Spermatogenesis and Sertoli cell activity in mice lacking Sertoli cell receptors for follicle stimulating hormone and androgen

Abel MH[a], Baker PJ[b], Charlton HM[a], Monteiro A[b], Verhoeven G[c], de Gendt K[c], Guillou F[d] and O'Shaughnessy PJ[b1]

[a]Department of Human Anatomy and Genetics
University of Oxford
South Parks Rd
Oxford OX1 3QX, UK

[b]Division of Cell Sciences
University of Glasgow Veterinary School
Bearsden Rd
Glasgow G61 1QH, UK

[c]Laboratory for Experimental Medicine and Endocrinology
Catholic University of Leuven
B-3000 Leuven, Belgium

[d]UMR 6175 Institut National de la Recherche Agronomique,
Centre National de Recherche Scientifique,
Université de Tours,
37380 Nouzilly, France

[1]Correspondence e-mail p.j.oshaughnessy@vet.gla.ac.uk
Telephone ++44 141 330 5793
Fax ++44 141 330 5797

Short title: Role of FSH and androgen in spermatogenesis

Gene names: *Aqp8*, Aquaporin 8; *Cst12*, Cystatin 12; *Dhh*, Desert hedgehog; *Espn*, Espin; *Gata1*, GATA binding protein 1; *Itgb1*, â1 Integrin; *Msi 1*, Musashi homolog 1; *Rhox5*, Reproductive homeobox 5; *Slc7a4*, Solute carrier family 7a4; *Slc38a5*, Solute carrier family 38a5; *Sox9*, SRY-box containing gene 9; *Tjp1*, Tight junction protein 1; *Trf*, Transferin; *Wt1*, Wilms tumor homolog

Summary

Spermatogenesis in the adult male depends upon the action of follicle stimulating hormone (FSH) and androgen. Ablation of either hormone has deleterious effects on Sertoli cell function and the progression of germ cells through spermatogenesis. In this study we have generated mice lacking both FSH receptors (FSHRKO) and androgen receptors on the Sertoli cell (SCARKO) to examine how FSH and androgen interact in the regulation of Sertoli cell function and spermatogenesis. Sertoli cell number in FSHRKO.SCARKO mice was reduced by about 50% but was not significantly different to FSHRKO mice. In contrast, total germ cell number in FSHRKO.SCARKO mice was reduced to 2% of control mice (and 20% of SCARKO mice) due to a failure to progress beyond early meiosis. Measurement of Sertoli cell-specific transcript levels showed that half were independent of hormonal action on the Sertoli cell while others were androgen-dependent or showed redundant control by FSH and androgen. Results show that there is both synergistic and redundant regulation of the Sertoli cell and spermatogenesis by FSH and androgen although the Sertoli cell retains a significant capacity for activity which is independent of direct hormonal regulation.

Key words: Sertoli, androgen, spermatogenesis, testis, FSH

Introduction

In the adult animal Sertoli cells act primarily to promote and maintain germ cell development. This is achieved by generation of a unique microenvironment within the seminiferous tubules through the formation of a Sertoli cell barrier and regulation of solute movement and secretion into the tubules (Dym and Fawcett 1970; Wong and Cheng 2005). In addition, they provide cytoarchitectural support and stimulation to the developing germ cells as they undergo proliferation and differentiation. Previous studies have shown that spermatogenesis is regulated by follicle-stimulating hormone (FSH) and testosterone and that these hormones act through specific receptors on the Sertoli cell (reviewed in (McLachlan et al.„ 2002)). The role that each of these hormones plays in the regulation of Sertoli cell function and spermatogenesis has become clearer through study of mice lacking specific hormones or hormone receptors. In animals lacking FSH (FSHâKO) or the FSH receptor (FSHRKO) there is a reduction in Sertoli cell number and germ cell number but the animals remain fertile (Kumar et al., 1997; Dierich et al., 1998; Abel et al.„ 2000; Johnston et al., 2004). In contrast, absence of the androgen receptor (AR), either ubiquitously through a mutation in the receptor (*Tfm*) or specifically in the Sertoli cells (SCARKO) will cause arrest of spermatogenesis in early meiosis (Lyon and Hawkes 1970; De Gendt et al., 2004; Holdcraft and Braun 2004;

Chang et al., 2004). Thus, FSH appears to act to induce proliferation of the Sertoli cells and germ cells and to "optimise" the progress of spermatogenesis while testosterone action on the Sertoli cell is critical for progression through meiosis.

While it is clear that both FSH and androgen are essential for normal spermatogenesis, what remains unknown is the nature and importance of interaction between the hormones. For example, the degree of overlap, or redundancy, is uncertain and also, therefore, the extent to which ablation of one hormone or its receptor may be compensated by the presence of the other hormone. Similarly, the degree to which Sertoli cell function and germ cell development is independent of direct hormonal stimulation remains unclear. To answer these questions directly we have generated mice lacking both FSHR and AR on the Sertoli cell (FSHRKO.SCARKO) and have determined the subsequent effects on Sertoli cell function and spermatogenesis.

Results

Phenotype

Adult FSHRKO.SCARKO mice were normally masculinised although testes from the double knockouts were significantly smaller than FSHRKO, SCARKO or control mice (Table 1). Seminal vesicle weights were also significantly smaller in FSHRKO.SCARKO mice (Table 1). Seminiferous tubule diameter decreased across the groups in the order control>FSHRKO>SCARKO>FSHRKO.SCARKO with an associated increase in the relative abundance of interstitial tissue (Fig 1). As expected from previous studies (Abel et al., 2000; Dierich et al., 1998; De Gendt et al., 2004), all stages of spermatogenesis were present in FSHRKO mice while, in SCARKO mice, the germ cells entered meiosis but there was apparent loss of pachytene spermatocytes with a marked reduction in the number of secondary spematocytes and with few round spermatids present (Fig 1). In FSHRKO.SCARKO mice germ cells entered meiosis but for most cells development stopped at early pachytene with no secondary spermatocytes or round spermatids apparent (Fig 1).

Morphometric analysis showed that Sertoli cell number in 8-week old FSHRKO.SCARKO mice was similar to FSHRKO mice and significantly less than control or SCARKO mice (Fig 2). Sertoli cells in SCARKO mice contained apparent large lipid droplets (not shown) that were clearly seen in other groups. The total germ cell number was reduced in FSHRKO and SCARKO mice compared to control and there was a further marked reduction in FSHRKO.SCARKO mice (Fig 2). The germ cell/Sertoli cell ratio was reduced, though not markedly, in FSHRKO mice compared to control. In contrast, in SCARKO mice the germ cell/Sertoli cell ratio was reduced to 10% of control and in the FSHRKO.SCARKO it was further reduced to 2% of control (Fig 2). Analysis of germ cell types in each group showed that spermatogonial numbers were reduced by about 60% in the

FSHRKO and FSHRKO.SCARKO mice (Fig 3). Spermatocyte number was also reduced by about 50% in FSHRKO and SCARKO mice but in the combined FSHRKO.SCARKO mouse there was very limited successful progression into meiosis with spermatocyte numbers reduced to about 4% of control (Fig 3). The number of round spermatids was reduced to about 40% in the FSHRKO mouse with very few (<0.5% of control) post-meiotic cells present in the SCARKO mouse and none in the FSHRKO.SCARKO mouse.

Hormone profile

Serum levels of LH were significantly elevated in FSHRKO mice but were not significantly affected in SCARKO mice. In FSHRKO.SCARKO mice LH levels were significantly greater than all other groups (Table 2). There were no significant differences in serum FSH or testosterone levels between groups.

Sertoli cell mRNA levels

To examine Sertoli cell function in the 4 groups the levels of 14 Sertoli cell specific mRNA transcripts were measured by real-time PCR relative to an external control and data normalised for Sertoli cell number (Johnston et al., 2004) (Fig 4). Six of the transcripts tested (*Sox9*, *Itgb1*,*Wt1*, *Dhh*, *Trf*, *Cst12*, and *Dhh*) showed no difference in abundance per Sertoli cell between the 4 groups (Fig 4A). Of the remaining 8 transcripts, 4 showed a significant alteration in levels related only to the presence of the AR (*Rhox5*, *Aqp8*, *Tjp1* and *Gata1*), one transcript responded to the loss of either FSHR or AR (*Slc38a5*) and 3 transcripts (*Espn*, *Msi1* and *Slc7a4*) showed redundant regulation by both receptors (Fig 4B).

Table 1

Organ weights in 8 week-old mice

Group	Testis (mg)*	SV (mg)
Control (n=53)	87.3 ± 1.9^{a}	196 ± 5^{a}
FSHRKO (n=36)	36.9 ± 1.3^{b}	183 ± 6^{ab}
SCARKO (n=65)	20.6 ± 0.4^{c}	190 ± 5^{a}
FSHRKO.SCARKO (n=38)	8.1 ± 0.2^{d}	172 ± 5^{b}

* Groups with different letter superscripts are significantly different

Figure 1

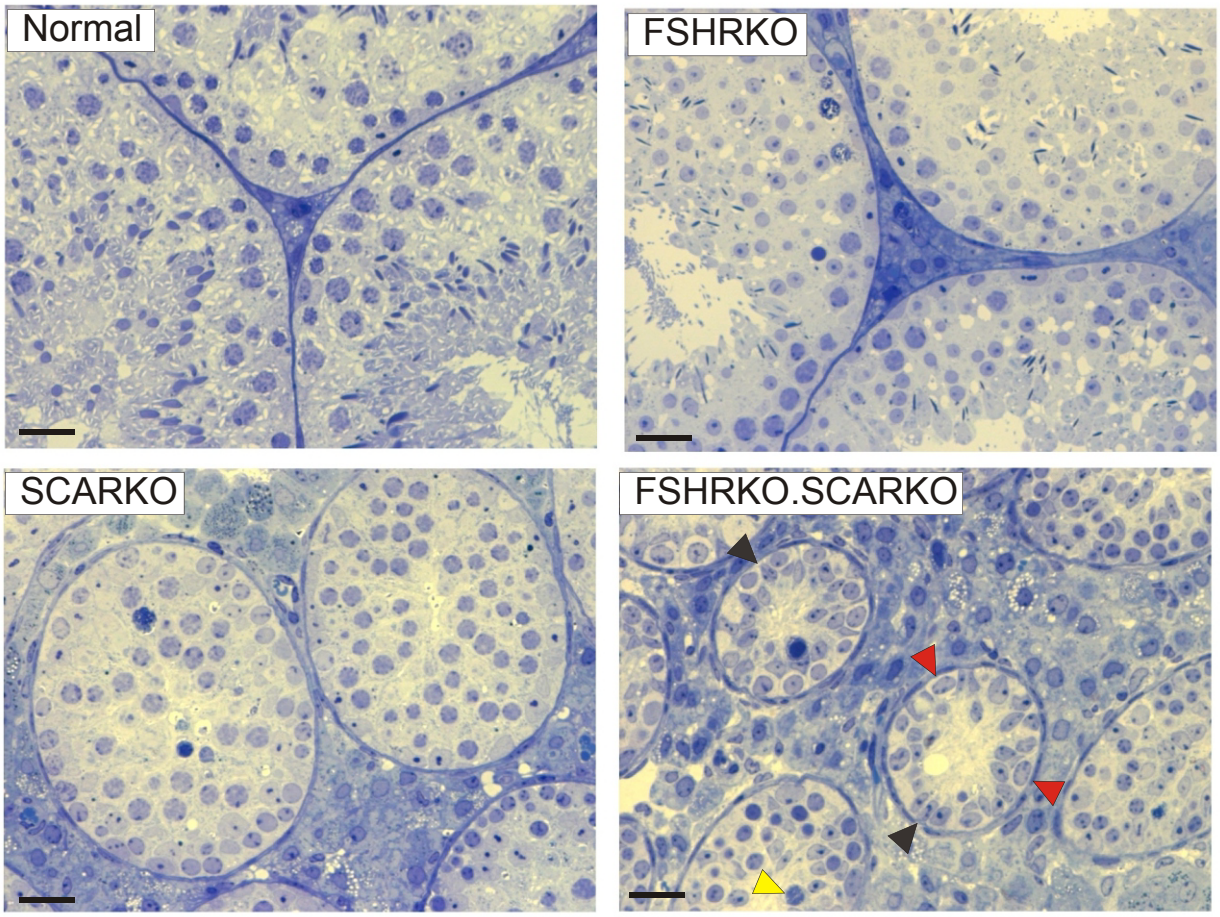


Fig 1 Semithin sections from testes of 8 week old Control, FSHRKO, SCARKO and FSHRKO.SCARKO mice. The FSHRKO mice contained all stages of spermatogenesis although germ cell number was reduced. In SCARKO mice spermatogenesis progressed through meiosis but there was progressive loss of pachytene spermatocytes and few secondary spermatocytes or round spermatids were observed. In FSHRKO.SCARKO mice the tubules were of a smaller diameter with large numbers of Sertoli cells (black arrowheads) and smaller numbers of spermatogonia (red arrowheads). Spermatogonia entered meiosis but development stopped at early pachytene in most cells (yellow arrowhead). The bar represents 20ìm.

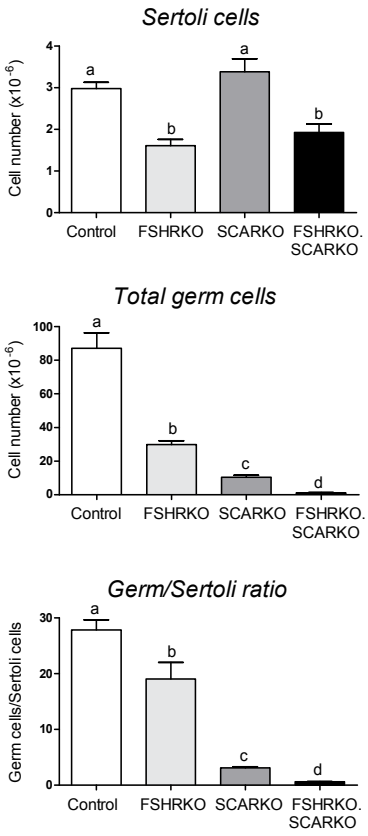
Table 2

Serum hormone levels in 8 week old animals

Group	Testosterone (pmol/ml)	LH* (ng/ml)	FSH (ng/ml)
Control	4.2 ± 1.7	0.064 ± 0.012[a]	35.7 ± 11.1
FSHRKO	5.1 ± 3.5	0.286 ± 0.085[b]	11.9 ± 3.8
SCARKO	8.5 ± 2.4	0.221 ± 0.065[ab]	32.6 ± 12.3
FSHRKO.SCARKO	3.0 ± 1.4	0.586 ± 0.086[c]	17.1 ± 5.46

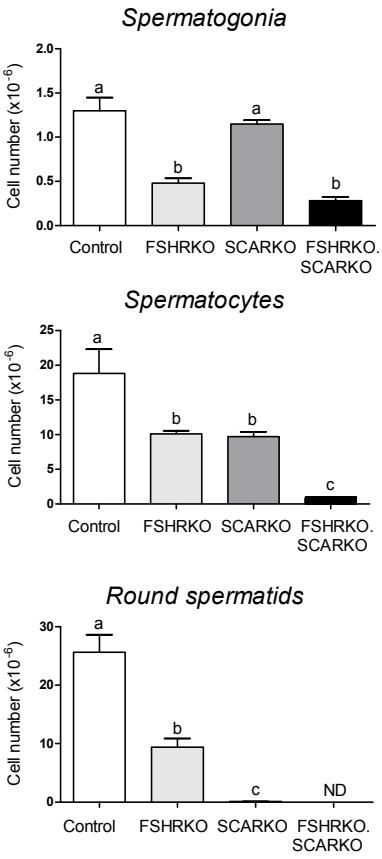
* Groups with different letter superscripts are significantly different

Figure 2



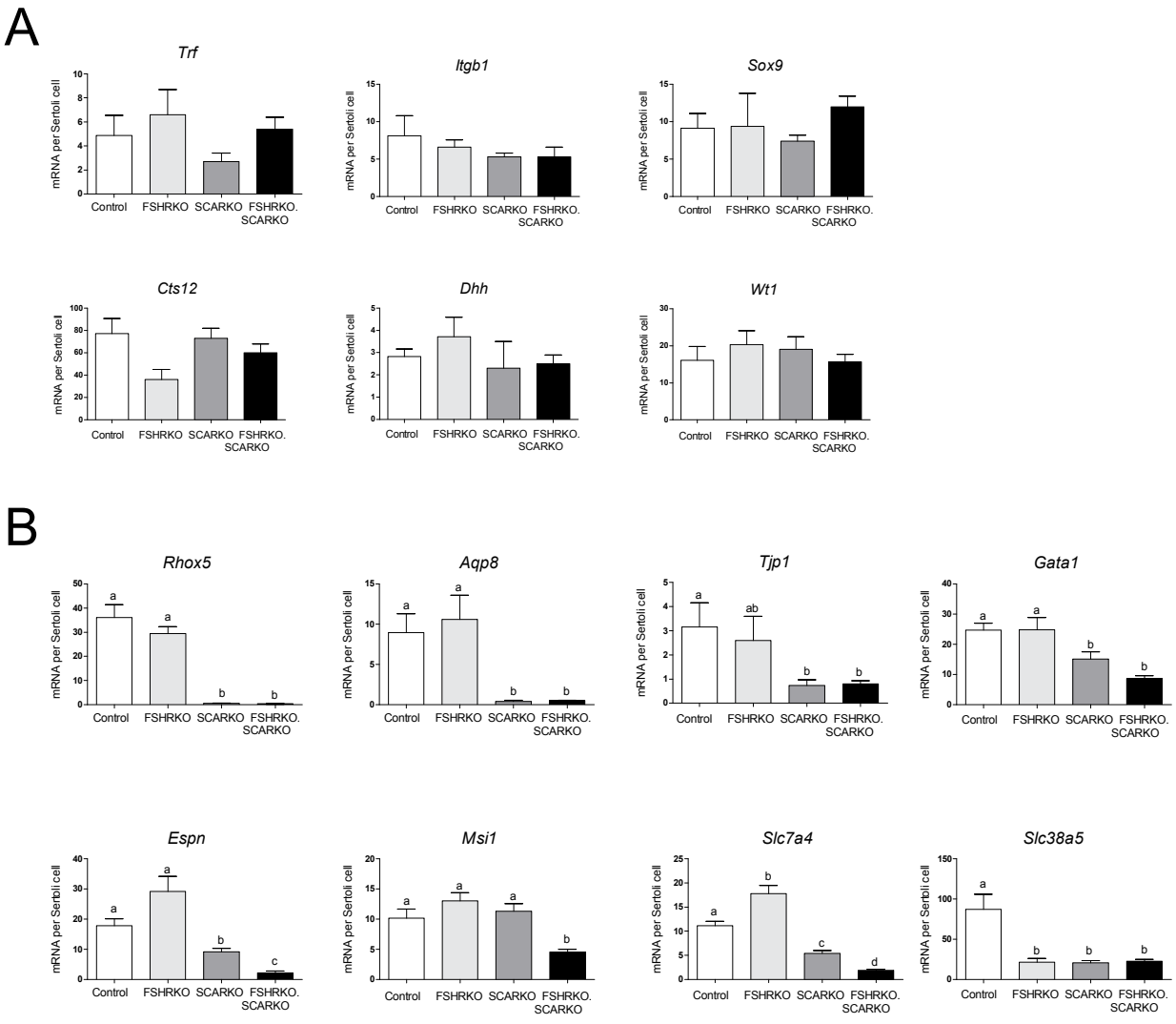
Morphometric analysis Sertoli and germ cell numbers in 8 week old testes from control, FSHRKO, SCARKO and FSHRKO.SCARKO mice. Cell number were measured using the optical disector method. Results show the mean ± sem of 4 animals per group. Groups with different letter superscripts are significantly different.

Figure 3



Morphometric analysis of germ cell types in 8 week old testes from control, FSHRKO, SCARKO and FSHRKO.SCARKO mice. Cell number were measured using the optical disector method. Results show the mean ± sem of 4 animals per group. Groups with different letter superscripts are significantly different.

Figure 4



Levels of Sertoli cell-specific mRNA transcripts in testes from 8 week old control, FSHRKO, SCARKO and FSHRKO.SCARKO mice. Transcripts levels were measured relative to an external standard by real-time PCR and corrected for Sertoli cell number as described in Methods. Results show the mean ± sem of 5 or 6 animals per group. Transcripts in A) showed no significant difference in expression per Sertoli cell between groups while transcripts in B) showed significant variation. In B) the groups with different letter superscripts are significantly different.

Discussion

Generation of the FSHRKO.SCARKO mouse provides us with a base from which to examine how FSH and androgen act and interact through the Sertoli cell to regulate testicular function. In addition, it allows us to determine what aspects of Sertoli cell function are independent of direct hormonal input. The phenotype shows that there is both synergistic and redundant regulation of the Sertoli cell and spermatogenesis by FSH and androgen but that regulation of a significant number of Sertoli cell transcripts (and, by implication, Sertoli cell function) is independent of direct hormonal control.

Interactions between the effects of FSH and androgens on testicular function have been studied previously using models such as the hypophysectomised rat or the hypogonadal (*hpg*) mouse which lacks gonadotrophin-releasing hormone (GnRH) with consequent loss of circulating gonadotrophins (Cattanach et al., 1977). These models have proved to be extremely valuable in our understanding of hormonal action in the testis but all suffer limitations. In the *hpg*, for example, low levels of circulating gonadotrophins and intratesticular testosterone remain, albeit at very low levels, while the testes are cryptorchid because of reduced androgen levels after birth. The germ cell/Sertoli cell ratio in the *hpg* mouse is reported to be about 1 (Myers et al., 2005) which is greater than that in the FSHRKO.SCARKO mouse and may be indicative of the low level hormonal stimulation. The advantage of the FSHRKO.SCARKO mouse, in contrast to other models, is the specificity and totality of the knockout effects. All current data suggests that the FSHR is confined to the Sertoli cells in the male (Heckert and Griswold 2002; Carpenter et al., 1998) while SCARKO mice have been generated to lack ARs only in the Sertoli cells (De Gendt et al., 2004). Thus, the targeted ablations affect only the Sertoli cells in this double knockout and while there may be knock-on effects on gonadotrophin levels or androgen production these are not complicating factors since the Sertoli cells are un-responsive.

Effectively, the FSHRKO.SCARKO mouse provides a baseline control from which the direct effects of FSH and androgen on Sertoli cell function can be assessed. Thus, in the SCARKO mouse the Sertoli cells are stimulated by FSH and not androgen, in the FSHRKO mouse they are stimulated by androgen and not FSH while in the normal mouse they are exposed to both hormones. From this perspective it can be seen that the action of FSH is to increase Sertoli cell number, total germ cell number and the number of germ cells associated with each Sertoli cell. This effect is achieved by increasing the number of spermatogonia and by enhancing the entry of these cells into meiosis. In contrast, androgens have no direct effect on Sertoli cell number but cause a marked increase in total germ cell number and, thus, the number of germ cells per Sertoli cell. The principal effect appears to be through increased entry into meiosis and, crucially, by enabling completion of meiosis. Together, the hormones have an additive effect on entry into meiosis but act synergisti-

cally to stimulate completion of meiosis and entry into spermiogenesis. In addition, it is clear from the double knockout that germ cells can initiate meiosis without direct hormonal stimulation through the Sertoli cell, supporting the conclusions of early *in vitro* studies (Hofmann and Dym 2005). The general description of hormone action on spermatogenesis is also consistent with earlier hormone replacement studies which have shown, for example, that FSH and androgen will increase or restore spermatocyte number (Haywood et al., 2003; Russell et al., 1993; McLachlan et al., 1995) while androgens will increase the number of post-meiotic germ cells in the *hpg* mouse (Singh et al., 1995; Haywood et al., 2003).

Development of a normal sized cohort of Sertoli cells is of importance for male fertility since each Sertoli cell can only support a finite number of germ cells. Previous studies have shown that both FSH and androgen can act to regulate the final Sertoli cell number in the adult animal (Sharpe et al., 2003; Johnston et al., 2004) with studies on the *Tfm* and ARKO mouse suggesting that the role of androgen is more critical (Johnston et al., 2004; Tan et al., 2005). Initial studies on the SCARKO mouse showed, however, that regulation of Sertoli cell number is more complex since androgen-dependent regulation does not appear to be through direct effects of androgen on the Sertoli cell (Johnston et al., 2004; De Gendt et al., 2004; Tan et al., 2005). In FSHRKO.SCARKO mice the number of Sertoli cells was identical to the FSHRKO mouse showing that only FSH is of importance in terms of direct effects on Sertoli cell proliferation with no synergistic effect of androgen apparent.

The significant increase in LH levels in FSHRKO mice has been reported previously (Baker et al., 2003) and attributed to reduced testosterone production by the testis. In FSHRKO.SCARKO mice the increase in LH was more marked and may be indicative of further dysfunction in the Leydig cells. Both SCARKO and FSHRKO mice have reduced Leydig cell number (Baker et al., 2003; De Gendt et al., 2005) and it is possible that the effects are additive in the FSHRKO.SCARKO mouse although this will require further study. Circulating testosterone levels were unchanged in both FSHRKO and FSHRKO.SCARKO but there was high variability between animals and a more reliable indicator of androgen levels may be the reduction in weight of the seminal vesicles in FSHRKO.SCARKO mice.

Despite our enhanced understanding of the role played by FSH and androgens in the regulation of spermatogenesis the cellular mechanisms involved are still in some doubt. It is clear that FSH can regulate levels of a large number of different mRNA transcripts (Sadate-Ngatchou et al., 2004a) and the overall effect of FSH may be to increase general Sertoli cell activity and, thereby, enhance germ cell progression and survival. In contrast, only a relatively small number of Sertoli cell genes have been shown unequivocally by array studies to be androgen-dependent in a number of different mouse models (Denolet et al., 2006; O'Shaughnessy et al., 2007; Eacker et al., 2007;

Sadate-Ngatchou et al., 2004b; Zhou et al., 2005). Somewhat confusingly, there is little overlap of identified androgen-dependent genes between these studies suggesting that androgen targets vary depending on the age, endocrine environment or previous hormonal exposure of the animals. Nevertheless, there is some accumulating evidence to suggest that androgens are required for functional generation of the Sertoli cell barrier and for development of the specialised tubular environment required for germ cell development (Meng et al., 2005; O'Shaughnessy et al., 2007). Thus, in contrast to FSH, androgen action may be mediated through a relatively small number of changes in Sertoli cell gene expression. To examine the effect of ablating both FSHR and AR on Sertoli cell activity we measured the abundance of 14 mRNA species known to be expressed specifically within the Sertoli cell population in the adult testis. The 14 transcripts divided into 3 groups, those showing no response to ablation of either or both receptors, those sensitive to loss of the AR and those sensitive to loss of both receptors. The relatively large number of transcripts (~40%) which were unaffected by loss of hormone responsiveness indicates that a significant proportion of Sertoli cell activity may be independent of hormone action. In addition, 20% of transcripts (*Espn*, *Msi1* and *Slc7a4*) showed a degree of redundancy between the effects of FSH and androgen. Overall, therefore, extrapolating from this data it appears likely that a significant number of Sertoli cell genes are either hormone independent or are regulated to an extent by both hormones which may act to ensure an adequate baseline of cell activity irrespective of fluctuations in hormone levels. When considering changes in Sertoli cell activity following alterations in hormone or hormone receptor levels a possible confounding factor is the effect of germ cell loss on Sertoli cell mRNA transcript levels (Jonsson et al., 1999; Maguire et al., 1993). All of the transcripts reported in this study, however, have been shown to be unaffected by germ cell ablation (O'Shaughnessy et al, submitted).

The transcripts shown in Fig 5 were selected for study largely because they are expressed specifically in the Sertoli cells and illustrate different aspects of hormonal regulation of the cell. Nevertheless, the altered expression of some of these transcripts, particularly in the SCARKO and FSHRKO.SCARKO mice, offers some further clues to the specific mechanism of hormonal action on the Sertoli cell. ESPN, for example, is an actin-bundling protein (Bartles et al., 1996) and an integral part of the ectoplasmic specialisations which are specific to the Sertoli cell and contribute to the Sertoli cell (blood testis) barrier (Sluka et al., 2006). The marked reduction of *Espn* levels in the FSHRKO.SCARKO mouse would be likely to contribute to the overall disruption of the Sertoli cell barrier seen in the absence of the AR alone (Meng et al., 2005). The marked loss of *Aqp8* transcripts in the SCARKO was surprising since previous array studies have not identified *Aqp8* as androgen dependent (Zhou et al., 2005; Denolet et al., 2006; O'Shaughnessy et al., 2007). This discrepancy may have come about, however, because earlier array studies used immature testes

(10d and 20d) and expression of Aqp8 only begins around day 16 in the rat testis (Kageyama et al., 2001). Loss of *Aqp8* in the SCARKO mouse and the pattern of expression of the solute carriers (*Slc7a4* and *Slc38a5*) in the different groups is further evidence that both FSH and androgens act to regulate the internal environment of the seminiferous tubules and, thereby, facilitate germ cell development (O'Shaughnessy et al., 2007).

This study shows that spermatogenesis is largely dependent upon the action of FSH and androgen on the Sertoli cell with only the initial onset of meiosis apparently independent of direct hormonal regulation. The two hormones can both act to maintain the meiotic germ cell population but there is an absolute need for androgen to complete meiosis. Spermiogenesis does not appear to require FSH since the ratio of round spermatids to mature sperm in the FSHRKO is similar to control (not shown) though the models are not informative on the role of androgen in this process. Since other cell types in the testis express the AR it is also possible that androgens may have additional indirect effects on spermatogenesis and this will require further study of FSHRKO.ARKO mice.

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. Mice with a specific Sertoli cell knockout of the AR have been previously generated by crossing male mice expressing AMH.Cre (Lecureuil et al., 2002) with female mice carrying an AR with a floxed exon 2 (AR^{fl}) (De Gendt et al., 2004). In order to produce male mice lacking both FSHR and AR within the Sertoli cell, mice carrying the AMH.Cre transgene (C57-BL6/SJL) and mice carrying the AR^{fl} allele (Swiss-Webster/129) were crossed with FSHRKO mice (C57-BL6/129) and interbred (Abel et al., 2000). The groups used for comparison with the double knockout FSHRKO.SCARKO mice were a) hemizygous FSHRKO/+ males (FSHRH) expressing AMH.Cre *or* AR^{fl} which were considered as control animals (the two groups were initially analysed separately but no significant difference between the AMH.Cre and AR^{fl} groups was seen and the data was combined) b) FSHRKO.Cre and FSHRKO.AR^{fl} which were considered as FSHRKO animals and c) FSHRH.SCARKO animals which were considered as SCARKO animals for this study. PCR genotyping was carried out as previously described (Hirst et al., 2004; De Gendt et al., 2004).

Mice were killed at eight weeks and testes 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 (0.1M, pH 7.2) for preparation of semi-thin sections.

Hormone measurements

Blood was collected by cardiac puncture under anaesthesia and the serum separated and stored at -20^{o}C until assayed. Serum and pituitary levels of FSH and LH were measured commercially using imunofluorimetric assays (Delfia, Wallac OY, Turku, Finland) as previously described (Haavisto et al., 1993; van Casteren et al., 2000). Serum levels of testosterone were measured by radioimmunoassay following ether extraction (O'Shaughnessy and Sheffield 1990).

Measurement of specific mRNA transcript levels

To quantify the content of specific mRNA species in testes from each group, a real-time PCR approach was used after reverse transcription (RT) of the isolated RNA. To allow specific mRNA levels to be expressed per Sertoli cell and to control for the efficiency of RNA extraction, RNA degradation, and the RT step, an external standard was used (Baker and O'Shaughnessy 2001a; O'Shaughnessy et al., 2002; Johnston et al., 2004). The external standard was luciferase mRNA (Promega UK, Southampton, UK), and 5 ng was added to each testis at the start of the RNA extraction procedure. Testis RNA was extracted using TRIzol (Life Technologies, Paisley, UK) and the RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript II, Life Technologies, Paisley, UK) as described previously (O'Shaughnessy and Murphy 1993). For real-time PCR the SYBR green method was used in a 96-well plate format using a Stratagene MX3000 cycler. Reactions contained 5 ìl 2 x SYBR mastermix (Stratagene, Amsterdam, Netherlands), primer (100 nM) and template in a total volume of 10 µl. 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 (Czechowski et al., 2004). The primers used have been described previously ((O'Shaughnessy et al., 2007) and O'Shaughnessy *et al* submitted). To correct for Sertoli cell number data from the real-time PCR studies was divided by the Sertoli cell number of each group as measured below.

Histology and stereology

To prepare semi-thin (1ìm) sections testes were embedded in araldite and sections stained with toluidine blue. For stereological analysis, testes were embedded in Technovit 7100 resin, cut into sections (20ìm), and stained with Harris' hematoxylin. 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 and germ cells in each testis. Each cell type was identified by previously described criteria (Russell L.D. et al., 1990; Baker and O'Shaughnessy 2001b). The

numerical density of each cell type was estimated using an Olympus BX50 microscope fitted with a motorized stage (Prior Scientific Instruments, Cambridge, UK) and Stereologer software (Systems Planning Analysis, Alexandria, VA, USA).

Statistical analysis

Data was analysed using analysis of variance followed by Fisher's pairwise comparisons. Data was log transformed where appropriate to avoid heterogeneity of variance.

Acknowledgements

This study was supported by the Wellcome Trust. We thank Prof DG de Rooij for assistance with identification of germ cell morphology.

References

Abel, M.H., Wootton, A.N., Wilkins, V., Huhtaniemi, I., Knight, P.G. and Charlton, H.M. (2000). The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* **141**, 1795-1803.

Baker, P.J. and O'Shaughnessy, P.J. (2001a). Expression of prostaglandin D synthetase during development in the mouse testis. *Reproduction* **122**, 553-559.

Baker, P.J. and O'Shaughnessy, P.J. (2001b). Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice. *Reproduction* **122**, 227-234.

Baker, P.J., Pakarinen, P., Huhtaniemi, I.T., Abel, M.H., Charlton, H.M., Kumar, T.R. and O'Shaughnessy, P.J. (2003). Failure of Normal Leydig Cell Development in Follicle-Stimulating Hormone (FSH) Receptor-Deficient Mice, But Not FSHbeta-Deficient Mice: Role for Constitutive FSH Receptor Activity. *Endocrinology* **144**, 138-145.

Bartles, J.R., Wierda, A. and Zheng, L. (1996). Identification and characterization of espin, an actin-binding protein localized to the F-actin-rich junctional plaques of Sertoli cell ectoplasmic specializations. *J. Cell Sci.* **109**, 1229-1239.

Carpenter, A.M., Lukyanenko, Y.O., Lee, V.H. and Hutson, J.C. (1998). FSH does not directly influence testicular macrophages. *J. Androl* **19**, 420-427.

Cattanach, B.M., Iddon, C.A., Charlton, H.M., Chiappa, S.A. and Fink, G. (1977). Gonadtrophin releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* **269**, 338-340.

Chang, C., Chen, Y.T., Yeh, S.D., Xu, Q., Wang, R.S., Guillou, F., Lardy, H. and Yeh, S. (2004). Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. *Proc. Natl. Acad. Sci. U.S.A* **101**, 6876-6881.

Czechowski, T., Bari, R.P., Stitt, M., Scheible, W.R. and Udvardi, M.K. (2004). Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant J.* **38**, 366-379.

De Gendt, K., Atanassova, N., Tan, K.A., De Franca, L.R., Parreira, G.G., McKinnell, C., Sharpe, R.M., Saunders, P.T., Mason, J., Hartung, S., Ivell, R., Denolet, E. and Verhoeven, G. (2005). Development and function of the adult generation of Leydig cells in mice with Sertoli cell-selective (SCARKO) or total (ARKO) ablation of the androgen receptor. *Endocrinology* **146**, 4117-4126.

De Gendt, K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lecureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M. and Verhoeven, G. (2004). A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proc. Natl. Acad. Sci. U.S.A* **101**, 1327-1332.

Denolet, E., De Gendt, K., Allemeersch, J., Engelen, K., Marchal, K., Van Hummelen, P., Tan, K.A., Sharpe, R.M., Saunders, P.T., Swinnen, J.V. and Verhoeven, G. (2006). The effect of a Sertoli cell-selective knockout of the androgen receptor on testicular gene expression in prepubertal mice. *Mol .Endocrinol.* **20**, 321-334.

Dierich, A., Sairam, M.R., Monaco, L., Fimia, G.M., Gansmuller, A., LeMeur, M. and Sassone-Corsi, P. (1998). Impairing follicle-stimulating hormone (FSH) signaling *in vivo*: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc. Natl. Acad. Sci. USA* **95**, 13612-13617.

Dym, M. and Fawcett, D.W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol.Reprod.* **3**, 308-326.

Eacker, S.M., Shima, J.E., Connolly, C.M., Sharma, M., Holdcraft, R.W., Griswold, M.D. and Braun, R.E. (2007). Transcriptional profiling of androgen receptor (AR) mutants suggests instructive and permissive roles of AR signaling in germ cell development. *Mol. Endocrinol.* **21**, 895-907.

Haavisto, A.M., Pettersson, K., Bergendahl, M., Perheentupa, A., Roser, J.F. and Huhtaniemi, I. (1993). A supersensitive immunofluorometric assay for rat luteinizing hormone. *Endocrinology* **132**, 1687-1691.

Haywood, M., Spaliviero, J., Jimemez, M., King, N.J., Handelsman, D.J. and Allan, C.M. (2003). Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. *Endocrinology* **144**, 509-517.

Heckert, L.L. and Griswold, M.D. (2002). The expression of the follicle-stimulating hormone receptor in spermatogenesis. *Recent Prog. Horm. Res.* **57**, 129-148.

Hirst, R.C., Abel, M.H., Wilkins, V., Simpson, C., Knight, P.G., Zhang, F.P., Huhtaniemi, I., Kumar, T.R. and Charlton, H.M. (2004). Influence of mutations affecting gonadotropin production or responsiveness on expression of inhibin subunit mRNA and protein in the mouse ovary. *Reproduction.* **128**, 43-52.

Hofmann, M-C.C. and Dym, M. (2005). Long-term culture of mammalian spermatogonia. In *Sertoli cell biology* (Eds MK Skinner and MD Griswold), pp. 449-470. San Diego: Elsevier.

Holdcraft, R.W. and Braun, R.E. (2004). Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. *Development* **131**, 459-467.

Johnston, H., Baker, P.J., Abel, M., Charlton, H.M., Jackson, G., Fleming, L., Kumar, T.R. and O'Shaughnessy, P.J. (2004). Regulation of Sertoli cell number and activity by follicle-stimulating hormone and androgen during postnatal development in the mouse. *Endocrinology* **145**, 318-329.

Jonsson, C.K., Zetterstrom, R.H., Holst, M., Parvinen, M. and Soder, O. (1999). Constitutive expression of interleukin-1alpha messenger ribonucleic acid in rat Sertoli cells is dependent upon interaction with germ cells. *Endocrinology* **140**, 3755-3761.

Kageyama, Y., Ishibashi, K., Hayashi, T., Xia, G., Sasaki, S. and Kihara, K. (2001). Expression of aquaporins 7 and 8 in the developing rat testis. *Andrologia* **33**, 165-169.

Kumar, T.R., Wang, Y., Lu, N. and Matzuk, M.M. (1997). Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.* **15**, 201-204.

Lecureuil, C., Fontaine, I., Crepieux, P. and Guillou, F. (2002). Sertoli and granulosa cell-specific Cre recombinase activity in transgenic mice. *Genesis.* **33**, 114-118.

Lyon, M.F. and Hawkes, S.G. (1970). X-linked gene for testicular feminization in the mouse. *Nature* **227**, 1217-1219.

Maguire, S.M., Millar, M.R., Sharpe, R.M. and Saunders, P.T. (1993). Stage-dependent expression of mRNA for cyclic protein 2 during spermatogenesis is modulated by elongate spermatids. *Mol. Cell Endocrinol.* **94**, 79-88.

Mayhew, T.M. (1992). A review of recent advances in stereology for quantifying neural structure. *J.Neurocytol.* **21**, 313-328.

McLachlan, R.I., O'Donnell, L., Meachem, S.J., Stanton, P.G., de Kretser, D.M., Pratis, K. and Robertson, D.M. (2002). Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Prog. Horm. Res.* **57**, 149-179.

McLachlan, R.I., Wreford, N.G., de Kretser, D.M. and Robertson, D.M. (1995). The effects of recombinant follicle-stimulating hormone on the restoration of spermatogenesis in the gonadotropin-releasing hormone-immunized adult rat. *Endocrinology* **136**, 4035-4043.

Meng, J., Holdcraft, R.W., Shima, J.E., Griswold, M.D. and Braun, R.E. (2005). Androgens regulate the permeability of the blood-testis barrier. *Proc. Natl. Acad. Sci. U.S.A* **102**, 16696-16700.

Myers, M., Ebling, F.J., Nwagwu, M., Boulton, R., Wadhwa, K., Stewart, J. and Kerr, J.B. (2005). Atypical development of Sertoli cells and impairment of spermatogenesis in the hypogonadal (hpg) mouse. *J. Anat.* **207**, 797-811.

O'Shaughnessy, P.J., Abel, M., Charlton, H.M., Hu, B., Johnston, H. and Baker. P,J, (2007). Altered expression of genes involved in regulation of vitamin a metabolism, solute transportation, and cytoskeletal function in the androgen-insensitive tfm mouse testis. *Endocrinology* **148**, 2914-2924.

O'Shaughnessy, P.J. and Murphy, L. (1993). Cytochrome P-450 17α-hydroxylase protein and mRNA in the testis of the testicular feminized (*Tfm*) mouse. *J.Mol.Endocrinol.* **11**, 77-82.

O'Shaughnessy, P.J. and Sheffield, J.W, (1990). Effect of testosterone on testicular steroidogenesis in the hypogonadal (*hpg*) mouse. *J. Steroid Biochem.* **35**, 729-734.

O'Shaughnessy, P.J,, Willerton, L. and Baker, P.J. (2002). Changes in Leydig cell gene expression during development in the mouse. *Biol. Reprod.* **66**, 966-975.

Russell, L.D., Ettlin, R.A., Sinha Hikim, A.P. and Clegg, E.D. (1990). In *Histological and Histopathological Evaluation of the Testis*, pp. 119-161. Clearwater: Cache River Press.

Russell, L.D., Corbin, T.J., Borg, K.E., De Franca, L.R., Grasso, P. and Bartke, A. (1993). Recombinant human follicle-stimulating hormone is capable of exerting a biological effect in the adult hypophysectomized rat by reducing the numbers of degenerating germ cells. *Endocrinology* **133**, 2062-2070.

Sadate-Ngatchou, P.I., Pouchnik, D.J. and Griswold, M.D. (2004a). Follicle-stimulating hormone induced changes in gene expression of murine testis. *Mol. Endocrinol.* **18**, 2805-2816.

Sadate-Ngatchou, P.I., Pouchnik, D.J. and Griswold, M.D. (2004b). Identification of testosterone-regulated genes in testes of hypogonadal mice using oligonucleotide microarray. *Mol. Endocrinol.* **18**, 422-433.

Sharpe, R.M., McKinnell, C., Kivlin, C. and Fisher, J.S. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction.* **125**, 769-784.

Singh, J., Oneill, C. and Handelsman, D.J. (1995). Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. *Endocrinology* **136**, 5311-5321.

Sluka, P., O'Donnell, L., Bartles, J.R. and Stanton, P.G. (2006). FSH regulates the formation of adherens junctions and ectoplasmic specialisations between rat Sertoli cells in vitro and in vivo. *J. Endocrinol.* **189**, 381-395.

Tan, K.A., De Gendt, K., Atanassova, N., Walker, M., Sharpe, R.M., Saunders, P.T., Denolet, E. and Verhoeven, G. (2005). The role of androgens in Sertoli cell proliferation and functional maturation: studies in mice with total (ARKO) or Sertoli cell-selective (SCARKO) ablation of the androgen receptor. *Endocrinology* **146**, 2674-2683.

van Casteren, J.I., Schoonen, W.G. and Kloosterboer, H.J. (2000). Development of time-resolved immunofluorometric assays for rat follicle-stimulating hormone and luteinizing hormone and application on sera of cycling rats. *Biol. Reprod.* **62**, 886-894.

Wong, C.H. and Cheng, C.Y. (2005). The blood-testis barrier: its biology, regulation, and physiological role in spermatogenesis. *Curr. Top. Dev. Biol.* **71**, 263-296.

Wreford, N.G. (1995). Theory and practice of stereological techniques applied to the estimation of cell number and nuclear volume of the testis. *Microsc. Res. Tech.* **32**, 423-436.

Zhou, Q., Shima, J.E., Nie, R., Friel, P.J. and Griswold, M.D. (2005). Androgen-regulated transcripts in the neonatal mouse testis as determined through microarray analysis. *Biol. Reprod,* **72**, 1010-1019.