Hormonal regulation of spermatogenesis

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Summary

Proper functioning of the mammalian testis is dependent upon an array of hormonal messengers acting through endocrine, paracrine, and autocrine pathways. Within the testis, the primary messengers are the gonadotrophins, follicle stimulating hormone and luteinizing hormone, and the androgens. Abundant evidence indicates that the role of the gonadotrophins is to maintain proper functioning of testicular somatic cells. It is the androgens, primarily testosterone, which act through the somatic cells to regulate germ cell differentiation. Despite extensive research in this area, little is known about the cell-specific requirements for androgens and even less is understood about the downstream effectors of androgen signalling. However, recent work using cell-specific ablation of androgen receptor function has demonstrated a clear requirement for androgen signalling at multiple, discrete time points during spermatogenesis. These models also provide useful tools for identifying the targets of androgen receptor activity. The purpose of this review is to provide a brief overview of recent advances in our understanding of hormonal regulation of spermatogenesis, with an emphasis on the role of testosterone within the testis, and to pose important questions for future research in this field.

Keywords: androgens, gonadotrophins, Sertoli, spermatogenesis

Gonadotrophic regulation of spermatogenesis

Spermatogenesis in mammals requires the actions of a complex assortment of peptide and steroid hormones, each of which plays an important role in the normal functioning of the seminiferous epithelium (Fig. 1 and Table 1). These hormonal messengers are critical not only for regulation of male germ cell development, but also for the proliferation and function of the somatic cell types required for proper development of the testis (Sharpe, 1994; McLachlan et al., 2002). These include the interstitial steroidogenic Leydig cells, whose primary function appears to be production of testosterone (Mendis-Handagama, 1997); the myoid cells that surround the seminiferous tubules and provide physical support and contractile motion to these structures (Maekawa et al., 1996); and the Sertoli cells, whose direct contact with proliferating and differentiating germ cells within the seminiferous tubules makes them essential for providing both physical and nutritional support for spermatogenesis (Griswold, 1998). Each of these cell types is a direct target for one or more of the hormones whose actions are essential for unimpaired male fertility.

Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are glycoprotein hormones secreted by the anterior pituitary that act directly on the testis to stimulate somatic cell function in support of spermatogenesis. These hormones, part of the transforming growth factor (TGF) β superfamily of secreted growth factors, share a common α-subunit and are distinguished by their hormone-specific...
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Most hormones shown can have both positive and negative effects, either at the level of receptor activation/desensitization or through activation and repression of downstream targets. GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; T, testosterone.

β-subunit (Pierce & Parsons, 1981). In males, FSH receptor expression (FSH-R) is limited to the testicular Sertoli cells (Rannikki et al., 1995), while LH receptors (LH-R) are found primarily in the Leydig cells, although receptor staining is also observed in spermatogenic cells (Ehlen et al., 2001; Lei et al., 2001).

Genetic and pharmacological studies in rodents indicate that the primary role of FSH in spermatogenesis is stimulation of Sertoli cell proliferation during prepubertal development (Heckert & Griswold, 2002). Sertoli cell number largely determines the number of germ cells (Sharpe, 1994). Targeted mutations in the murine FSH-R and FSHβ subunit genes lead to dramatically reduced testis weights and epididymal sperm numbers, although males are fertile in both cases (Kumar et al., 1997; Dierich et al., 1998). These results are consistent with hormone depletion-replacement studies in human males (Matsumoto & Brenner, 1985; Matsumoto et al., 1986), although unlike in rodents, FSH can also rescue spermatogenesis in gonadotrophin-suppressed men independently of T (Matsumoto et al., 1983). However, the authors of this study suggest this effect is because of increased FSH-induced Sertoli cell sensitivity to residual T production within the testis.

Regulation of testosterone (T) synthesis seems to be the only indispensable function of LH within the adult testis. Treatment of LH-R knockout mice with exogenous T is able to fully rescue spermatogenesis in the absence of LH-R function (Lei et al., 2001). Testosterone treatment also confers a qualitative recovery of spermatogenesis in hpg mice, which lack both FSH and LH because of an inactivating mutation at the GnRH locus (Singh et al., 1995). In the absence of T replacement, spermatogenesis is arrested during meiosis in hpg males (Cattanach et al., 1977). It is also clear that this effect of T replacement is not dependent upon stimulation of other Leydig cell products by T or LH, as T alone completely rescues spermatogenesis in rats treated with the Leydig cell-specific cytotoxin ethane dimethane sulphonate (EDS) (Kerr et al., 1993).

Steroid function in spermatogenesis

Testosterone and its metabolites, dihydrotestosterone (DHT) and estradiol (E2), are collectively referred to as the sex hormones. This is because of their primary role in the regulation of gonadal and germ cell development in both males and females as well as in the sexual differentiation of males. In the male, T assumes the lead role in both morphological development and reproductive function, although E2 and its receptor estrogen receptor (ERx), but not ERβ, clearly play some role in the maintenance of male fertility. However, these effects appear to be indirect and secondary. Disruption of ERβ has no apparent effect in males, as XY animals are morphologically normal and fertile (Krege et al., 1998). Initial observations of Erα null male mice suggested a primary role for this gene in regulating spermatogenesis, as animals presented with reduced fertility and dramatically decreased epididymal sperm counts (Lubahn et al., 1993). However, it is now clear that the primary function of ERα in the male reproductive tract is the regulation of luminal fluid reabsorption in the rete testis and efferent ducts linking the testis and epididymis (Hess et al., 2000; Lee et al., 2000). As might be expected, males homozygous for mutations in both Erα and β have a similar phenotype to Erα−/−/β−/− males (Couse et al., 1999).

Targeted disruption of Cyp19, whose gene product converts T to E2, results in progressive loss of fertility and disruption of spermatogenesis, although males are initially fertile. It is unclear why loss of E2 synthesis via mutation of Cyp19 has a less severe reproductive phenotype than disruption of Erα. However, it is suggestive of the existence of an as yet unidentified Cyp19 paralog or an E2 independent function of ER within the Cyp19−/− male. Surprisingly, despite the complete absence of measurable aromatase activity from Cyp19−/− ovaries, there appears to be no difference in serum concentrations of E2 between wild-type and Cyp19 mutant animals of either sex (Fisher et al., 1998).

In contrast to the role of estrogens in males, androgens and androgen receptor (AR) function are essential for proper sexual differentiation and the maintenance of normal spermatogenesis. AR activity is regulated by T and DHT, whose binding initiates nuclear translocation and the
transcriptional regulatory function of AR (Lindzey et al., 1994). In humans, DHT is crucial for the development of the male reproductive tract, although DHT plays little or no role in the development of male mice (Walsh et al., 1974; Mahendroo et al., 2001). As discussed above, T production is regulated by LH, whose release from the pituitary stimulates the steroidogenic Leydig cells in the testis (Mendis-Handagama, 1997). Not surprisingly, AR itself plays an important role in the regulation of T levels through autocrine feedback on the Leydig cells, via endocrine effects on GnRH production, and through inhibition of LH synthesis and secretion by the pituitary (Amory & Bremner, 2001).

In both humans and mice, males carrying a hemizygous null mutation in the X chromosome-linked Ar gene exhibit complete Androgen Insensitivity Syndrome (cAIS), characterized by pseudohermaphroditism and sterility (Lyon & Hawkes, 1970; Brown, 1995). This syndrome is recognized by the presentation of XY individuals with a stereotypically male phenotype. The Table 1 below provides a summary of animal models of hormonal regulation of spermatogenesis:

<table>
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<th>Model</th>
<th>Organism</th>
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| Gnhr


/ hpg Mouse | Loss of LH, FSH, and T secondary to GnRH deficiency; cryptorchid; spermatogenic arrest at pachytene, rescued by LH and T | Cattanach et al. (1977), Singh et al. (1995) |
| Fsh-r/- Mouse | Loss of FSH-R signalling in Sertoli cells; decreased testis weight and sperm output because of reduced Sertoli cell proliferation; fertile | Dierich et al. (1998) |
| Fshβ/- Mouse | Loss of FSH; decreased testis weight and sperm output as for Fsh-r/-; fertile | Kumar et al. (1997) |
| Lh-r/- Mouse | T deficiency because of loss of LH-R function in Leydig cells; T still detectable in serum; failure of postnatal male sexual development, cryptorchid; spermatogenic arrest at round spermatid stage, rescued by T | Lei et al. (2001) |
| EDS Rat | T deficiency because of selective destruction of Leydig cells; phenotype similar to hypophysectomy; rescued by T | Sharpe et al. (1990), Kerr et al. (1993) |
| Cyp19/-/- Mouse | E2 deficiency because of lack of aromatase enzyme; abnormal male sexual behaviour; progressive disruption of spermatogenesis, although fertile for several months | Fisher et al. (1998), Robertson et al., (1999), Robertson et al., (2001) |
| Erz/-/- Mouse | Indirect effect on spermatogenesis from loss of ERz signalling in rete testis and efferent ducts; disrupted spermatogenesis because of lack of luminal fluid reabsorption causing increased pressure in seminiferous tubules | Lubahn et al. (1993), Hess et al. (2000), Lee et al. (2000) |
| Erβ/-/- Mouse | No effect on spermatogenesis because of loss of ERβ | Krege et al. (1998) |
| Arflox/Y Mouse | Null allele of Ar; pseudohermaphroditism; cryptorchid; meiotic arrest at pachytene | Lyon & Hawkes (1970) |
| Arflox(ex1-neo) Y Mouse | Hypomorphic Ar allele, reduced AR levels in all tissues; significantly elevated LH, FSH, and T levels; spermiogenic arrest during elongating steps | Holdcraft & Braun (2004) |
| Arflox(ex1-neo) Y; Amh-cre Mouse | Sertoli cell-specific ablation of AR function, hypomorphic for AR in all other cell types; spermiogenic arrest at the transition from round to elongating spermatids | Holdcraft & Braun (2004) |
| SCARKO Mouse | Sertoli cell-specific ablation of AR function; meiosis is arrested during pachytene with corresponding reduction in testis mass; males otherwise indistinguishable from wild-type | De Gendt et al. (2004) |
| S-AR/-/Y Mouse | Sertoli cell-specific ablation of AR function; decreased testis mass with meiotic arrest at diplotene; hypotesteronemic with elevated serum LH level | Chang et al. (2004) |
female external appearance, usually diagnosed at puberty in connection with primary amenorrhea (Brown, 1995). The vagina is incompletely formed and ends abruptly, forming a blunt-ended pouch. Small, abdominally positioned testes are the only internal reproductive organs present with spermatogenesis blocked early in meiosis and some seminiferous tubules having depletion of spermatogonia (Lyon & Hawkes, 1970; Vanha-Perttula et al., 1970).

While the phenotype of cAIS rodents and patients clearly demonstrates the crucial requirement for AR in male development, the suitability of AIS as a model for studying the spermatogenic function of Ar is poor. Testicular descent fails in mice with cAIS, and the spermatogenic phenotype mimics that of cryptorchidism in an otherwise normal male, namely early meiotic arrest (Lyon & Hawkes, 1970). Thus it is impossible to discern the contribution to the phenotype of loss of AR function separate from that because of the abdominal positioning of the testes.

Mice homozygous for a mutation in the gonadotrophin releasing hormone gene (Gnrh<sup>hpg/hpg</sup>), which have dramatically lowered serum testosterone levels (Singh et al., 1995), present a testicular phenotype similar to cAIS (Cattanach et al., 1977). Spermatogenesis in these animals can be qualitatively rescued by androgen replacement therapy (Singh et al., 1995). This occurs in the absence of appreciable levels of LH and FSH. Further, FSH alone fails to significantly rescue spermatogenesis beyond the meiotic stages (Singh & Handelsman, 1996; Haywood et al., 2003), providing further support for the contention that T and/or DHT is the major hormonal regulator of spermatogenesis. Interestingly, one study has shown that oestrogen supplementation in Gnrh<sup>hpg/hpg</sup> males may also rescue spermatogenesis (Ebling et al., 2000). However, the mechanism by which this might occur is unclear.

An absolute requirement for androgens in spermatogenesis is further supported by classic hormone withdrawal experiments in rats. Removal of androgens from adult rats by hypophysectomy (Hx) leads to an acute, stage-specific regression of the seminiferous epithelium (Russell & Clermont, 1977; Ghosh et al., 1991). Elimination of T is manifested initially as loss of mid-stage round spermatids and mature, elongated spermatzoa, indicative of an affect of androgens on spermatiation and the transition from round to elongating steps of spermiogenesis. Mid-stage meiotic spermatocytes also undergo an immediate and obvious regression. After long term Hx and elimination of residual testosterone activity by flutamide or EDS treatment, spermatogenesis rarely proceeds beyond meiosis, with very few round spermatids observed and elongated spermatids nearly nonexistent (Kerr et al., 1992; Franca et al., 1998). As with Gnrh null mice, androgen or LH replacement leads to qualitative recovery of spermatogenesis while FSH has little direct stimulatory effect (Elkington & Blackshaw, 1974; Russell & Clermont, 1977; El Shennawy et al., 1998). Similar results are seen in response to suppression of Gnrh activity (Szende et al., 1990), and destruction of Leydig cells with the Leydig-specific cytotoxin EDS (Sharpe et al., 1990; Kerr et al., 1993).

While androgens have a positive regulatory influence on differentiating germ cells, there is a well-established negative effect of androgens on the differentiation of spermatogonial stem cells. This observation stems from work aimed at understanding the prolonged suppression of spermatogenesis in men following radiation or chemotherapy treatment for cancer (Meistrich, 1998). Following treatment, spermatogonia are present, but fail to proliferate or differentiate. It was found that in irradiated rats, stimulation of spermatogenesis occurred following treatment with GnRH agonist or T, which both act to suppress intratesticular T concentration. This work was later extended to show that a GnRH antagonist stimulates spermatogonial proliferation and inhibits apoptosis following irradiation (Shuttlesworth et al., 2000). In addition, recent studies have shown that this effect is because of inhibition of androgen function, as the androgen antagonist flutamide stimulates, while testosterone inhibits, spermatogonial proliferation and differentiation (Shetty et al., 2000, 2002). A similar effect has been demonstrated in juvenile spermatogonial depletion (jsd) mice, whose germ cells regress to a spermatogonia-only phenotype following the first wave of spermatogenesis. Stimulation of spermatogonial proliferation and differentiation, along with completion of spermatogenesis, is observed in the testes of these animals following treatment with GnRH antagonist or flutamide. The stimulatory action of GnRH antagonist is prevented by co-administration of T, while flutamide reverses the repressive effect of testosterone (Shetty et al., 2001).

In the testis, AR protein is expressed in the somatic Leydig, myoid, and Sertoli cells (Bremner et al., 1994; Vornberger et al., 1994; Suarez-Quian et al., 1999; Zhou et al., 2002). Some groups have reported the presence of AR within mouse fetal and postnatal germ, human spermatogonia, and rat spermatids (Kimura et al., 1993; Vornberger et al., 1994; Zhou et al., 2002). However, it is clear from work with AR-null chimeric mice and from germ cell transplantation experiments that AR is not required in male germ cells for normal fertility (Lyon et al., 1975; Johnston et al., 2001). While expression in Leydig and myoid cells is continuous, Sertoli cell expression of AR occurs in a stage dependent fashion. Although it is apparent that other factors must be involved to confer stage-specific expression in the complex environment of the testis, hormone withdrawal-replacement experiments have shown that testosterone can support stage-specific Sertoli cell AR expression (Bremner et al., 1994; Zhu et al., 2000). In addition, a DHT responsive promoter has been defined upstream of the Ar gene itself, providing further support for androgen/AR auto-regulation of testicular AR expression (Grossmann et al., 1994). Interestingly, stages VII–VIII, during which AR expression in Sertoli cells is highest, correspond directly with those most acutely affected by androgen withdrawal (Russell &
Sertoli cells are also the only somatic cell type in direct contact with differentiating germ cells. They provide both physical and nutritional support for spermatogenesis, which occurs in the intercellular spaces between Sertoli cells (Grishold, 1998). Adhesion between germ cells and Sertoli cells may be androgen dependent, as testosterone withdrawal leads not only to retention and phagocytosis of mature, elongated spermatids, but also to the premature release of round spermatids (Russell & Clermont, 1977; Kerr et al., 1993; O’Donnell et al., 1996). Taken together, these observations have led to the general belief that Sertoli cells are the primary mediators of AR regulation of spermatogenesis.

To examine the consequences of loss AR function specifically in Sertoli cells, several groups have recently created conditional alleles of the Ar gene, and used them to generate Sertoli cell-specific ablation of Ar (Chang et al., 2004; De Gendt et al., 2004; Holdcraft & Braun, 2004). The results of these studies suggest there are multiple AR-dependent steps during spermatogenesis. In the first study, a conditional allele of Ar was created by flanking exon 2 with loxP sites (De Gendt et al., 2004). In the absence of Cre, the allele has full wild-type AR function. However, when crossed to mice expressing the Cre recombinase driven by the Sertoli cell-specific anti-Müllerian hormone (Amh) promoter, the germ cells undergo meiotic arrest, predominantly during the pachytene stage, although some dipotene spermatocytes, secondary spermatocytes, and round spermatids are produced. Other than sterility, the males appear to be normal with fully descended testes, and a grossly normal appearance.

In a second study, a hypomorphic conditional allele of Ar was created by introducing a neomycin-resistance gene cassette in the first intron of Ar, and flanking the first exon of Ar with inverted loxP sites (Holdcraft & Braun, 2004). The hypomorphic allele, which results in reduced levels of wild-type Ar mRNA in all AR-expressing cells, causes severe oligospermia, despite fully descended testes, indicating maximal sensitivity to loss of AR function during spermatid elongation. The males also exhibit severe alterations in serum hormone levels, with significant increases in LH, FSH, and T concentrations. Crossing the allele to an Amh-Cre line causes a more severe spermatogenic phenotype than hypomorphism alone. Although the males have no apparent defect in meiosis, the germ cells are defective in making the transition from round to elongating spermatids, as indicated by a significant reduction in elongated spermatids, and the continued presence of round spermatids in stage IX tubules. The elevated hormone levels in mice carrying the hypomorphic allele may explain the apparently contradictory results between the two studies, as elevated T and/or intratesticular E2 levels may be suppressing meiotic arrest. While serum E2 levels are normal in the males, it is possible that elevated intratesticular E2 levels, because of increased conversion of supraphysiological T levels, suppress the meiotic arrest phenotype. In support of this possibility, one previous study of Gush-null males has shown that E2 can support qualitatively normal spermatogenesis in the absence of testosterone (Ebling et al., 2000). Alternatively, lack of meiotic arrest may be the result of residual AR activity in a subset of Sertoli cells because of incomplete inversion of the first exon. This latter hypothesis is consistent with the phenotype of LHβ mice (R. Kumar, personnel communication), and LH receptor-null mice (Lei et al., 2001), in which a round spermatid arrest is observed as a consequence of basal levels of androgen action in the absence of LH signalling (Lei et al., 2001).

A third conditional Ar allele has recently been reported that has the second exon flanked by loxP sites (Chang et al., 2004). Recombination-induced deletion of the allele in Sertoli cells, again by crossing to Amh-Cre mice, also causes azoosperma. In this model spermatogenesis is primarily blocked at the dipotene spermatocyte stage, instead of during pachytene (De Gendt et al., 2004). It is unclear why the two alleles have different meiotic arrest phenotypes, although it could be because of the genetic backgrounds of the mice, the efficiency of recombination, or changes in steroid levels. The mice also exhibit hypogonadism, while at the same time displaying significantly increased serum LH levels. The authors suggest that the decreased concentration of serum T is because of upregulation of anti-Müllerian hormone expression by Sertoli cells, a conclusion supported by their own observation of elevated Amh mRNA in these animals, as well as by studies showing lowered serum T, Leydig cell hypoplasia, and inhibition of steroidogenesis, all induced by AMH (Behringer et al., 1990; Lyet et al., 1995; Fynn-Thompson et al., 2003).

Taken together, the observations from these studies reveal a differential requirement for AR activity in Sertoli cells for at least three steps of spermatogenesis. AR is first required for progression through meiosis I, again during the transition from the round to elongating steps of spermatogenesis, and finally during the terminal stages of spermiogenesis. Surprisingly, it is the meiotic requirement for AR function that appears to be least sensitive to absolute levels of AR activity, as evidenced by the differential effects on meiosis observed between these models of Sertoli cell AR ablation. Whether AR function in Sertoli cells also affects steroidogenesis remains an open question.

**Future studies**

Many important questions remain to be answered regarding the androgenic regulation of male reproduction.
As discussed above, little is known about the phenomenon of stage-specific regulation of Sertoli cell AR expression beyond a presumptive role for androgens and for AR itself. Identification of potential co-activators or repressors that act in conjunction with AR at these stages, or the discovery of other transcription factors that regulate stage-specific AR expression, would provide insight into the important functional differences between Sertoli cells during the different stages of spermatogenesis. It would also be of interest to determine the role that the specific germ cell complement of tubules at different stages plays in regulation of Sertoli cell function. Additionally, the discovery of novel factors that regulate AR expression or unique targets of AR transcriptional regulation within Sertoli cells would provide intriguing candidates for future male contraceptive design.

While androgens, acting through AR, are clearly essential for normal spermatogenesis, the crucial downstream targets of AR activation in the testis remain elusive. Androgens may also mediate effects in Sertoli cells via a non-classical receptor pathway, as androgens have been shown to activate signalling cascades in multiple cell types independently of a direct androgen-AR-DNA interaction (Walker, 2003). Few AR-responsive genes have been defined in the testis, and none of these have been demonstrated to play a role in spermatogenesis. The homeobox transcription factor PEM has been shown to be positively regulated by androgens in Sertoli cells specifically during the androgen-sensitive stages of spermatogenesis (Lindsey & Wilkinson, 1996). However, targeted disruption of the Pem gene in the mouse produces no discernible phenotype (Pitman et al., 1998). Overexpression of PEM in Sertoli cells was shown to increase the frequency of DNA strand breaks in adjacent germ cells, but again no effect was observed on the reproductive fitness of these males (Wayne et al., 2002). The advent of microarray technology now allows for the rapid identification of novel factors that regulate stage-specific AR expression, would provide insight into the important role of AR in Leydig and peritubular myoid cells. The proximity of myoid cells to the spermatogonial stem cells, presents the possibility that AR may regulate some aspect of stem cell proliferation or differentiation via this cell type. This is a particularly intriguing possibility in the context of androgenic suppression of stem cell differentiation and spermatogenic recovery in jsd males and following chemotherapeutic insult. Similarly, conditional removal of AR function if Leydig cells will allow dissection of the role of AR in the maturation of Leydig cells during puberty (O’Shaughnessy et al., 2002), and in feedback regulation of steroidogenesis in the adult.

Finally, a long-term goal in the field should be the delineation of AR function as having either an instructive, supportive, or dual role in the differentiation and development of male germ cells. To date, it is unclear whether the ultimate outcome of AR function is to produce a healthy environment within which germ cells can thrive, or whether the downstream effect of AR activity is the generation of a signal(s) that impinges directly upon germ cell migration, differentiation and survival. The development of these new genetic models of AR regulation of spermatogenesis might provide the tools necessary to answer this question and many others in the fields of androgen and Ar biology.

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References
stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proceedings of the National Academy of Sciences of the USA* 95, 13612–13617.


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