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# Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors

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## Abstract

**FSH and androgen act to stimulate and maintain spermatogenesis. FSH acts directly on the Sertoli cells to stimulate germ cell number and acts indirectly to increase androgen production by the Leydig cells. In order to differentiate between the direct effects of FSH on spermatogenesis and those mediated indirectly through androgen action, we have crossed hypogonadal (*hpg*) mice, which lack gonadotrophins, with mice lacking androgen receptors (AR) either ubiquitously (ARKO) or specifically on the Sertoli cells (SCARKO). These *hpg*.ARKO and *hpg*.SCARKO mice were treated with recombinant FSH for 7 days and testicular morphology and cell numbers were assessed. In untreated *hpg* and *hpg*.SCARKO mice, germ cell development was limited and did not progress beyond the pachytene stage. In *hpg*.ARKO mice, testes were smaller with fewer Sertoli cells and germ cells compared to *hpg* mice. Treatment with FSH had no effect on Sertoli cell number but significantly increased germ cell numbers in all groups. In *hpg* mice, FSH increased the numbers of spermatogonia and spermatocytes, and induced round spermatid formation. In *hpg*.SCARKO and *hpg*.ARKO mice, in contrast, only spermatogonial and spermatocyte numbers were increased with no formation of spermatids. Leydig cell numbers were increased by FSH in *hpg* and *hpg*.SCARKO mice but not in *hpg*.ARKO mice. Results show that in rodents 1) FSH acts to stimulate spermatogenesis through an increase in spermatogonial number and subsequent entry of these cells into meiosis, 2) FSH has no direct effect on the completion of meiosis and 3) FSH effects on Leydig cell number are mediated through interstitial ARs.**

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## Introduction

Sertoli cell function and spermatogenesis are dependent upon the actions of FSH and androgen. This is clearly seen in hypogonadal (*hpg*) mice, which lack circulating FSH and LH and are infertile with associated failure of the germ cells to progress beyond early meiosis (Cattanach *et al*. 1977). Treatment of *hpg* mice with FSH stimulates germ cell proliferation, with an increase in spermatogonial and spermatocyte numbers, and induces spermatid formation (Singh & Handelsman 1996). This is similar to the effects of FSH in the GNRH-immunised or hypophysectomised rat (Vihko *et al*. 1991, Russell *et al*. 1993, McLachlan *et al*. 1995), and in tandem with studies on FSH receptor knockout (FSHRKO) mice, our current understanding is that the primary function of FSH is to maintain germ cell numbers and to promote germ cell progression through meiosis (Dierich *et al*. 1998). One major problem with the study of FSH action in these models, however, is dissociating the effects of FSH from those of androgen. In early studies, the FSH preparations used contained low levels of LH, which could induce androgen production by the Leydig cells. Use of recombinant FSH has overcome this problem but there remains the issue that FSH has been shown to induce Leydig cell function, probably indirectly through stimulation of the Sertoli cells (Johnson & Ewing 1971, Chen *et al*. 1976, Vihko *et al*. 1991). We have shown that FSH will increase androgen levels in the *hpg* testis and that it will induce expression of androgen-dependent Sertoli cell genes such as *Rhox5* (Abel *et al*. 2009). It remains likely, therefore, that some of the effects of FSH on spermatogenesis, seen in models such as the *hpg* or hypophysectomised animal, are mediated indirectly through stimulation of androgen production. To investigate the role of androgen in mediating FSH action and to identify the direct effects of FSH, we have generated *hpg* mice


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lacking androgen receptors (AR) either ubiquitously (*hpg*.ARKO) or specifically on the Sertoli cells (*hpg*.SCARKO). Treatment of these mice with FSH allows us to dissect the direct effects of FSH on testicular function from those mediated by androgen action through the Sertoli cell or other androgen-responsive cells in the testis.

## Results

### *Testis volume, seminal vesicle weight and testosterone levels*

Testicular volume was similar in *hpg* and *hpg*.SCARKO mice but was significantly reduced in *hpg*.ARKO mice (Fig. 1). Treatment of the animals with FSH for 7 days increased testis volume significantly in all three groups. Seminal vesicle weights were similar in *hpg* and *hpg*.SCARKO mice, and were significantly increased by FSH (Table 1). The *hpg*.ARKO mice do not develop seminal vesicles. Intratesticular testosterone levels were significantly increased in *hpg* mice after 7 days treatment with FSH but were unaffected in *hpg*.SCARKO or *hpg*.ARKO mice (Fig. 1). For comparison, intratesticular testosterone levels in normal adult mice are about 50 pmol/testis (Baker *et al*. 2003, O'Shaughnessy *et al*. 2008).

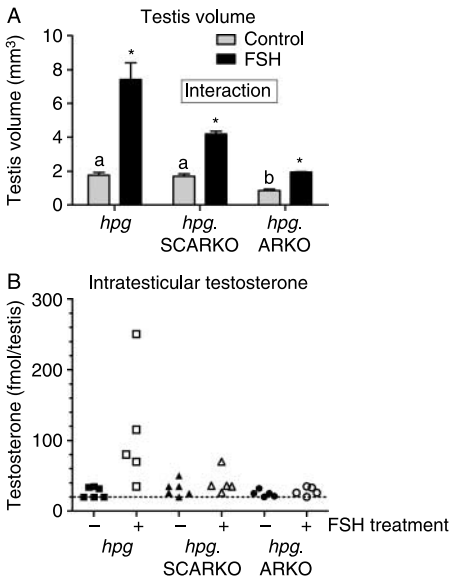


**Figure 1** Effect of FSH on (A) testis volume and (B) intratesticular testosterone in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. In (A), data were analysed by two-factor ANOVA followed by *t*-tests as described in Materials and Methods. An interaction indicates that the effect of FSH was significantly different in the three animal groups. In untreated animals, groups with different letter superscripts were significantly different ($P<0.05$). If FSH had a significant effect on a particular animal group, this is indicated by *. Mean±S.E.M. is shown. Animal numbers, *hpg* $n=7$; *hpg*+FSH $n=3$; *hpg*.SCARKO $n=5$; *hpg*.SCARKO+FSH $n=4$; *hpg*.ARKO $n=3$; *hpg*.ARKO+FSH $n=3$. In (B), individual values from each animal are shown. The limit of detection of the assay is indicated by the broken horizontal line. Data were analysed by the Kruskal–Wallis and Mann–Whitney tests. FSH had a significant effect ($P<0.05$) in the *hpg* mice but had no effect in the other animal groups.

**Table 1** Seminal vesicle (SV) weights in *hpg* and *hpg*.SCARKO mice.

| | **SV weight** (mg) | |
|---|---|---|
| **Group** | Control | plus FSH |
| *hpg* | 2.94±0.16 ($n=13$) | 3.40±0.13 ($n=30$) |
| *hpg*.SCARKO | 3.18±0.15 ($n=12$) | 3.65±0.16 ($n=19$) |

There was no significant difference between SV weights in *hpg* and *hpg*.SCARKO mice, but there was a significant ($P<0.05$) effect of FSH in both groups. Mean±S.E.M. is reported.

### *Morphology*

As described previously, and with the variation noted in Materials and Methods, spermatogenesis in untreated *hpg* mice was severely disrupted with spermatocyte progression only as far as the pachytene stage and no spermatids present (Fig. 2). Few Leydig cells were apparent within the interstitial space (Fig. 2). Testes from untreated *hpg*.SCARKO mice had a similar morphology with spermatogenesis progressing to the primary spermatocyte stage (Fig. 2). In *hpg*.ARKO mice, the tubules were smaller, and while spermatogenesis progressed to the same stage as the *hpg* mice, fewer spermatocytes were apparent. Crystalline structures with the appearance of microliths were present in some tubules, as previously reported (O'Shaughnessy *et al*. 2009).

Treatment of *hpg* mice with FSH caused an increase in seminiferous tubule diameter (Figs 2 and 3), clear establishment of a tubular lumen and an increase in germ cell number (Fig. 2). In some tubules, spermatogenesis progressed to the round spermatid stage (Fig. 2). In *hpg*.SCARKO and *hpg*.ARKO mice, there was also an increase in tubule diameter, although not as marked as in the *hpg* (Figs 2 and 3) and, while there was a clear increase in germ cell number, there was no apparent progression beyond the primary spermatocyte stage.

FSH treatment caused an apparent increase in interstitial space and cell numbers in *hpg* and *hpg*.SCARKO mice (Fig. 2). There was no clear effect of FSH on the interstitium of the *hpg*.ARKO mouse (Fig. 2).

### *Stereology*

Sertoli cell number was similar in *hpg* and *hpg*.SCARKO mice but was significantly reduced in *hpg*.ARKO mice (Fig. 3). Treatment with FSH had no significant effect on Sertoli cell number in any group. Leydig cell number was similar in *hpg* and *hpg*.SCARKO mice but was slightly reduced in *hpg*.ARKO mice compared with the *hpg*.SCARKO (Fig. 3). Treatment with FSH increased Leydig cell number in *hpg* and *hpg*.SCARKO mice but had no effect in *hpg*.ARKO mice.

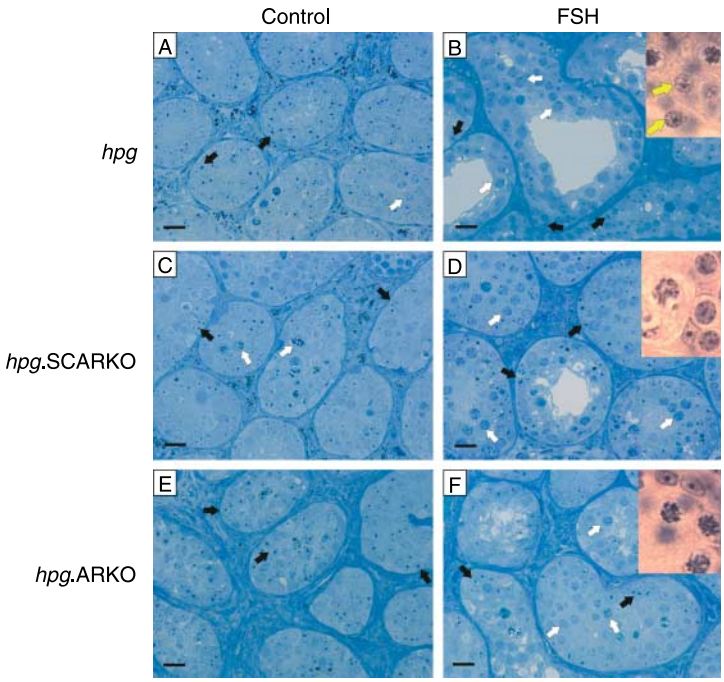


**Figure 2** Semi-thin sections showing testicular morphology in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice and in animals treated with FSH. In untreated animals (A, C and E), spermatogenesis was severely disrupted with only spermatogonia and some spermatocytes present. In *hpg*.ARKO (E) mice, tubule diameter was smaller and relative interstitial space was larger. Treatment with FSH (B, D and F) increased tubule diameter and germ cell numbers in all mice, although the effect was most marked in the *hpg*. After FSH treatment, round spermatids developed in *hpg* mice (yellow arrow, inset B) but not in either *hpg*.SCARKO or *hpg*.ARKO mice (insets D and F). Representative Sertoli cells and spermatocytes are indicated by black and white arrows respectively. Photomicrographs are from semi-thin sections of testes fixed in paraformaldehyde/gluteraldehyde apart from the insets, which are from Bouin's-fixed testes. The bar represents 20 μm.

Spermatogonial, spermatocyte and total germ cell numbers were similar in *hpg* and *hpg*.SCARKO mice but were significantly reduced in *hpg*.ARKO mice (Fig. 4). Treatment with FSH increased total germ cell number in all three groups by three- to fourfold (Fig. 4). Statistical analysis showed no interaction between the effects of FSH and animal phenotype, indicating that the effect of FSH was similar in all three groups. Further analysis of germ cell types showed that spermatogonial and spermatocyte numbers were increased by FSH in all groups with no significant interaction. As indicated above, FSH treatment induced development of round spermatids only in *hpg* mice and not in *hpg*.SCARKO or *hpg*.ARKO mice.

## Discussion

The function that FSH plays in the regulation of spermatogenesis in rodents and higher mammals has been the subject of considerable study, and continuing uncertainty, since the early pioneering work in the 1930s, which showed that FSH could partially restore spermatogenesis in rats after hypophysectomy (Greep *et al*. 1936). Problems with LH contamination of FSH preparations limited the progress for a number of years, but a major development in our understanding of FSH action in the rodent came with the generation of mice lacking FSH (FSHβKO) or the FSH receptor (FSHRKO; Kumar *et al*. 1997, Abel *et al*. 2000, Krishnamurthy *et al*. 2000). These animals were fertile, but they showed that FSH was required for normal development of Sertoli cell and germ cell numbers (Kumar *et al*. 1997, Abel *et al*. 2000, Krishnamurthy *et al*. 2000). In addition, more recent study of mice lacking both FSHR and AR on the Sertoli cells showed that FSH acts to increase the number of spermatogonia and the entry of these cells into meiosis (Abel *et al*. 2008). From these studies, it was clear that FSH was required for normal testicular development, but it still remained uncertain how many of the effects of FSH were mediated directly through FSH action and how many were dependent on indirect alteration of androgen levels. This study was designed, therefore, to determine which effects of FSH on testicular function are direct, which are dependent on androgen action and whether those effects of androgen are mediated through the Sertoli cell.

Sertoli cell numbers in the mouse are normally determined by around post-natal day 15. Factors regulating Sertoli cell number are not fully understood but androgens, probably acting through the peritubular myoid cells (PMCs), stimulate proliferation *in utero*, while FSH is required post-natally (Johnston *et al*. 2004, Tan *et al*. 2005). In the adult *hpg* mouse, Sertoli cell numbers are about 50% of normal (Baker & O'Shaughnessy 2001, Haywood *et al*. 2003), reflecting the loss of both FSH and androgen post-natally in these mice. Interestingly, there was a reduction in Sertoli cell numbers in the *hpg*.ARKO mice compared with the *hpg*. Androgen production by the *hpg* is minimal post-natally but is normal *in utero* (O'Shaughnessy *et al*. 1998), and Sertoli cell numbers are normal at birth (Baker & O'Shaughnessy 2001, Johnston *et al*. 2004). This contrasts with ARKO or androgen-resistant *Tfm* mice that have reduced Sertoli cell number at birth (Johnston *et al*. 2004, Tan *et al*. 2005) suggesting that differences in Sertoli cell numbers between *hpg* and

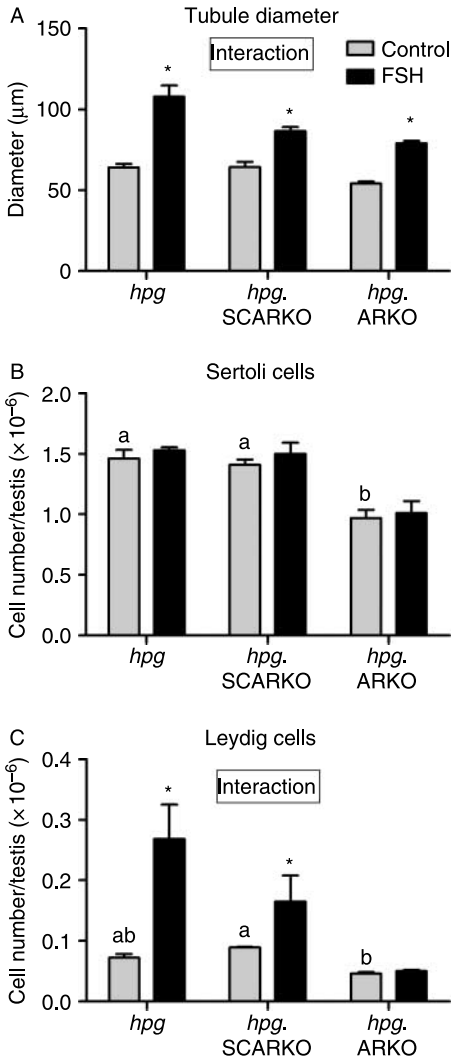


**Figure 3** Effect of FSH on (A) tubule diameter, (B) Sertoli cell number and (C) Leydig cell number in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. An interaction indicates that the effect of FSH was significantly different in the three animal groups. In untreated animals, groups with different letter superscripts were significantly ($P<0.05$) different. If FSH had a significant effect on a particular animal group, this is indicated by *. Mean±S.E.M. is shown. Animal numbers, *hpg* $n=6$; *hpg*+FSH $n=3$; *hpg*.SCARKO $n=4$; *hpg*.SCARKO+FSH $n=3$; *hpg*.ARKO $n=3$; *hpg*.ARKO+FSH $n=3$.

*hpg*.ARKO mice are likely to be due to androgen action in the foetal testis. The number of Sertoli cells in *hpg*.SCARKO mice was the same as in the *hpg*, consistent with data showing that SCARKO mice have a normal contingent of Sertoli cells (De Gendt *et al.* 2004, Abel *et al.* 2008). This provides further confirmation that androgen effects on Sertoli cell numbers are independent of direct androgen action on the Sertoli cell (Johnston *et al.* 2004, Tan *et al.* 2005). Failure of FSH to affect Sertoli cell number in any group in this study is consistent with earlier findings (O'Shaughnessy *et al.* 1992, Singh & Handelsman 1996) and indicates that the Sertoli cells in the adult *hpg* are no longer sensitive to the mitogenic effects of FSH.

Differences in germ cell numbers between untreated *hpg* and *hpg*.ARKO mice could be due to the presence of very low levels of androgen in the post-natal *hpg* testis or, as above, to the effects of androgen action *in utero*. Androgen action *in utero* appears more likely since the presence of endogenous testicular androgen post-natally would probably lead to a difference in germ cell number between *hpg* and *hpg*.SCARKO mice, as androgen action through the Sertoli cell is clearly required for normal germ cell development (De Gendt *et al.* 2004). If the effects are due to androgen action *in utero*, differences between *hpg* and *hpg*.SCARKO mice would not arise since Sertoli cells do not express ARs until after birth (Bremner *et al.* 1994, Zhou *et al.* 1996). In the foetal testis, ARs are expressed predominantly on PMCs, which would suggest that the differences in germ cell number between adult *hpg* and *hpg*.ARKO mice are due to androgen action through the PMCs *in utero*. Interestingly, it has recently been shown that androgen action through the PMCs is essential post-natally for the development of normal spermatogenesis (Welsh *et al.* 2009). It has been reported that primordial germ cells express the AR, which would offer an alternative mode of action of androgens *in utero* (Merlet *et al.* 2007). The direct effect of androgen on the germ cells is reported to be inhibitory, however (Merlet *et al.* 2007), suggesting that this is unlikely to explain differences between *hpg* and *hpg*.ARKO mice.

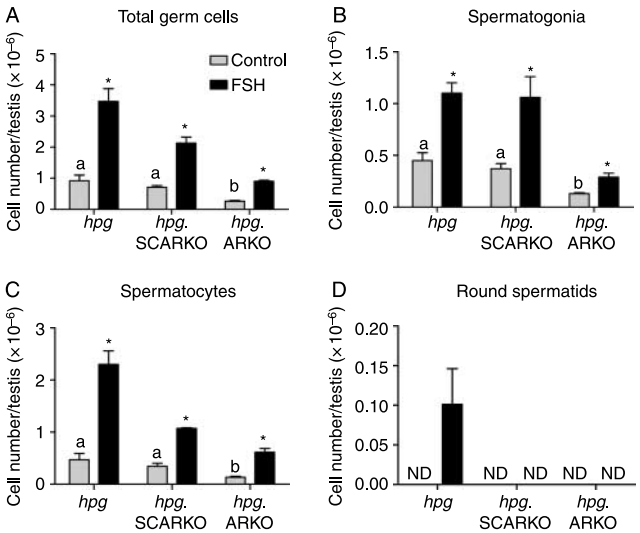


**Figure 4** Effect of FSH on numbers of (A) total germ cell, (B) spermatogonia, (C) spermatocytes and (D) round spermatids per testis in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. In untreated animals, groups with different letter superscripts were significantly different ($P<0.05$). If FSH had a significant effect on a particular animal group, this is indicated by *. Mean±S.E.M. is shown. Animal numbers, *hpg* $n=6$; *hpg*+FSH $n=3$; *hpg*.SCARKO $n=4$; *hpg*.SCARKO+FSH $n=3$; *hpg*.ARKO $n=3$; *hpg*.ARKO+FSH $n=3$.

A number of previous studies, using a variety of different models including the *hpg* mouse, *hpg* mouse expressing FSH, GNRH-immunised rat and hypophysectomised rat, have reported that FSH acts to increase the numbers of spermatogonia, spermatocytes and round spermatids (Vihko *et al*. 1991, Bremner *et al*. 1994, McLachlan *et al*. 1995, Russell *et al*. 1998, Haywood *et al*. 2003). In the *hpg*, *hpg*.SCARKO and *hpg*.ARKO models, FSH increased the total germ cell number and spermatogonial and spermatocyte numbers, consistent with earlier studies, and showed that these effects of FSH are independent of androgen action through the Sertoli cell or any other androgen-responsive cell in the testis. FSH treatment also stimulated round spermatid formation in the *hpg* testis, as previously reported (Singh & Handelsman 1996, Haywood *et al*. 2003), although spermatid numbers were only about 5% of spermatocyte numbers. In contrast, FSH failed to stimulate the generation of round spermatids in the *hpg*.SCARKO and *hpg*.ARKO mice showing that this effect of FSH is entirely dependent on androgen action through the Sertoli cells. This is consistent with earlier studies using hypophysectomised rats, which showed that stimulation of post-meiotic germ cell formation by FSH was partially inhibited by the AR antagonist flutamide (Russell *et al*. 1998) or ethane dimethane sulphonate, which acts to destroy Leydig cells (Matikainen *et al*. 1994). One caveat to these studies is that the *hpg* mice used here will have developed in a gonadotrophin-free environment and may not, therefore, show the same response to FSH as the normal adult animal. The consistency between results using the *hpg* models and other data described above using different animal models would suggest, however, that these results are relevant to normal spermatogenesis. Overall, therefore, the results from this and earlier studies show that, in rodents FSH acts to stimulate spermatogenesis through an increase in spermatogonial number and subsequent entry of these cells into meiosis. Completion of meiosis appears to be absolutely dependent on the action of androgen.

Generally, insofar as it has been studied, the effects of FSH appear to be similar across different mammalian species. In rhesus and cynomolgus monkeys, FSH appears to act primarily to increase the number of spermatogonia (Marshall *et al*. 1986, 1995, Simorangkir *et al*. 2009), while in sheep immunisation against FSH reduces spermatogonial numbers (Kilgour *et al*.1998). The role of FSH in human spermatogenesis remains somewhat unclear since there is a conflict between the effects of FSHβ deletion and FSHR deletion (Tapanainen *et al*. 1997, Lindstedt *et al*. 1998, Phillip *et al*. 1998, Layman *et al*. 2002) and because treatment of infertile hypogonadotrophic men is based on treatment with hCG making it difficult to establish effects of FSH. Nevertheless, the prevailing evidence suggests that data from rodents are relevant generally and that the primary effect of FSH is to maintain spermatogenesis quantitatively through effects on spermatogonial numbers.

Numerous studies have shown that FSH will stimulate Leydig cell function through an indirect mechanism, which is assumed to involve release of paracrine factors from the Sertoli cells following direct stimulation of the FSH receptor (Chen *et al*. 1976, Vihko *et al*. 1991). In this study, intratesticular testosterone levels were only increased by FSH in the *hpg* group and not the *hpg*.SCARKO or *hpg*.ARKO groups. This contrasts with the increase in seminal vesicle weights after FSH treatment in both *hpg* and *hpg*.SCARKO groups suggesting that there is an increase in testosterone in the *hpg*.SCARKO at the start of treatment, but that this is not maintained up to 7 days. Baines *et al*. (2008) have shown previously that FSH will increase Leydig cell number in the adult *hpg*. Our results confirm this observation and show that the effects of FSH on Leydig cell number in the *hpg* mouse are mediated through androgen action not involving the Sertoli cells. Since Leydig cells express ARs (Zhou *et al*. 2002), the simplest explanation is that FSH indirectly stimulates androgen production by the Leydig cells, which, in turn, acts directly on the Leydig cells to induce proliferation or, possibly, differentiation from precursor stem cells. This is consistent with earlier data showing that Leydig cell number is reduced in *Tfm* and ARKO mice (O'Shaughnessy *et al*. 2002, De Gendt *et al*. 2005).

In conclusion, the design of this study has allowed us to dissect the direct effects of FSH away from those of androgen and to show that FSH acts only during the initial stages of spermatogenesis to optimise germ cell number. Results also demonstrate that FSH cannot stimulate completion of meiosis, which is entirely dependent on androgen action.

## Materials and Methods

### *Animals and treatments*

All mice were bred and all procedures carried out under UK Home Office Licence and with the approval of a local ethical review committee. SCARKO and ARKO mice have been previously generated by crossing female mice carrying an *Ar* with a floxed exon 2 ($Ar^{fl}$) with male mice expressing *Cre* under the regulation of the Sertoli cell-specific promoter *Amh* or the ubiquitous promoter *Pgk1* (Lecureuil *et al*. 2002, De Gendt *et al*. 2004). In order to produce *hpg*.SCARKO mice, *hpg* mice (C3HE/HeH-101/H) were initially crossed with mice carrying the $Ar^{fl}$ allele (Swiss-Webster/129) and with mice carrying the *Amh-Cre* transgene (C57-BL6/SJL). From these crosses, female mice heterozygous for the GNRH deletion (*hpg*/+) and homozygous for the $Ar^{fl}$ allele were crossed with *hpg*/+*Amh-Cre* males (heterozygous or homozygous for *Cre*) to generate *hpg*.SCARKO mice. The *hpg* deletion and $Ar^{fl}$ allele were detected by PCR analysis of ear clip lysates (Lang 1991) and excision of the floxed *Ar* confirmed at termination by PCR of testicular DNA (De Gendt *et al*. 2004). The generation of

*hpg*.ARKO mice was similar except that *Pgk-Cre* (C57-BL6/SJL) replaced *Amh-Cre*. The *hpg*.ARKO males were detected by PCR of ear clip lysates for *Sry* and deletion of GNRH and confirmed at termination by the absence of epididymides, seminal vesicles and ductus deferens. The *hpg* mice used in this study were generated from the same litters producing *hpg*.SCARKO and *hpg*.ARKO mice.

To determine the effects of FSH treatment, adult (10 weeks of age) male *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice were injected s.c. with 8 IU recombinant human FSH (Serono Ltd) in 0.2 ml PBS (pH 7.4, Sigma–Aldrich) once daily for 7 days. The manufacturer's datasheet states that the hormone preparation contains no LH activity. The dose used was based on preliminary dose–response studies showing that 8 IU/day caused a maximum increase in testis weight over a 1-week period. Mice were killed on day 8 (24 h after the last injection), and testes were snap frozen in liquid nitrogen or fixed overnight. Fixation was either in Bouin's for subsequent morphometric analysis or 4% paraformaldehyde/1% gluteraldehyde in phosphate buffer (0.1 M, pH 7.4) for preparation of semi-thin sections.

Testicular morphology in the *hpg* mouse has been described previously in a number of publications (Cattanach *et al*. 1977, Singh & Handelsman 1996, Myers *et al*. 2005, Lim *et al*. 2008). Spermatogenesis can progress to the pachytene spermatocyte stage in the *hpg* mouse, and numbers of spermatocytes and spermatogonia are similar (Singh & Handelsman 1996, Myers *et al*. 2005, Lim *et al*. 2008). In the *hpg* mice produced for this study, ~80% were of this phenotype but the remaining 20% of animals had <5% of the expected number of spermatocytes present. All the mice used in this study are generated by crossing mouse lines that are on different backgrounds, and it appears likely that the altered phenotype in some animals is caused by background effects. Mice with a clear, marked reduction in spermatocyte numbers were not used in the study reported here.

### *Hormone measurements*

Intratesticular levels of testosterone were measured by RIA following ethanol extraction, as previously described (O'Shaughnessy & Sheffield 1990). The limit of detection of the assay was 40 fmol/ml, which equates to 20 fmol/testis after extraction. The intra- and inter-assay coefficients of variation were 6.8 and 12.1% respectively. Cross reactivity with androstenedione and 5α-androstane-3α,17β-diol was 3.0 and 8.1% respectively.

### *Histology and stereology*

To prepare semi-thin (1 μm) sections, testes were embedded in araldite and sections were stained with toluidine blue. For stereological analysis, testes were embedded in Technovit 7100 resin, cut into sections (20 μm) and stained with Harris's haematoxylin. The total testis volume was estimated using the Cavalieri principle (Mayhew 1992). The optical disector technique (Wreford 1995) was used to count the number of Sertoli cells, germ cells and Leydig cells in each testis. Each cell type was identified by previously described criteria (Russell *et al*. 1990, Baker & O'Shaughnessy 2001). The numerical density of each cell type was estimated using an Olympus BX50 microscope fitted with a motorised stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA, USA). Tubule diameter was measured directly in a total of at least 36 tubules from three sections.

### *Statistical analysis*

Most data sets were analysed using two-factor ANOVA with effects of FSH and AR deletion as the factors. Where the interaction between factors was significant, this indicates that the effect of FSH was altered by deletion of the AR. To determine whether differences between individual groups were significant, *t*-tests were employed using the pooled variance from the ANOVA. Data were log transformed where appropriate to avoid heterogeneity of variance. Data on intratesticular testosterone were analysed by the non-parametric Kruskal–Wallis test followed by the Mann–Whitney test.

## 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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