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, N4a1). 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, Spinl1, Tubb3 and Tsex) and this trend continued up to 72 h. By contrast with the somatic cells, only five germ cell transcripts (Dkk1, 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 or androgens will increase testicular growth 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 SCARCO 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-5 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

(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.

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Effects of FSH on testicular transcript levels

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, 0·1M, pH 7·2, at 4°C for 3–4 h, washed in PBS (pH 7·4), cut into 1 mm sections 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 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 2X SYBR mastermix (Stratagene, Amsterdam, Nether lands), 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).

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

| Fold change | Gene symbol | Gene title | Fold change | Gene symbol | Gene title |
|-------------|-------------|------------|-------------|-------------|------------|
| 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        | Fapb5       | 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 |
| 7-8         | Slc38a5     | Solute carrier family 38, member 5 | 7-2         | Apbb2       | Amyloid precursor-binding protein B2 |
| 7-7         | Aqp5        | Aquaporin 5 | 6-7         | Rassf5      | Ras association domain family 5 |
| 7-6         | 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         |  |  |
| 5-0         | Spinl1      | Eppin | 5-9         | Fcgr2b      | Fc receptor, IgG, low affinity Ib |
| 4-6         | Sosr1       | Odd-skipped related 1 | 5-8         | Trim47      | Tripartite motif protein 47 |
| 4-5         | Fads2       | Fatty acid desaturase 2 | 5-7         | Spsb1       | splice 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         | Scarac5     | Steroidogenic acute regulatory protein class A5 | 5-5         | Vnn1        | Vanin 1 |
| 4-1         | Pscdbp      | Pleckstrin homolog binding protein | 5-5         | Fcgr2b      | Fc receptor, IgG, low affinity Ib |
| 4-0         | Gpt2        | Glutamic pyruvate transaminase 2 | 5-4         | Ptprd       | Protein tyrosine phosphatase, receptor D |
| 4-0         | Rpslka2     | Ribosomal protein S6 kinase 2 | 5-3         | FCGR2B      | Fc receptor, IgG, low affinity Ib |
| 4-0         | Gpd1        | Glyceraldehyde-3-phosphate dehydrogenase 1 | 5-1         | Ctgf        | Connective tissue growth factor |
| 3-8         | Igf1        | Insulin-like growth factor 1 | 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

| Gene symbol | Gene title | Fold change | Gene symbol | Gene title |
|-------------|------------|-------------|-------------|------------|
| 3-7         | Fah        | 4-9         | Hsd17b11    | Hydroxysteroid (17β) dehydrogenase 11 |
| 3-7         | Mpz21      | 4-9         | Cabc1       | Chaperone, ABC1 complex-like |
| 3-6         | InsI3      | 9630031F1-2Rik | RIKEN cDNA 9630031F12 gene |
| 3-4         | Wif1       | 4-8         | Ptc1        | Patched homolog 1 |
| 3-3         | Inha       | 4-7         | Smoc2       | SPARC related modular calcium binding 2 |
| 3-2         | Col18a1    | 4-7         | Fhod3       | Formin homology 2 domain containing 3 |

**Transcripts up-regulated 72 h after start of treatment**

| Symbol | Gene title | Fold change |
|--------|------------|-------------|
| S56E-5 | Procollagen, type XVIII, α 1 | 3.2 |
| Inha   | Inhibin α  | 3.3 |
| 3.4 Wif1 | Wnt inhibitory factor 1 | 3.4 |
| 3.5 Hdc | Histidine decarboxylase | 3.5 |
| 3.6 Insl3 | Insulin-like 3 | 3.6 |
| 3.7 Fah | Fumarylcoacetate hydrolase | 3.7 |
| 3.8 Inha | Inhibin α  | 3.8 |
| 3.9 Dmkn | Dermokine | 3.9 |
| 4.0 Klk1b | Kallikrein 1b | 4.0 |
| 4.1 Sept6 | Septin 6 | 4.1 |
| 4.2 Igf1 | Insulin-like growth factor 1 | 4.2 |
| 4.3 Pscdbp | Pleckstrin homology binding protein | 4.3 |
| 4.4 Tgfb1 | Transforming growth factor, β 1 | 4.4 |
| 4.5 Tsx | Testis specific X-linked gene | 4.5 |
| 4.6 Zcchc18 | Zinc finger, CCHC domain 18 | 4.6 |
| 4.7 Scara5 | Scavenger receptor class A, member 5 | 4.7 |
| 4.8 Sept6 | Septin 6 | 4.8 |
| 4.9 D17H6-5 | Chr 17, human D6S56E 5 | 4.9 |
| 4.9 Papp | Pregnancy-associated plasma protein A | 4.9 |
| 4.9 Krak | Kallikrein 1 | 4.9 |
| 4.9 Tp85G1 | Tumour protein D52-like 1 | 4.9 |
| 4.9 Inha | Inhibin α  | 4.9 |
| 4.9 Scl25a5 | Solute carrier family 25, member 5 | 4.9 |
| 4.9 Car3 | Carbonic anhydrase 3 | 4.9 |
| 4.9 Pde4b | Phosphodiesterase 4B, cAMP specific | 4.9 |

**Transcripts down-regulated 72 h after start of treatment**

| Symbol | Gene title | Fold change |
|--------|------------|-------------|
| S56E-5 | Procollagen, type XVIII, α 1 | 3.2 |
| Inha   | Inhibin α  | 3.3 |
| 3.4 Wif1 | Wnt inhibitory factor 1 | 3.4 |
| 3.5 Hdc | Histidine decarboxylase | 3.5 |
| 3.6 Insl3 | Insulin-like 3 | 3.6 |
| 3.7 Fah | Fumarylcoacetate hydrolase | 3.7 |
| 3.8 Inha | Inhibin α  | 3.8 |
| 3.9 Dmkn | Dermokine | 3.9 |
| 4.0 Klk1b | Kallikrein 1b | 4.0 |
| 4.1 Sept6 | Septin 6 | 4.1 |
| 4.2 Igf1 | Insulin-like growth factor 1 | 4.2 |
| 4.3 Pscdbp | Pleckstrin homology binding protein | 4.3 |
| 4.4 Tgfb1 | Transforming growth factor, β 1 | 4.4 |
| 4.5 Tsx | Testis specific X-linked gene | 4.5 |
| 4.6 Zcchc18 | Zinc finger, CCHC domain 18 | 4.6 |
| 4.7 Scara5 | Scavenger receptor class A, member 5 | 4.7 |
| 4.8 Sept6 | Septin 6 | 4.8 |
| 4.9 D17H6-5 | Chr 17, human D6S56E 5 | 4.9 |
| 4.9 Papp | Pregnancy-associated plasma protein A | 4.9 |
| 4.9 Krak | Kallikrein 1 | 4.9 |
| 4.9 Tp85G1 | Tumour protein D52-like 1 | 4.9 |
| 4.9 Inha | Inhibin α  | 4.9 |
| 4.9 Scl25a5 | Solute carrier family 25, member 5 | 4.9 |
| 4.9 Car3 | Carbonic anhydrase 3 | 4.9 |
| 4.9 Pde4b | Phosphodiesterase 4B, cAMP specific | 4.9 |

**Effects of FSH on testicular transcript levels**

Kinna 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, Ldr and Nr4a1; Deschepper et al. 1986, Le Goasogne 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, Spinh2 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, Ins3 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, Ttx, Drd4, Spinul1, Effo) 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, Ttx, Drd4 and Spinul1) 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, Patpa, Wnt4 and Shbg), one had not shown any significant change (Tif, Wt1, Amh, Spata2, Tjpl and Gdnf), two had shown a significant decrease (Fshr and Rgs11) after FSH treatment and one transcript (Dfb19) 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 one transcript (Dfb19) 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 (Tif, Wt1 and Amh; Fig. 3A). Two of these transcripts (Tif 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, Dkk1 and Spol1), spermatocytes (Mybl1, Zfp541) or spermatids (1700021K02Rik, Hdc and Tpl1) was measured following FSH treatment (Fig. 4). Expression levels of Stra8, Spol1, Mybl1 and Tpl1 were unaffected by treatment but there was a transient increase in Pou5f1 at 12 h while Dkk1 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.
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 (\textit{Wif1}, \textit{Dmkn}, \textit{Dkk3}) were measured in \textit{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 \textit{hpg} mouse
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...
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 study...
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 (Mek) 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 Agr5 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 the Sertoli cell to become active enough to support germ cell receptors for follicle-stimulating hormone and androgen. Endocrinology 149 3275–3285.

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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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