Effects of follicle stimulating hormone on testicular mRNA transcript levels in the hypogonadal mouse

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Abstract

Follicle stimulating hormone (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 72h after the start of treatment. Approximately 400 transcripts were significantly altered at each time point by FSH treatment. At 12h there was a clear increase in the levels of a number of transcripts known to be expressed in the Sertoli cells (eg *Fabp5*, *Lgals1*, *Tesc*, *Scara5, Aqp5*). Additionally, level of Leydig cell transcipts were also markedly increased (eg *Ren1*, *Cyp17a1*, *Akr1b7*, *Star*, *Nr4a1*). This was associated with a small but significant rise in testosterone at 24 and 72h. At 24h, androgen-dependent Sertoli cell transcripts were upregulated (eg *Rhox5*, *Drd4*, *Spinlw1*, *Tubb3* and *Tsx*) and this trend continued up to 72h. Surprisingly, only 4 germ cell trancripts (*Dkkl1*, *Hdc*, *Oct4* and *1700021K02Rik*) were altered by FSH within the time-course of the experiment. Pathway analysis showed changes in cell cycle and cell proliferation pathways at all times and a general decline in transcripts related to formation and regulation of tight junctions. Results show 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.

Introduction

Postnatal testicular growth, spermatogenesis and fertility are dependent upon the pituitary gonadotrophins follicle-stimulating hormone (FSH) and luteinising hormone (LH). LH acts directly on Leydig cells to stimulate androgen production while androgens and FSH stimulate spermatogenesis through direct action on the Sertoli cells (1). The role of gonadotrophins is clearly seen in the hypogonadal (*hpg*) mouse which lacks gonadotrophin releasing hormone (GnRH) (2) and, consequently, has undetectable circulating levels of LH and FSH (3). The gonads of the *hpg* mouse remain in a pre-pubertal state throughout life, with spermatogenesis blocked at early meiosis (3;4) although treatment with exogenous gonadotrophins or androgens will increase testicular growth and restore germ cell development (5-8). 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 regulation of testicular function. In particular, study of mice lacking androgen receptors (AR) in the Sertoli cells (SCARKO (9)) have shown that androgens are essential for spermatocyte progression through meiosis. In contrast, mice lacking FSH (FSHβKO (10)) or the FSH-receptor (FSHRKO) (11;12) are fertile with all stages of spermatogenesis present. Nevertheless, in FSHRKO and FSHβKO mice there is a reduction in sperm number and quality (13;14) suggesting that FSH may act to optimise 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 (15). This is achieved by an increase in the number of spermatogonia and enhanced entry of these cells into meiosis (15).

Previous studies have identified a number of Sertoli cell products or mRNA transcripts that are FSH-sensitive including, for example, inhibin, AR, transferrin, DMRT, androgen binding protein and CREM (16-20). In addition, a previous study Sadate-Ngatchou *et al* (21) has examined the short term effects of FSH on testicular gene expression *in vivo* using arrays. In this series of experiments adult *hpg* mice were given a single injection of FSH and transcript levels were shown to peak 4h after injection and diminish thereafter (21). This study shows the acute response of the testis to FSH stimulation but we continue to lack a clear understanding of how FSH acts to regulate testicular development and function over the longer term. To extend and complement the earlier work of Sadate-Ngatchou *et al* (21), therefore, we have carried out a more comprehensive review of the effects of FSH 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 (3) were bred at Oxford. The *hpg* mutation was identified by PCR analysis of tail DNA as previously reported (22). 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, were injected sub-cutaneously with 8 IU recombinant human FSH (Serono Ltd. Middlesex, UK) in 0.2 ml PBS (phosphate buffered saline, pH 7.4, Sigma Aldrich, Dorset, UK) every 12 hours for 12, 24, or 72 hours. This dose of recombinant hormone had previously been shown to induce a significant increase in testis weight in *hpg* mice when given for one week (Abel and Charlton unpublished). Mice were killed one hour 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 sacrificed at each timepoint. 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 for 24hrs at 4°C and embedded in araldite. Semi-thin, 1µ 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 timepoint and the RNA from testes of individual animals extracted on RNeasy columns (QIAGEN, Valencia, CA). RNA was quantified using a NanoDrop ND-1000 (NanoDrop Wilmington, DE) and RNA quality was checked using the Agilent bioanalyzer 2100 (Agilent, Santa Clara, CA). Samples of total RNA (8µg) from individual animals were reverse transcribed and then *in vitro* transcribed and hybridised to mouse MOE430A arrays (Affymetrix, Santa Clara, CA) (n = 3 or 4 for each group) according to the Genechip expression technical manual (Affymetrix) as previously reported (23). 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, 12h and 72h FSH groups were extracted and hybridised to the arrays and in a subsequent experiment control and 24h 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, parametric test, variance not assumed equal, $p<0.05$. To generate data for cluster analysis the control groups were pooled. Clustering was carried out using a heuristic iterative two-step algorithm (24) (www.esat.kuleuven.ac.be/~dna/BioI/Software.html). Further analysis of potential interactions between significantly altered transcripts was partly 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, Paisley, UK) and residual genomic DNA was removed by DNAse treatment (DNA-*free,* Ambion Inc supplied by AMS Biotechnology, Abingdon, UK). RNA (1µg) was reverse transcribed using random hexamers (Ambion) and Moloney murine leukaemia virus reverse transcriptase (Life Technologies) as previously described (25).

Quanitative 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 manufacturers recommendations in a 25µl volume. For SYBR green, each reaction contained 5µl 2 x SYBR mastermix (Stratagene, Amsterdam, Netherlands), primer (100nM) and template in a total volume of 10µl. The thermal profile used for amplification was 95°C for 8min followed by 40 cycles of 95°C for 25 secs, 63°C for 25 sec and 72°C for 30 sec. 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, Warrington, UK) using parameters previously described (26). 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. 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 rFSH as above and intratesticular levels of testosterone measured by radioimmunoassay following ethanol extraction as previously described (27). 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 rFSH treatment.

There was a significant increase in testis weight within 12h of the start of FSH treatment

and weight continued to increase up to 24h (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 hours which became more marked by 72h (Fig 1B). This was confirmed on electron micrographs with several small vacuoles apparent within the cytoplasm at 24h and larger vacuoles present at 72 hours (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 (<25fmol/testis, n=8) and increased to low but consistently detectable levels 24h after the start of treatment with FSH (65.0 ± 12.4 fmol/testis, n=4) and remained detectable up to 72h (76.2 ± 45.0 fmol/testis, 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 72h after the start of FSH treatment and 162, 411 and 215 significantly decreased at the same times. Cluster analysis showed that there were 5 clusters containing more than 70% of the significantly altered transcripts and the three predominant clusters are shown in Supplementary Fig 1.

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 is shown in Supplementary Table 2. At 12h 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 (eg *Fabp5*, *Lgals1*, *Tesc*, *Scara5*) (28-31) and, perhaps surprisingly, in the Leydig cells (eg *Ren1*, *Cyp17a1*, *Akr1b7*, *Star*, *Ldlr*, *Nr4a1*) (32-35) (Table 1). Other transcripts listed in Table 1 do not have a known site of expression in the testis although 6 of these transcripts are known to be secreted from the cell (*Dmkn*, *Wif1*, *Col4a1*, *Pappa*, *Svs5*, *Dkk3*) and may be involved in the paracrine regulation induced by FSH. By 24h after the start of FSH treatment, androgen-dependent Sertoli cell transcripts appeared in the list of up-regulated transcripts (eg *Rhox5*, *Drd4*, *Spinlw1*, *Tubb3* and *Tsx*) (36-39) and this trend became more marked by 72h. Surprisingly, 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 72h respectively) (40) and *1700021K02Rik* (Spatial) (increased 3.1-fold at 72 h) (41) are significantly altered by FSH (Table 1 and Supplementary Table 2).

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 (42). 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 *Ren*) (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*) which 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 72h.

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 (36;38;39) (Fig 2B). In agreement with results from the array studies 4 transcripts (*Rhox5*, *Tsx*, *Drd4* and *Spinlw1*) showed increased expression 24-72h after FSH treatment while 1 transcript (*Igfbp3*) showed a significant decrease in expression (Fig 2B).

Sertoli cell genes

In addition to androgen-dependent Sertoli cell genes described above, 14 other known Sertoli cell transcripts were measured by real-time PCR following FSH treatment of adult *hpg* mice (Fig 3). Of these Sertoli cell transcripts 5 had shown significantly increased expression on the arrays (*Tesc*, *Lgals1*, *Aqp5*, *Dhh* and *Shbg*), 6 had not shown any significant change (*Trf*, *Wt1*, *Amh*, *Spata2*, *Tjp1* and *Gdnf*), 2 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 levels of transcript for 4 out of the 5 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 3 of the 6 transcripts which 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 2-fold increase on the arrays but had not reached significance.

The inhibin subunits are expressed in a number of cell types in the testis (43) 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

Failure of FSH treatment to have a marked effect on germ cell transcripts in the *hpg* testis was tested using real-time PCR. Expression of 8 germ cell transcripts known to be expressed predominantly in spermatogonia (*Stra8*, *Oct4*, *Dkkl1* and *Spo11*) spermatocytes (*Mybl1*) or spermatids (*1700021K02Rik* (spatial), *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 *Oct4* at 12h while *Dkkl1* showed a variable but significant increase at 72h. 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 6 of these transcripts (*Wif1*, *Dmkn*, *Dkk3*, *Pappa*, *Wnt4* and *Tfrc*) 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.

Pathway analysis

Analysis of significantly regulated transcripts (>1.5 fold) showed that there were alterations in cell cycle and cell proliferation pathways at all times (Supplementary Fig 2). At 12h after the first injection up-regulation of the pathway components predominated but as treatment continued there was a shift to down-regulation, particularly at 72h. Analysis of canonical pathways showed that components of the cholesterol biosynthetic pathway were significantly increased at 12h but not at other times (Supplementary Table 3A) 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 uncertain remains about how it acts to regulate Sertoli cell activity and spermatogenesis. The *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* (21) using a longer treatment period, different array chips with a larger characterised gene set (14,000 vs 6000 characterised genes) and with a larger animal cohort. Together, the two studies complement each other and serve to identify transcripts regulated by FSH over the short and medium term. The studies are not directly comparable, however, because of differences in time-course and treatment regime but the transcript lists can be compared with some caution at 12h. At this time the main difference is that animals used for this study will have been treated twice with FSH while those used by Sadate-Ngatchou *et al* (21) have been injected once. This comparison shows that at 12h after the start of FSH treatment there are 44 transcripts in common between the studies, 25 upregulated and 19 downregulated (Supplementary Table 3).

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 upregulated more than 2 fold at all times (Supplementary Table 4A) indicating that there was a changing pattern of expression as the exposure to FSH was maintained. There were also 63 transcripts

downregulated more than 2 fold at all times (Supplementary Table 4B) 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* (28-30) although some transcripts (eg *Shbg*, *Tjp1* (44;45)) 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.

Among the transcripts which showed decreased levels in response to FSH were a number encoding tight junction components. This is consistent with a recent 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 (46). Other transcripts which showed a significant decrease in levels after FSH treatment included *Fshr* and *Rgs11*. It is well established that FSH will cause downregulation of its receptor by decreasing transcript levels (47;48) 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 (49). 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 12h (32). Results from the arrays and real-time PCR show that all components of the androgen biosynthetic pathway were induced at 12h apart from *Hsd3b6*. Since *Hsd3b1* is already highly expressed in the adult *hpg* testis (50) 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 12h 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. The *hpg* mouse testis is likely to contain both adult and fetal-type Leydig cells (50) and *Hsd3b6* is a marker of adult Leydig cell differentiation (51). This might imply that FSH is acting to induce activity in the fetal Leydig cell population but *Sult1e1* is also a marker of adult Leydig cells (52) and is increased 72h 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 (53) 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 (50) and the effects are also very rapid since Sadate-Ngatchou *et al* (21) saw a marked increase in *Cyp17a1* after only 4h 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 (54-56). One report has suggested that the active factors are TIMP1 and Procathepsin L (57) 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 (58). 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 bioavailabitity and activity of IGF1 (59) although the effect of altered *Igfbp3* may be more complex (60). 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 (61) 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 starting, in most cases, 24h after the first FSH injection. 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 72h. Changes in the Sertoli cell and tubule diameter correlate with a marked rise in *Aqp5* at 12hours suggesting that increased water movement across the Sertoli cell membrane may contribute to increased tubular diameter and testis weight. The failure of FSH to have a marked effect on spermatogenesis within the time-course of the experiment was reflected in a relative lack of effect on germ cell transcripts on the arrays. Subsequent real-time PCR studies of a small number of known germ cell genes largely confirmed the array data but showed a significant increase in *Oct4* and *Dkkl1* not seen on the arrays. OCT4 has been shown to be necessary for primordial germ cell survival (62) and the increase in response to FSH, albeit small, may facilitate an increase in spermatogonial number within the testis. Both *Hdc* and *Spatial* are associated with round spermatids and the later stages of spermatogenesis (40;41) suggesting that the expression detected in this study must either be associated with earlier stages of spermatogenesis or with a different, somatic cell type. It has been shown previously that more prolonged treatment of *hpg* mice with FSH will stimulate an increase in germ cell number and development (5;63;64) but results from this study suggest that FSH has little stimulatory effect in the short term. In the longer term, 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 (27;65).

In this study, FSH treatment of *hpg* mice for up to 72h 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 (12;15) it had little effect on germ cell transcript levels up to 72h suggesting that longer-term action of FSH is required to affect spermatogenesis.

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Figure legends

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

Figure 2 Real-time PCR measurements of mRNA transcript levels in testes from adult *hpg* mice treated for 0 (control), 12, 24, or 72h with FSH. Data show results from Leydig cell-specific transcripts (A) and from Sertoli cell-specific, androgen-dependent transcripts (B). The mean ± sem of 3 or 4 animals per group is shown. Groups with different letter superscripts are significantly different.

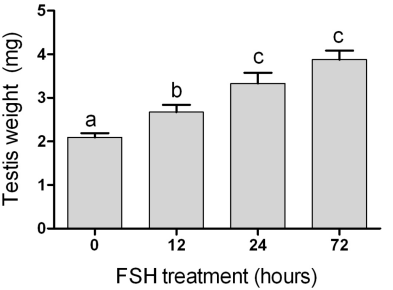
Figure 3 Real-time PCR measurements of Sertoli cell-specific mRNA transcript levels in testes from adult *hpg* mice treated for 0 (control), 12, 24, or 72h with FSH. Results show the mean ± sem of 3 or 4 animals per group. Groups with different letter superscripts are significantly different, where no superscripts are shown there was no difference between groups.

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 72h with FSH. Results show the mean ± sem of 3 or 4 animals per group. Groups with different letter superscripts are significantly different, where no superscripts are shown there was no difference between groups.

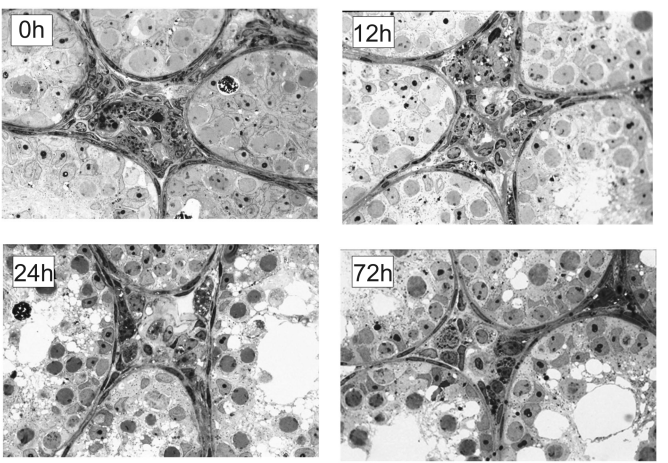
Figure 5 Real-time PCR measurements of transcript levels mRNA species with unknown testicular origin. Levels were measured in testes from adult *hpg* mice treated for 0 (control), 12, 24, or 72h with FSH. Results show the mean ± sem of 3 or 4 animals per group. Groups with different letter superscripts are significantly different.

Fig 1

A)


Testis weight (mg)
5
4
3
2
1
0
a
b
c
c
0
12
24
72
FSH treatment (hours)

B)


0h
12h
24h
72h

C)

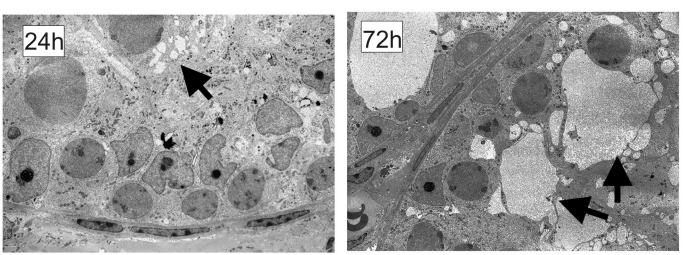
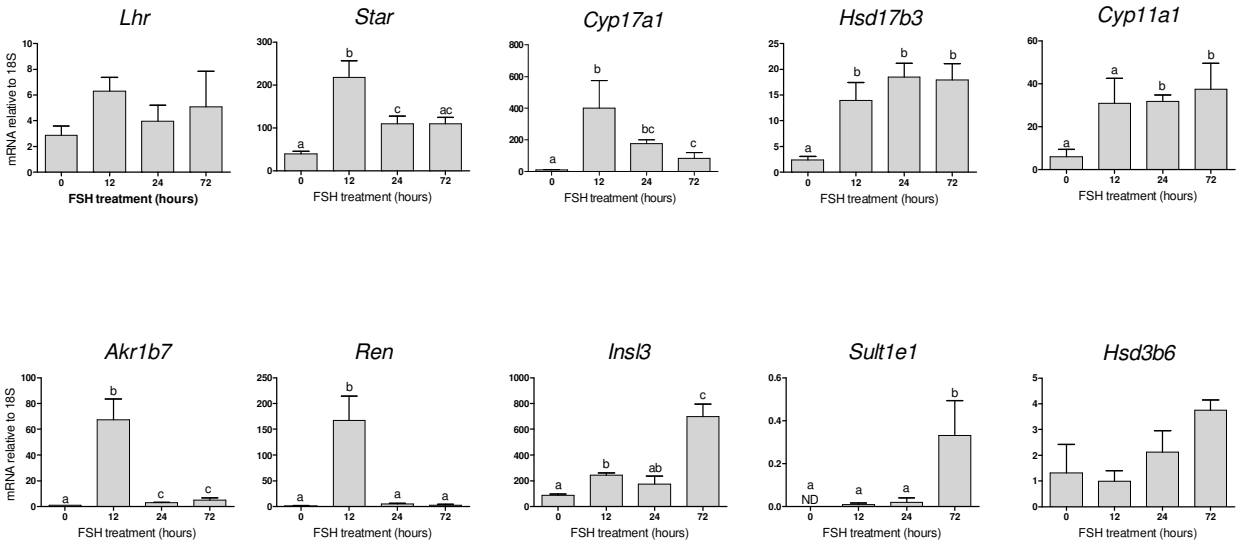

24h
72h

Fig 2

A)


Lhr
Star
Cyp17a1
Hsd17b3
Cyp11a1
Akr1b7
Ren
Insl3
Sult1e1
Hsd3b6
mRNA relative to 18S
FSH treatment (hours)
ND

B)

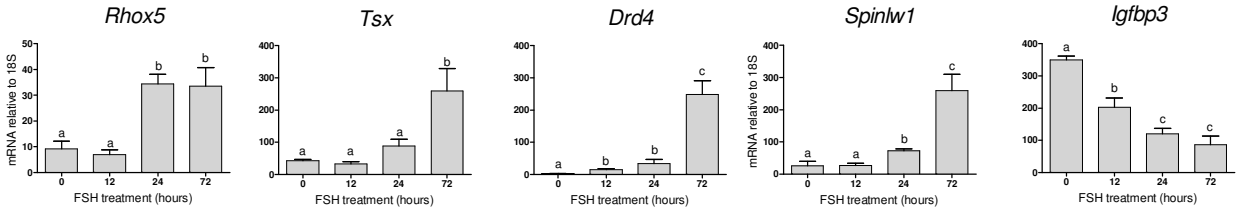
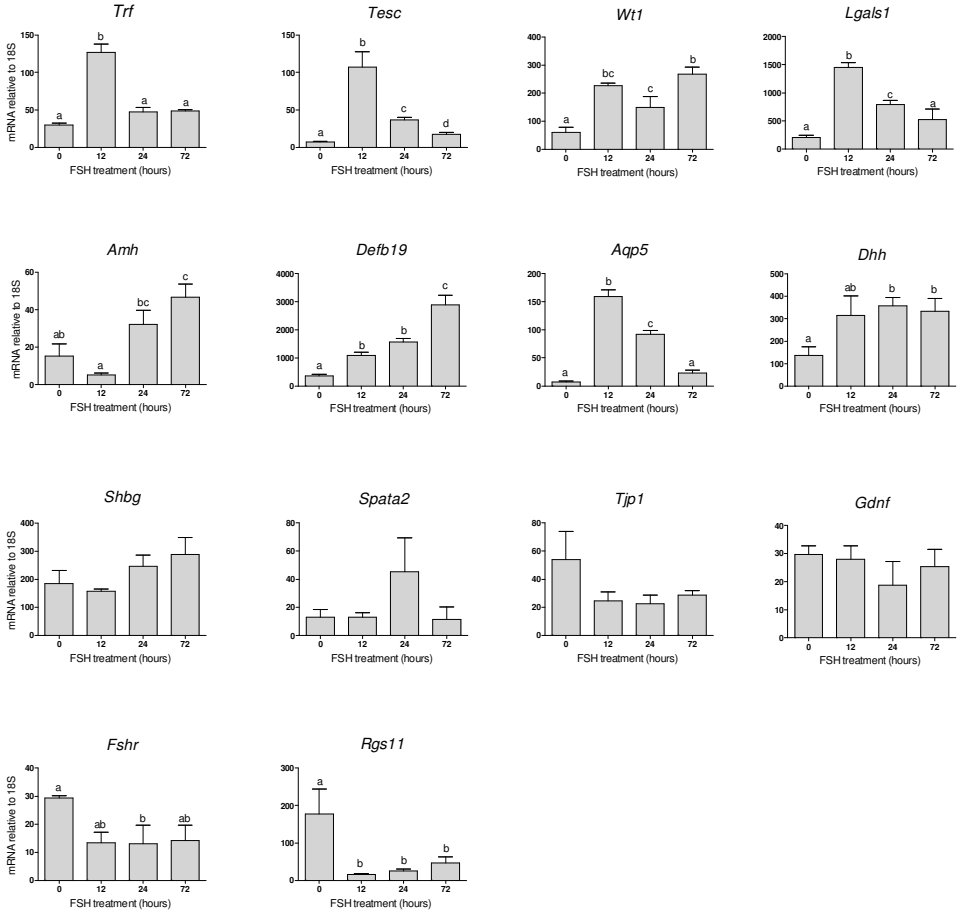

Rhox5
Tsx
Drd4
Spinlw1
Igfbp3
mRNA relative to 18S
FSH treatment (hours)

Fig 3

A)


Trf
Tesc
Wt1
Lgals1
Amh
Defb19
Aqp5
Dhh
Shbg
Spata2
Tjp1
Gdnf
Fshr
Rgs11
mRNA relative to 18S
FSH treatment (hours)
0
12
24
72

B)

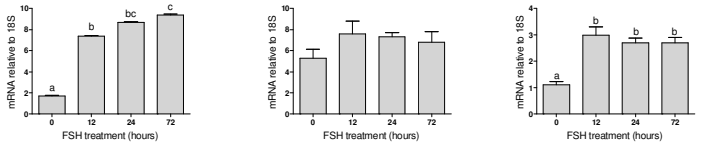

Inha
Inhba
Inhbb
mRNA relative to 18S
FSH treatment (hours)
0
12
24
72

Fig 4

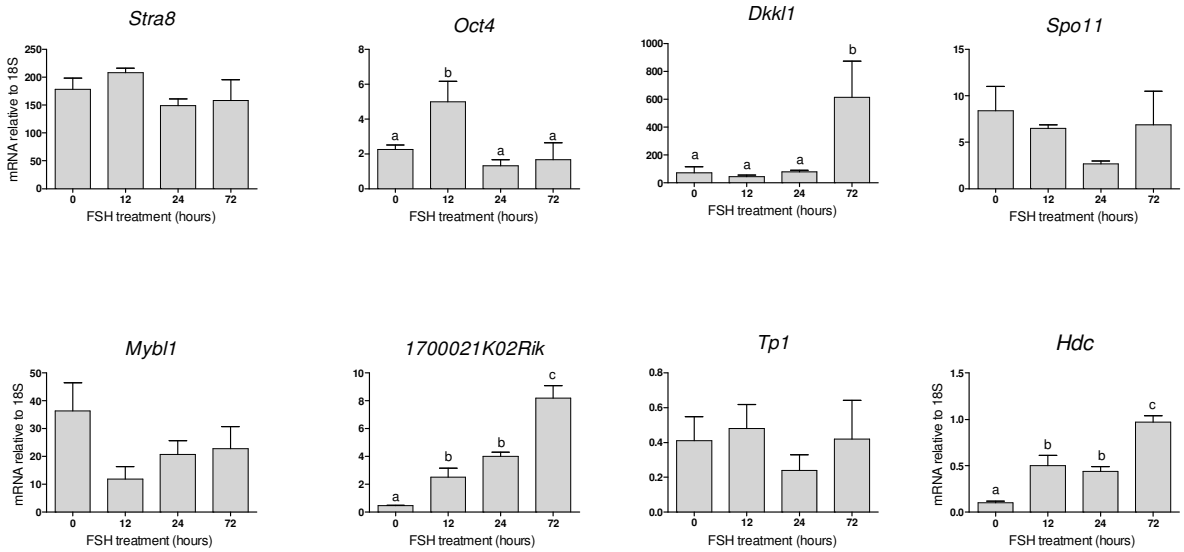

Stra8
Oct4
Dkkl1
Spo11
Mybl1
1700021K02Rik
Tp1
Hdc
mRNA relative to 18S
FSH treatment (hours)
0
12
24
72
a
b
c

Fig 5

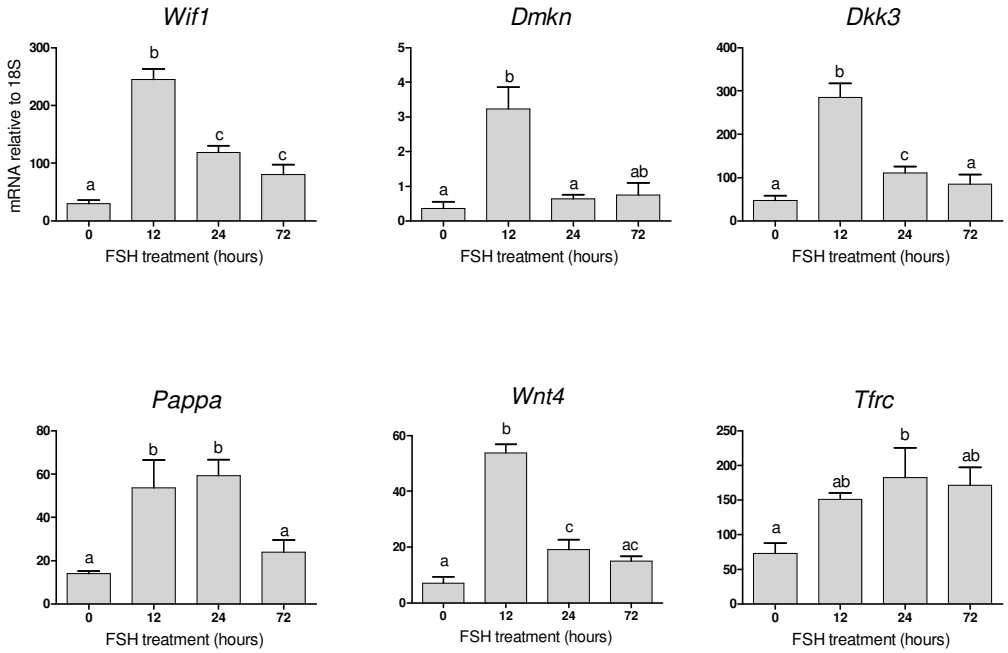

Wif1
mRNA relative to 18S
300
200
100
0
a
b
c
c
0
12
24
72
FSH treatment (hours)
Dmkn
5
4
3
2
1
0
a
b
a
ab
0
12
24
72
FSH treatment (hours)
Dkk3
400
300
200
100
0
a
b
c
a
0
12
24
72
FSH treatment (hours)
Pappa
80
60
40
20
0
a
b
b
a
0
12
24
72
FSH treatment (hours)
Wnt4
60
40
20
0
a
b
c
ac
0
12
24
72
FSH treatment (hours)
Tfrc
250
200
150
100
50
0
a
ab
b
ab
0
12
24
72
FSH treatment (hours)

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

Transcripts up-regulated 12h after start of treatment

Fold change	Gene symbol	Gene Title
20.7	Ren1	renin 1 structural
18.2	Dmkn	dermokine
13.3	Aqp5	aquaporin 5
12.1	Wif1	Wnt inhibitory factor 1
11.0	Fabp5	fatty acid binding protein 5, epidermal
6.6	Tubb3	tubulin, beta 3
6.5	Cyp17a1	cytochrome P450, family 17a 1
6.3	Akr1b7	aldo-keto reductase family 1 B7
6.0	Lgals1	lectin, galactose binding, soluble 1
5.9	Col4a1	procollagen, type IV, alpha 1
5.7	Pappa	pregnancy-associated plasma protein A
5.2	Star	steroidogenic acute regulatory protein
4.6	Ldlr	low density lipoprotein receptor
4.6	Tesc	tescalcin
4.6	Rps6ka2	ribosomal protein S6 kinase 2
4.6	Scara5	scavenger receptor class A 5
4.4	Hgsnat	Heparan N-acetyltransferase
4.4	Hs3st1	heparan sulfate 3-O-sulfotransferase 1
4.2	Syne1	synaptic nuclear envelope 1
4.1	Slc38a5	solute carrier family 38, member 5
4.0	Gpd1	glycerol-3-phosphate dehydrogenase 1
3.9	Dos	downstream of Stk11
3.8	Svs5	seminal vesicle secretory protein 5
3.8	Bhmt	betaine-homocysteine methyltransferase
3.7	D9Ertd280e	Chr 9, ERATO Doi 280
3.7	Tnfrsf12a	tumor necrosis factor receptor 12a
3.7	1200016E24Rik	RIKEN cDNA 1200016E24
3.7	Nr4a1	nuclear receptor subfamily 4, group A1
3.6	Dkk3	dickkopf homolog 3
3.6	Cyp51	cytochrome P450, family 51

Transcripts down-regulated 12h after start of treatment

Fold change	Gene symbol	Gene Title
7.27	Myh8	myosin, heavy polypeptide 8
6.65	Rin2	Ras and Rab interactor 2
5.03	Pdgfc	platelet-derived growth factor C
4.55		Transcribed locus*
4.53	Bcan	brevican
4.46	Rgs11	regulator of G-protein signaling 11
4.45	Tmem37	transmembrane protein 37
4.43	Fhod3	formin homology 2 domain containing 3
4.38	Derl3	Der1-like domain family, member 3
4.01	Ddit4l	DNA-damage-inducible transcript 4-like
3.98	BC013672	cDNA sequence BC013672
3.97	Scin	scinderin
3.96	Ddit4l	DNA-damage-inducible transcript 4-like
3.89	Cabc1	chaperone, ABC1 complex like
3.86	Krt20	keratin 20
3.74	Tmem140	transmembrane protein 140
3.71	Dbp	D site albumin promoter binding protein
3.71	Rnasel	ribonuclease L
3.70	Spsb1	splA receptor domain and SOCS box 1
3.67	Tnni3	troponin I, cardiac
3.65	Cdo1	cysteine dioxygenase 1, cytosolic
3.57	Stard8	START domain containing 8
3.55	Slc40a1	solute carrier family 40, member 1
3.52	Hdac5	histone deacetylase 5
3.32	Dbp	D site albumin promoter binding protein
3.23	Chdh	choline dehydrogenase
3.23	8030411F24Rik	RIKEN cDNA 8030411F24 gene
3.23	Per3	period homolog 3 (Drosophila)
3.22	Ctnna2	catenin, alpha 2
3.19	Trim47	tripartite motif protein 47

Transcripts up-regulated 24h after start of treatment

Fold change	Gene symbol	Gene title
28.8	Lin7c	lin-7 homolog C (C. elegans)
18.0	Cyp17a1	cytochrome P450, family 17a1
13.6	Cyp11a1	cytochrome P450, family 11a1
11.0	Fabp5	fatty acid binding protein 5, epidermal
10.1	Rhox5	reproductive homeobox 5
9.4	Star	steroidogenic acute regulatory protein
8.7	Slc38a5	solute carrier family 38, member 5
7.9	Aqp5	aquaporin 5
7.4	Tubb3	tubulin, beta 3
7.0	Drd4	dopamine receptor 4
6.3	Tesc	tescalcin
5.5	Lgals1	lectin, galactose binding, soluble 1
5.0	Spinlw1	eppin
4.6	Osr1	odd-skipped related 1
4.5	Fads2	fatty acid desaturase 2
4.2	Pappa	pregnancy-associated plasma protein A
4.2	Scara5	scavenger receptor class A5
4.2	Pscdbp	pleckstrin homology binding protein
4.1	Plac8	placenta-specific 8
4.0	Gpt2	glutamic pyruvate transaminase 2
4.0	Rps6ka2	ribosomal protein S6 kinase 2
4.0	Gpd1	glycerol-3-phosphate dehydrogenase 1
4.0	Hdc	histidine decarboxylase
3.8	Igf1	insulin-like growth factor 1
3.7	Fah	fumarylacetoacetate hydrolase
3.7	Mpzl2	myelin protein zero-like 2
3.6	Insl3	insulin-like 3
3.4	Wif1	Wnt inhibitory factor 1
3.3	Inha	inhibin alpha
3.2	Col18a1	procollagen, type XVIII, alpha 1

Transcripts down-regulated 24h after start of treatment

Fold change	Gene Smbol	Gene title
9.9	Ddit4l	DNA-damage-inducible transcript 4-like
9.9	Rin2	Ras and Rab interactor 2
9.1	Slc40a1	solute carrier family 40, member 1
8.0	Rgs11	regulator of G-protein signaling 11
7.6	Igfbp3	insulin-like growth factor binding protein 3
7.4	Myh6	myosin, heavy polypeptide 6 alpha
7.2	Apbb2	amyloidprecursor protein-binding B2
6.7	Rassf5	Ras association domain family 5
6.3	Tmem37	transmembrane protein 37
6.3	Chdh	choline dehydrogenase
6.2	Itga9	integrin alpha 9
6.2	Thbd	thrombomodulin
5.9	Fcgr2b	Fc receptor, IgG, low affinity IIb
5.8	Trim47	tripartite motif protein 47
5.7	Spsb1	splA receptor domain and SOCS box 1
5.6	Ddit4l	DNA-damage-inducible transcript 4-like
5.5	Vnn1	vanin 1
5.5	Fcgr2b	Fc receptor, IgG, low affinity IIb
5.4	Ptprd	protein tyrosine phosphatase, receptor D
5.4	Nkx3-1	NK-3 transcription factor, locus 1
5.3	Fcgr2b	Fc receptor, IgG, low affinity IIb
5.1	Ctgf	connective tissue growth factor
5.0	H19	H19 fetal liver mRNA
5.0	Pdgfc	platelet-derived growth factor C
4.9	Hsd17b11	hydroxysteroid (17β) dehydrogenase 11
4.9	Cabc1	chaperone, ABC1complex like
4.9	9630031F12Rik	RIKEN cDNA 9630031F12 gene
4.8	Ptch1	patched homolog 1
4.7	Smoc2	SPARC related modular calcium binding 2
4.7	Fhod3	formin homology 2 domain containing 3

Transcripts up-regulated 72h after start of treatment

Fold change	Gene symbol	Gene title
35.0	Drd4	dopamine receptor 4
21.1	Slc38a5	solute carrier family 38a5
14.9	Rhox5	reproductive homeobox 5
13.9	Fabp5	fatty acid binding protein 5, epidermal
8.4	Spinlw1	eppin

Transcripts down-regulated 72h after start of treatment

Fold change	Gene symbol	Gene title
13.4	Igfbp3	insulin-like growth factor binding protein 3
8.2	Rgs11	regulator of G-protein signaling 11
6.6	Rin2	Ras and Rab interactor 2
6.5	Clca1	chloride channel calcium activated 1
6.0	Slc40a1	solute carrier family 40 1

7.6	Klk1b24	kallikrein 1-related peptidase b24
6.6	Tubb3	tubulin, beta 3
5.9	Hdc	histidine decarboxylase
5.5	Fah	fumarylacetoacetate hydrolase
5.5	Tsx	testis specific X-linked gene
5.4	Zcchc18	zinc finger, CCHC domain 18
4.6	Sct	secretin
4.6	Gpd1	glycerol-3-phosphate dehydrogenase 1
4.5	Myh1	myosin, heavy polypeptide 1
4.5	Fabp4	fatty acid binding protein 4, adipocyte
4.5	St8sia2	ST8 sialyltransferase 2
4.5	Tgfb1	transforming growth factor, beta 1
4.3	Pscdbp	pleckstrin homology binding protein
4.2	Igf1	insulin-like growth factor 1
4.1	Scara5	scavenger receptor class A, member 5
4.1	Sept6	septin 6
4.0	D17H6S56E-5	Chr 17, human D6S56E 5
4.0	Pappa	pregnancy-associated plasma protein A
4.0	Klk1	kallikrein 1
3.9	Dmkn	dermokine
3.8	Tpd52l1	tumor protein D52-like 1
3.8	Inha	inhibin alpha
3.8	Slc25a5	solute carrier family 25, member 5
3.7	Car3	carbonic anhydrase 3
3.7	Pde4b	phosphodiesterase 4B, cAMP specific
5.6	Spsb1	splA receptor domain and SOCS box 1
4.9	Ifitm1	interferon induced transmembrane 1
4.7	Myh8	myosin, heavy polypeptide 8
4.6	Fhod3	formin homology 2 domain containing 3
4.4	Bcan	brevican
4.4	Tmem140	transmembrane protein 140
4.4	BC013672	cDNA sequence BC013672
4.3	Tmem37	transmembrane protein 37
4.0	Erbb3	v-erb-b2 homolog 3
3.9	Dst	dystonin
3.9	Xist	inactive X specific transcripts
3.8	Arhgdig	Rho GDP dissociation inhibitor gamma
3.7	Edn1	endothelin 1
3.7	6330403K07Rik	RIKEN cDNA 6330403K07 gene
3.7	Jun	Jun oncogene
3.6	Pla2g5	phospholipase A2, group V
3.6	Apbb2	amyloid beta precursor protein-binding B2
3.6	H19	H19 fetal liver mRNA
3.6	Hspb1	heat shock protein 1
3.6	Fcgr2b	Fc receptor, IgG, low affinity IIb
3.5		Transcribed locus**
3.5	Adi1	acireductone dioxygenase 1
3.4	H2-T23	histocompatibility 2, T region locus 23
3.4	Scin	scinderin
3.3	Rnasel	ribonuclease L

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