Direct action through the Sertoli cells is essential for androgen stimulation of spermatogenesis

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Abstract

Androgens act to stimulate spermatogenesis through androgen receptors (AR) on the Sertoli cells and peritubular myoid cells (PTM). Specific ablation of the AR in either cell type will cause a severe disruption of spermatogenesis. To determine whether androgens can stimulate spermatogenesis through direct action on the PTM alone or whether action on the Sertoli cells is essential we have crossed hypogonadal (*hpg*) mice which lack gonadotrophins and intratesticular androgen 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 testosterone (T) or dihydrotestosterone (DHT) for 7 days and testicular morphology and cell numbers assessed. Androgen treatment did not affect Sertoli cell numbers in any animal group. Both T and DHT increased numbers of spermatogonia and spermatocytes in *hpg* mice but DHT has no effect on germ cell numbers in *hpg*.SCARKO and *hpg*.ARKO mice. T increased germ cell numbers in *hpg*.SCARKO and *hpg*.ARKO mice but this was associated with stimulation of FSH release. Results show that androgen stimulation of spermatogenesis requires direct androgen action on the Sertoli cells.

Introduction

Spermatogenesis is regulated by follicle-stimulating hormone (FSH) and by androgen released from the Leydig cells in response to luteinising hormone (LH). In mice lacking androgen receptors (AR) either through a natural mutation (*Tfm*) or through genomic manipulation (ARKO) there is severe disruption to spermatogenesis with a failure of germ cells to progress beyond the early stages of meiosis (1;2). In contrast, mice lacking FSH or the FSH-receptor are fertile, albeit with reduced germ cell number (3-6), indicating that FSH acts primarily to optimise spermatogenesis and germ cell number while androgens are critical for the completion of meiosis and, therefore, fertility.

In the adult animal testicular ARs are expressed in Sertoli cells, Leydig cells and peritubular myoid cells (PTMs) (2). The primary function of the Sertoli cells is to promote and maintain germ cell development through direct interaction with the cells and through generation of a unique microenvironment within the lumen of the seminiferous tubules. In mice lacking ARs only in the Sertoli cells (SCARKO) spermatogenesis is largely blocked at meiosis (2;7;8) although the disruption to spermatogenesis in the SCARKO is less marked than in the *Tfm* or ARKO mouse. In addition to the SCARKO mouse, it has recently been reported that mice lacking ARs in the peritubular myoid cells (PTM-ARKO) show severe depletion of germ cell numbers and are infertile (9). This shows that androgen action through the PTMs is also essential for spermatogenesis and reveals a more complex interplay between endocrine stimulation, somatic cell activity and spermatogenesis than previously envisioned.

To examine these interactions more closely and to determine whether androgen can acutely stimulate spermatogenesis without a direct effect on the Sertoli cell we have

generated mice lacking gonadotrophins and Sertoli cell androgen receptors by crossing SCARKO mice with hypogonadal (*hpg*) mice. The *hpg* mouse lacks circulating gonadotrophins (10) with a consequent loss of androgen production (11) and disruption to spermatogenesis (10). Treatment of *hpg*.SCARKO mice with androgen will directly stimulate all androgen-sensitive testicular cells apart from the Sertoli cell and show whether direct androgen action on the Sertoli cells is essential for germ cell development.

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 *Pgk-1* (2;12). The *hpg*.SCARKO and *hpg*.ARKO mice were generated as previously described (6;13).

Adult mice (10 weeks) were treated with androgen by subcutaneous implantation of a 2cm silastic capsule containing testosterone (T) or the non-aromatisable androgen dihydrotestosterone (DHT). Margaret? Mice were killed one week after the start of treatment and testes snap frozen in liquid nitrogen or fixed overnight. Fixation was either in 4% paraformaldehyde/1% gluteraldehyde in phosphate buffer (0.1M, pH 7.4) for preparation of semi-thin sections or in Bouin's for subsequent morphometric analysis or immunohistochemistry.

Hormone measurement

Serum levels of FSH were measured by ELISA (IDS, Bolden, UK).

Histology, stereology and immunohistochemistry

To prepare semi-thin (1μm) 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 (20μm), and stained with Harris' hematoxylin. The total testis volume was estimated using the Cavalieri principle (14). The optical disector technique (15) was used to count the number of Sertoli cells and germ cells in each testis. Each cell type was identified by previously described criteria (16;17). 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). Control data for this study includes data from a previous study (6) plus additional animals.

For immunohistochemistry, testes were embedded in paraffin and sections (5 μm) were mounted on glass slides, dewaxed, and rehydrated. Sections were incubated with rabbit anti-AR antiserum (sc-816, Santa Cruz Biotechnology) following heat-induced antigen retrieval (2). Bound primary antibody was detected using a peroxidase-conjugated secondary antibody, followed by a fluorescyl-tyramide amplification step with visualization using 3,3-diaminobenzidine tetrahydrochloride (Dako UK Ltd, Cambridgeshire). For negative control samples, nonimmune serum replaced primary antiserum.

Statistical analysis

Data were analysed using two factor analysis of variance (anova) with hormone treatment and animal group as the two variables. To show 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 FSH levels were analysed by the non-parametric Kruskal-Wallis test followed by the Mann-Whitney test.

Results

Androgen receptor expression

Immunohistochemistry was used to localise ARs in the testes of *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice (Fig 1). In the *hpg* testis ARs were clearly present in the PTMs and interstitial tissue with lighter staining apparent in the Sertoli cells (Fig 1A). In *hpg*.SCARKO mice there was clear staining for ARs in the PTMs and interstitial tissue while no specific staining was seen in the *hpg*.ARKO mice (Fig 1B & C). For comparison, testes from normal mice were included and AR expression was apparent in Sertoli cells, PTMs and interstitial tissue (Fig 1D).

Effect of androgen on testis size, testis morphology and SV weight

Hypogonadal mice are cryptorchid due to lack of post-natal androgen production. Prolonged androgen-treatment of these animal will induce testicular descent but the duration of treatment in the experiments described here was insufficient to affect testicular localisation. Treatment of *hpg* and *hpg*.SCARKO mice with T or DHT did, however, increase testicular volume with the effect on *hpg* mice being more marked (Fig

2). Surprisingly, T also increased the volume of *hpg*.ARKO mice but there was no effect of DHT (Fig 2).

Treatment of *hpg* mice with T or DHT caused an increase in seminiferous tubule diameter (Figs 2 & 3), clear establishment of a tubular lumen and an apparent increase in germ cell number (Fig 3). In some tubules spermatogenesis progressed to the round spermatid stage following treatment with T (Fig 3). Testosterone also increased tubule diameter in *hpg*.SCARKO and *hpg*.ARKO mice but DHT only had an effect in *hpg*.SCARKO mice (Fig 2 & 3).

Seminal vesicle weights were significantly increased by T and DHT in both *hpg* and *hpg*.SCARKO mice (Fig 2). Due to a lack of ARs seminal vesicles do not develop in *hpg*.ARKO mice.

Stereology

Sertoli cell number was unaffected by androgen treatment in any animal group (Fig 4). Total germ cell number was increased by T in all three animal groups (Fig 5). In contrast, DHT increased total germ cell number in *hpg* mice but had no effect on *hpg*.SCARKO or *hpg*.ARKO mice (Fig 5). Similarly, T and DHT both increased spermatogonial and spermatocyte numbers in *hpg* mice but only T was effective in *hpg*.SCARKO and *hpg*.ARKO mice. Formation of round spermatids was limited mainly to *hpg* mice treated with T (Fig 5). A very small number of round spermatids were formed in *hpg* mice treated with DHT and *hpg*.SCARKO mice treated with T (Fig 5).

FSH levels

Serum FSH levels in *hpg* and *hpg*.ARKO mice were all below 1.5 ng/ml and in *hpg*.SCARKO mice all levels were below the level of detection of the assay (0.2ng/ml) (Fig 6). For comparison, serum FSH levels in normal adult male mice are about 25ng/ml (18). Treatment of *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice with T caused a significant increase in serum FSH but DHT had no clear effect (Fig 6).

Discussion

Studies with mice lacking functional ARs (*Tfm* or ARKO) have shown clearly that androgen is essential for the onset of spermatogenesis (1;2). In addition, there is strong evidence from other models to indicate that androgens are critical for the maintenance of spermatogenesis. For example, testosterone withdrawal appears to induce apoptosis of pachytene spermatocytes and round spermatids (19) and there is growing evidence that androgens are required for post-meiotic germ cell adhesion to the Sertoli cell (20-22). Since Sertoli cells express AR (23), germ cells do not express AR (23;24) and Sertoli cells are intimately involved in germ cell development it has appeared likely for some time that the major effects of androgen are mediated through the Sertoli cells. This has been re-enforced by the testicular phenotype of the SCARKO mouse which lacks germ cell development beyond the first meiotic division (2). It is of considerable interest, therefore, that a recent study has reported major disruption to spermatogenesis in mice lacking AR in the PTMs (9). Total loss of germ cells in these animals was greater than in SCARKO mice and appeared to be largely through disruption of the premeiotic cells (9). The results are all the more surprising since Cre expression (and, therefore, AR ablation) occurred in less than 50% of PTMs. It is now clear, therefore, that androgens act to

promote spermatogenesis through actions on both Sertoli cells and PTMs. To study this dual control in more detail we have used animal models based on the *hpg* mouse which lacks germ cell development and which is highly sensitive to androgen action (25;26). By combining *hpg* mice with SCARKO mice we have been able to determine whether androgens can stimulate spermatogenesis without direct effects through AR in the Sertoli cell. Results show that (non-aromatisable) androgen action through the PTMs (and Leydig cells) alone is insufficient to stimulate spermatogenesis in these animals and that direct androgen action on the Sertoli cells is essential.

The requirement for direct androgen action on the Sertoli cell to stimulate spermatogenesis may appear to be obvious from the phenotype of the SCARKO mouse (2). The major defect in this animal, however, is loss of post-meiotic cells and this does not rule out additional direct spermatogenic effects of androgen through other cell types. In particular, pre-meiotic germ cell numbers are not markedly affected in the SCARKO mouse (spermatogonial numbers are normal and spermatocyte numbers are about 50-60% of normal (2;27)) indicating that androgen action through other cells combined with FSH action on the Sertoli cells is sufficient to maintain reasonable pre-meiotic germ cell numbers. The phenotype of the FSHRKO and PTM-ARKO mice and the effects of FSH on the *hpg* would also be consistent with this hypothesis (3;6;9). In data reported here, however, the failure of DHT to stimulate tubule and germ cell development in the *hpg*.SCARKO mouse, in comparison to the *hpg* mouse, shows that androgen stimulation of spermatogonial and spermatocyte development requires direct action on the Sertoli cells. These experiments do not rule out the possibility that androgen action through the PTMs is also essential to stimulate spermatogenesis and studies using *hpg*.PTM-ARKO

mice would clarify this. In addition, longer-term direct effects of androgen through the PTMs, without direct effects on the Sertoli cells, cannot be excluded. The 7-day time-course was chosen for studies reported here because longer periods of androgen treatment will induce testicular descent in *hpg* and *hpg*.SCARKO mice which, in itself, will affect germ cell development.

A previous study has reported that T and DHT have similar effects on the development of spermatogenesis in the *hpg* mouse over an 8-week time period (25). Our results largely confirm this report although DHT was less effective than T for the induction of round spermatid formation which may be due to the relatively short duration of the current study with attendant lack of testicular descent. Unexpectedly, however, we observed marked differences in the effects of T and DHT on spermatogenesis in *hpg*.SCARKO and *hpg*.ARKO mice. These differences appear to be due to the induction of FSH release from the pituitary by T but not DHT. In normal adult males androgens act to decrease circulating FSH primarily through effects on hypothalamic GnRH. In the *hpg* mouse, however, the primary effect of exogenous steroids is likely to be at the pituitary and there is evidence from *in vivo* and *in vitro* studies that T can stimulate pituitary FSH and *Fshb* levels (28-31). More pertinently, so far as this study is concerned, it has also been shown that oestrogen will increase circulating FSH levels in male *hpg* mice (32;33). The differential induction of FSH levels by T, but not by DHT, makes it highly likely that in our *hpg* models aromatisation of T to oestrogen leads to stimulation of pituitary FSH release. Differences between the effects of T and DHT on spermatogenesis in *hpg*.SCARKO and *hpg*.ARKO mice can be attributed, therefore, to the effects of FSH

which has been shown to stimulate spermatogenesis in both *hpg*.SCARKO and *hpg*.ARKO mice (6).

In summary, non-aromatisable androgen does not stimulate spermatogenesis in *hpg* mice lacking androgen receptors on the Sertoli cells. Androgen stimulation of spermatogenesis therefore requires direct androgen action on the Sertoli cells.

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Figure Legends

Figure 1

Localisation of AR expression.. Immunohistochemistry was used to localize AR expression in adult testes from (A) *hpg*, (B) *hpg*.SCARKO, (C) *hpg*.ARKO and (D)normal mice. In the *hpg* testis ARs were clearly present in the PTMs (black arrows) and interstitial tissue with lighter staining apparent in the Sertoli cells (white arrows, cells identified by their nuclear shape and more prominent nucleolus). In *hpg*.SCARKO mice there was clear staining for ARs in the PTMs (black arrows) and interstitial tissue while no specific staining was seen in the *hpg*.ARKO mice. Apparent staining in the tubules is background staining. In normal mice AR expression was apparent in Sertoli cells, PTMs and interstitial tissue. The bar represents 20μm.

Figure 2

Effect of androgen on A) testis volume, B) tubule diameter and C) seminal vesicle weight in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. Adult mice were treated with testosterone (T) or dihydrotestosterone (DHT) for 7 days. Results show the mean ± SEM for 3-6 animals per group in A) and B) and 9-36 animals per group in C). Within a particular animal type, groups with different letter superscripts were significantly ($P<0.05$) different.

Figure 3

Semi-thin sections showing the effect of androgen on testicular morphology in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. In untreated animals spermatogenesis was severely disrupted with only spermatogonia and some spermatocytes present. Treatment with T increased germ cell numbers in all mice although the effect was most marked in the *hpg*. Treatment with DHT increased germ cell number only in the *hpg*. In the hpg.ARKO group black arrows indicate the presence of microliths (13). The bar represents 20μm.

Figure 4

Effect of androgen on Sertoli cell number in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. Adult mice were treated with testosterone (T) or dihydrotestosterone (DHT) for 7 days and cell numbers counted as described in *Materials and Methods*. Results show the mean ± SEM for 3-6 animals per group. Within a particular animal type, groups with different letter superscripts were significantly ($P<0.05$) different.

Figure 5

Effect of androgen treatment on the number of A) Total germ cells, B) Spermatogonia, C) Spermatocyte and D) Spermatids in *hpg*, *hpg*.SCARKO and *hpg*.ARKO mice. Adult mice were treated with testosterone (T) or dihydrotestosterone (DHT) for 7 days and cell numbers counted as described in *Materials and Methods*. Results show the mean ± SEM for 3-6 animals per group. Within a particular animal type, groups with different letter superscripts were significantly ($P<0.05$) different. No statistical analysis was applied to spermatid numbers. ND = not detected.

Figure 6

Serum levels of FSH in *hpg*, *hpg*.SCARKO and *hpg*.ARKO males treated with testosterone (T) or dihydrotestosterone (DHT) for 7 days. Results show values from individual animals in each group. The effect of testosterone was significant ($P<0.05$) in all three groups.

Figure 1

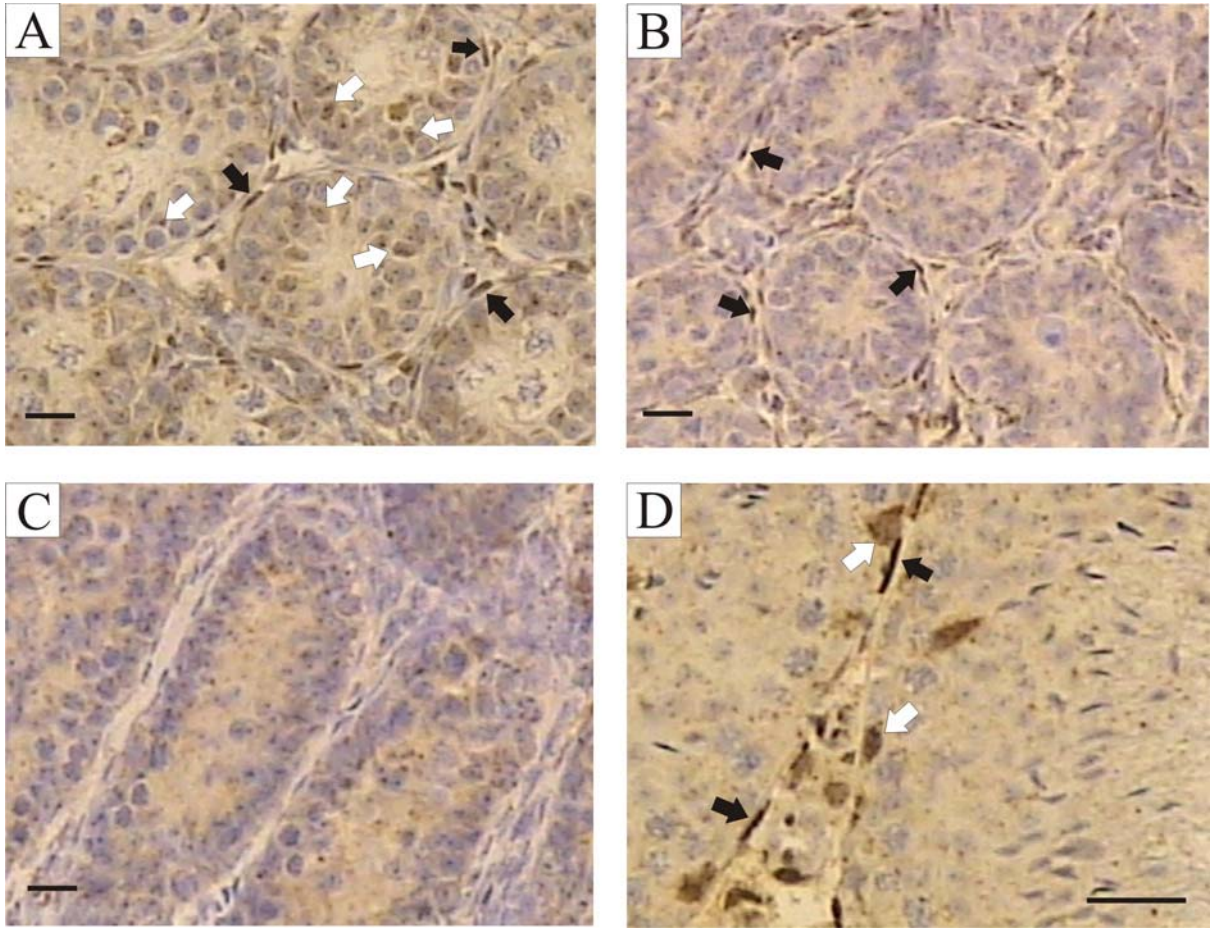


Figure 2

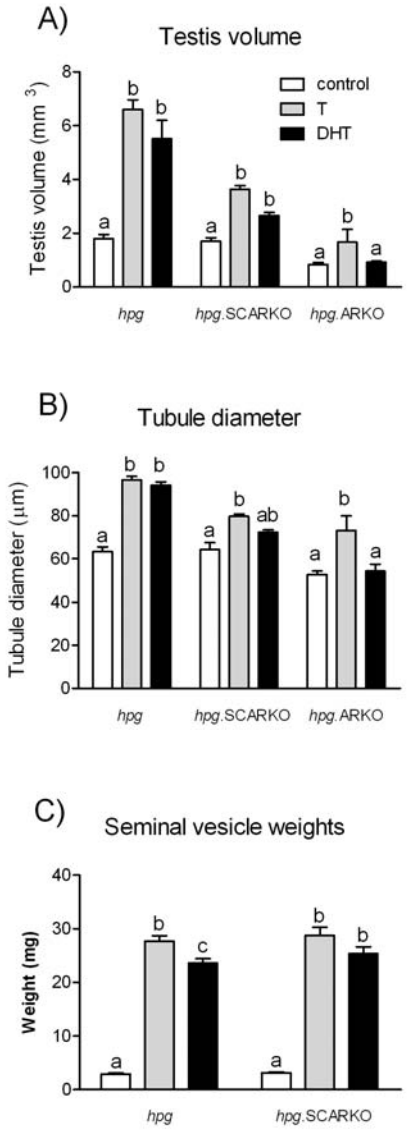
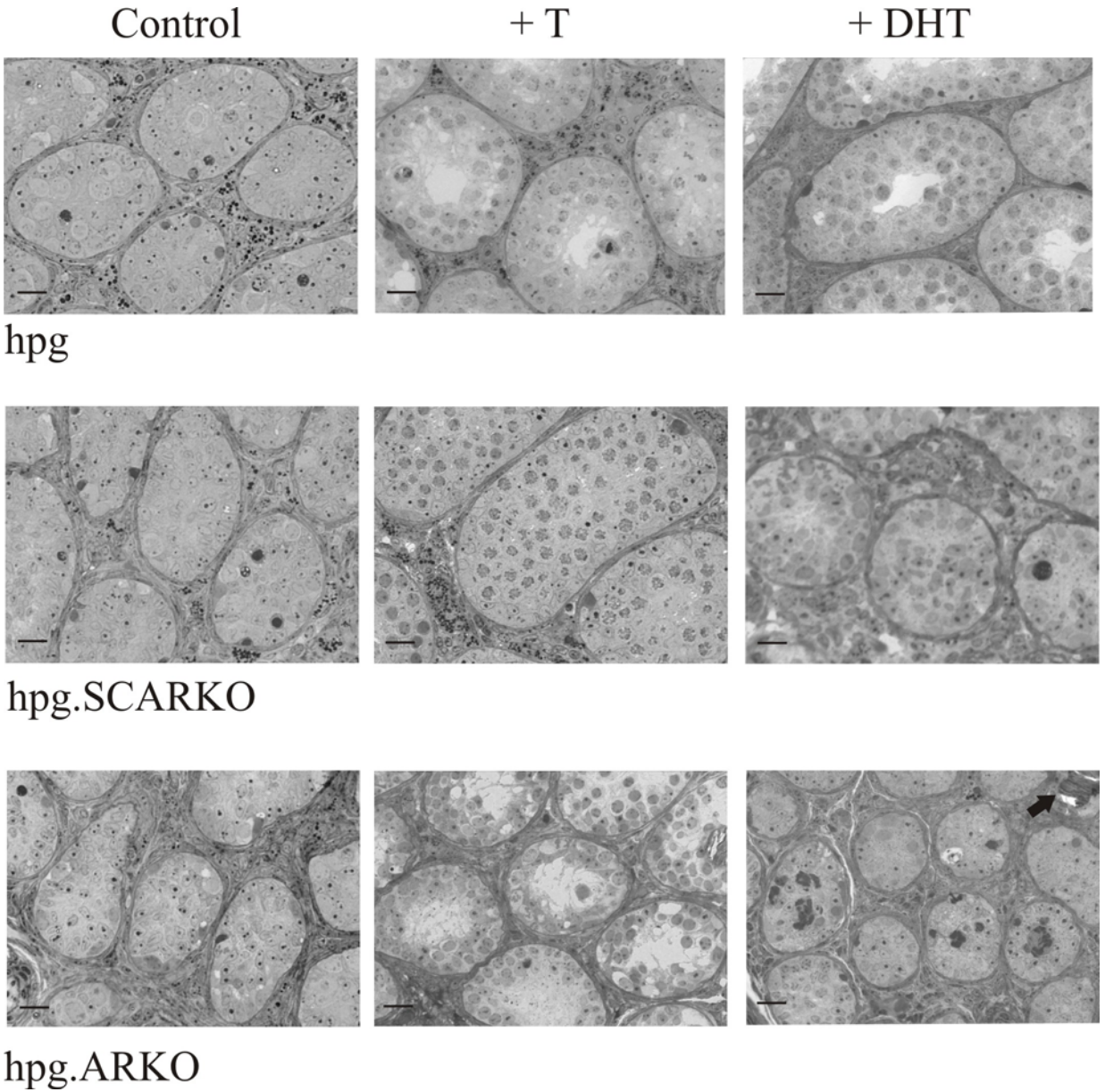
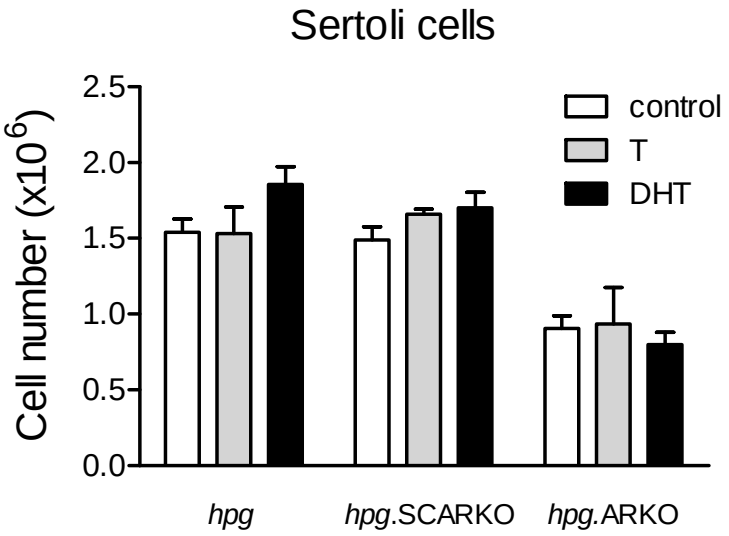

A)
Testis volume
Testis volume (mm 3)
control
T
DHT
hpg
hpg.SCARKO
hpg.ARKO
B)
Tubule diameter
Tubule diameter (µm)
hpg
hpg.SCARKO
hpg.ARKO
C)
Seminal vesicle weights
Weight (mg)
hpg
hpg.SCARKO

Figure 3




Sertoli cells
Cell number (x10^6)
2.5
2.0
1.5
1.0
0.5
0.0
control
T
DHT
hpg
hpg.SCARKO
hpg.ARKO

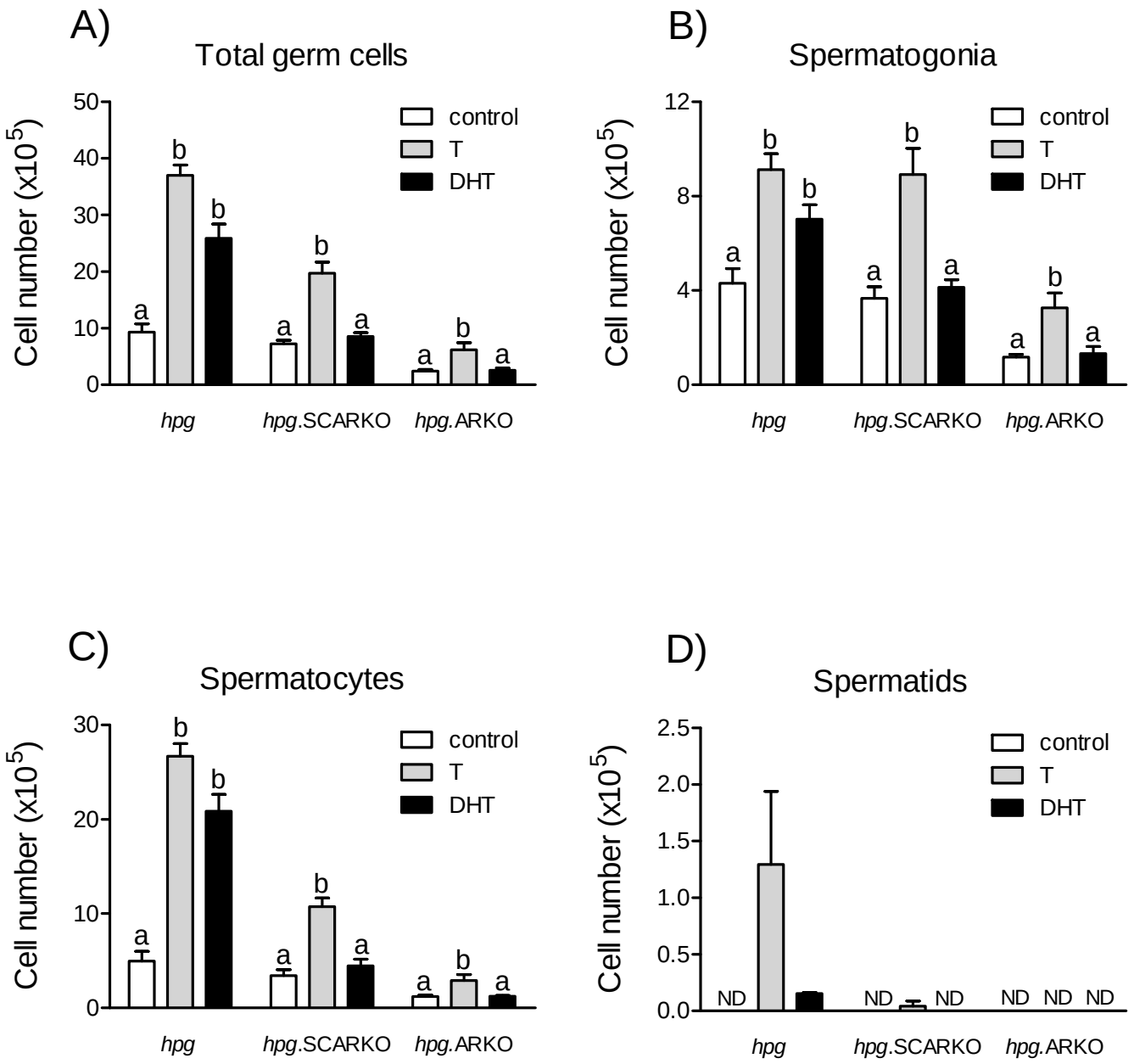
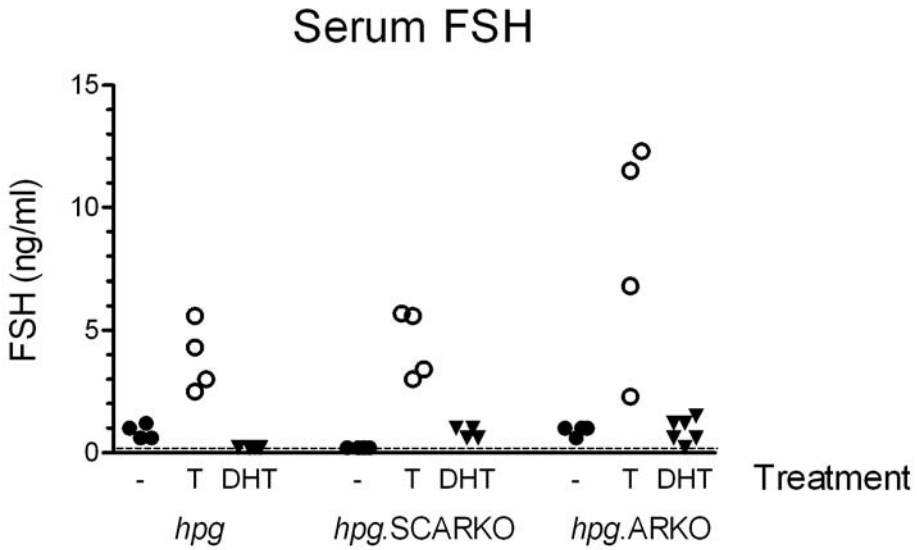

A)
Total germ cells
Cell number (x10^5)
50
40
30
20
10
0
control
T
DHT
a
b
b
a
b
a
a
b
a
hpg
hpg.SCARKO
hpg.ARKO
B)
Spermatogonia
Cell number (x10^5)
12
8
4
0
control
T
DHT
a
b
b
a
b
a
a
b
a
hpg
hpg.SCARKO
hpg.ARKO
C)
Spermatocytes
Cell number (x10^5)
30
20
10
0
control
T
DHT
a
b
b
a
b
a
a
b
a
hpg
hpg.SCARKO
hpg.ARKO
D)
Spermatids
Cell number (x10^5)
2.5
2.0
1.5
1.0
0.5
0.0
control
T
DHT
ND
ND
ND
ND
ND
ND
hpg
hpg.SCARKO
hpg.ARKO

Figure 6


Serum FSH
FSH (ng/ml)
15
10
5
0
- T DHT - T DHT - T DHT
Treatment
hpg
hpg.SCARKO
hpg.ARKO