *hpg* mice treated for 0 (control), 12, 24 or 72 h with FSH. Results show the mean±S.E.M. of three or four animals per group. Groups with different letter superscripts are significantly different; where no superscripts are shown there was no difference between groups.

up-regulated more than twofold at all times indicating that there was a changing pattern of expression as the exposure to FSH was maintained. There were also 63 transcripts down-regulated more than twofold at all times suggesting that the inhibitory effects of FSH are more consistent. Results from the arrays and from real-time PCR showed that FSH treatment caused a general increase in many transcripts encoding known Sertoli cell-specific products such as *Tesc*, *Lgals1*, *Fabp5* and *Aqp5* (Kingma *et al.* 1998, Perera *et al.* 2001, Dettin *et al.* 2003) although some transcripts (e.g. *Shbg*, *Tjp1* (Wang *et al.* 1989, Byers *et al.* 1991)) were unaffected while others were decreased (see below) indicating that the effect of FSH was not simply to increase the overall activity of the cells. Comparison of the up-regulated transcripts in this study with the list of Sertoli cell transcripts generated by Chalmel *et al.* (2007) confirms that, at least initially, a high proportion of the affected transcripts are likely to be of Sertoli cell origin. The declining proportion of Sertoli cell transcripts at later times is likely to be due to increasing activity in other cells such as the Leydig cell. The lower proportion of down-regulated transcripts that match to the Sertoli cell list of Chalmel *et al.* (2007) may reflect the GeneChip sensitivity involved in generating that list since many of these down-regulated transcripts might be expected to have a low level of expression in the normal animal.

Among the transcripts that showed decreased levels in response to FSH were a number encoding tight junction components. This is consistent with a recent

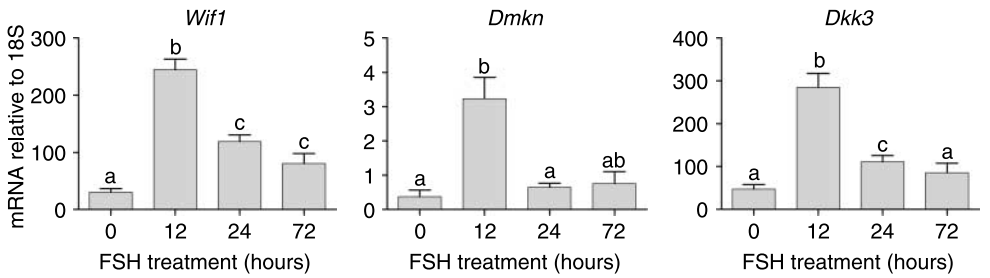

Figure 5 Real-time PCR measurements of mRNA transcript levels of species with unknown testicular origin. Levels were measured in testes from adult *hpg* mice treated for 0 (control), 12, 24 or 72 h with FSH. Results show the mean±S.E.M. of three or four animals per group. Groups with different letter superscripts are significantly different.

study which reported that gonadotrophins reduce transcript levels of Sertoli cell barrier components but that FSH may act at the level of protein organisation to induce barrier functionality (Tarulli *et al.* 2008). Other transcripts that showed a significant decrease in levels after FSH treatment included *Fshr* and *Rgs11*. It is well established that FSH will cause down-regulation of its receptor by decreasing transcript levels (O'Shaughnessy 1980, Themmen *et al.* 1991) and a reduction in *Fshr* is to be expected. RGS11, in contrast, belongs to the regulator of G protein-signalling family which are GTPase-activating proteins that act to inhibit signal transduction and thus play a role in desensitisation (Chasse & Dohlman 2003). The role of RGSs in normal hormonal signalling is not well established but the declining levels of *Rgs11* after FSH treatment may act to enhance signal transduction despite a reduction in receptor levels.

In addition to changes in Sertoli cell transcripts induced by FSH, it was clear from the rise in testicular androgen and the array and real-time PCR data that FSH was also acting to induce Leydig cell function. This effect was marked and rapid with a Leydig cell transcript (*Ren1*) showing the greatest fold change at 12 h (Deschepper *et al.* 1986). Results from the arrays and real-time PCR show that all components of the androgen biosynthetic pathway were induced at 12 h apart from *Hsd3b6*. Since *Hsd3b1* is already highly expressed in the adult *hpg* testis (Baker *et al.* 2003) lack of HSD3B6 is unlikely to affect the steroidogenic potential of the cells. In addition to the steroidogenic enzymes, pathway analysis showed that most components of the cholesterol biosynthetic pathway were induced 12 h after FSH treatment while *Ldlr* levels are increased. This shows that FSH is acting to increase the capacity of the Leydig cells to produce and sequester cholesterol and to convert cholesterol to androgen. Interestingly, one of the critical components of the cholesterol biosynthetic pathways (*Mvk*) also acts to inhibit *Lhr* translation (Nair & Menon 2004) and this may serve to regulate further Leydig cell sensitivity to LH. The *hpg* mouse testis is likely to contain both adult and fetal-type Leydig cells (Baker *et al.* 2003) and *Hsd3b6* is a marker of adult Leydig cell differentiation (Baker *et al.* 1999). This might imply that FSH is acting to induce activity in the fetal Leydig cell population but *Sult1e1* is a marker of adult Leydig cells (Song *et al.* 1997) and is increased 72 h after FSH suggesting that the effects of FSH are probably being mediated through the adult Leydig cells.

In the testis, receptors for FSH are only found in the Sertoli cells (Heckert & Griswold 2002) and the effects of FSH must be mediated by a factor or factors released by the Sertoli cells which act on the Leydig cells. In the short-term, the effects of FSH on Leydig cell function in the *hpg* appear to be more marked than effects of hCG (Baker *et al.* 2003) and the effects are also very rapid since Sadate-Ngatchou *et al.* (2004) saw a marked increase in *Cyp17a1* after only 4 h of FSH treatment. FSH appears, therefore, to be able to induce a powerful and rapid response in Leydig cells presumably through stimulation of release of potent trophic factors by the Sertoli cells. The presence of such factors has been postulated for a number of years since early studies on perfused testes or hypophysectomised animals treated with FSH (Johnson & Ewing 1971, Chen *et al.* 1976, Vihko *et al.* 1991). One report has suggested that the active factors are TIMP1 and Procathepsin L (Boujrad *et al.* 1995) but this has not been confirmed and we saw no evidence of changes in these factors in our study. Following FSH treatment, our array data showed that there was an increase in *Igf1* levels and IGF1 has been suggested to play a role in Leydig cell differentiation (Morera *et al.* 1987). Interestingly, there was a marked decline in *Igfbp3* and an increase in *Pappa* levels after FSH treatment. Increased *Pappa* would be expected to increase the bioavailability and activity of IGF1 (Conover *et al.* 2004) although the effect of altered *Igfbp3* may be more complex (Modric *et al.* 2001). The time-course of changes in expression of *Igf1* levels does not appear to fit well with a role in the stimulation of Leydig cell function after FSH treatment although it is possible that early changes in *Pappa* and *Igfbp3* may alter early IGF1 bioavailability. Other secreted molecules showing a marked increase in transcript levels after FSH include *Wif1*, *Dkk3* and *Dmkn*. Both WIF1 and DKK3 act to regulate WNT signalling and the WNT/CTNNB1 pathway is critical for normal Sertoli cell development (Boyer *et al.* 2008) although its function in the Leydig cell remains uncertain.

Following the increase in Leydig cell activity after FSH treatment, there was a significant change in the levels of known androgen-dependent Sertoli cell-specific transcripts. It is possible that changes in these transcripts are due to direct effects of FSH treatment but the known androgen-dependence of the transcripts makes it more likely that changes are related to increased Leydig cell androgen production induced by FSH. The rise in intratesticular androgen after FSH treatment was significant but levels remained very low, probably because FSH stimulates synthesis of the components of the steroidogenic pathway without being able to stimulate the pathway itself. The apparent effect of these low levels of androgen on Sertoli cell transcript levels suggests that the Sertoli cells are extremely sensitive to androgen stimulation.

Treatment of *hpg* mice with FSH increased vacuolation in the Sertoli cells and induced formation of a lumen within the seminiferous tubules but had little apparent effect on germ cell morphology or progression up to 72 h. Changes in the Sertoli cell and tubule diameter correlate with a marked rise in *Aqp5* at

12 h suggesting that increased water movement across the Sertoli cell membrane may contribute to increased tubular diameter and testis weight. The absence of spermatogenic progression over the time-course studied is likely to reflect the inactive state of the Sertoli cell in the adult *hpg* testis and the time required for the Sertoli cell to become active enough to support germ cell maturation. Real-time PCR studies of a small number of known germ cell genes showed that there was a variable response of germ cell transcripts to FSH stimulation. POU5F1 has been shown to be necessary for primordial germ cell survival (Kehler *et al.* 2004) and the increase in response to FSH, albeit small, may facilitate an increase in spermatogonial number within the testis. DKKL1 and ZFP541 are expressed in both spermatocytes and spermatids (Kohn *et al.* 2005, Choi *et al.* 2008) while HDC and SPATIAL are associated with round spermatids and the later stages of spermatogenesis (Safina *et al.* 2002, Irla *et al.* 2003). Lack of a general increase in germ cell transcripts (data from both arrays and real-time PCR) would indicate that the effects seen are not due to an overall increase in germ cell number but are more likely to be part of an early specific response to FSH stimulation. It has been shown previously that more prolonged treatment of *hpg* mice with FSH will stimulate an increase in germ cell number and development (O'Shaughnessy *et al.* 1992, Singh & Handelsman 1996*b*, Baines *et al.* 2008) but the stimulatory effect of FSH on the Leydig cells makes interpretation of the FSH effects on the germ cells difficult because of the known stimulatory effect of testosterone on germ cell development in the *hpg* mouse (O'Shaughnessy & Sheffield 1990, Singh *et al.* 1995).

In this study, FSH treatment of *hpg* mice for up to 72 h induced significant changes in Sertoli cell transcript levels and led to indirect stimulation of Leydig cell function. The changes in Leydig cell activity probably induced further changes in androgen-dependent Sertoli cell transcripts. While FSH is known to be required for optimal germ cell development (Abel *et al.* 2000, 2008), treatment of *hpg* mice for 72 h did not have a marked effect on germ cell differentiation suggesting that longer-term action of FSH is required to induce germ cell proliferation and progression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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