Spermatogenesis

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Spermatogenesis is a complex process involving mitotic cell division, meiosis and the process of spermiogenesis. The regulation of spermatogenesis involves both endocrine and paracrine mechanisms. The endocrine stimulation of spermatogenesis involves both follicle stimulating hormone (FSH) and luteinizing hormone, the latter acting through the intermediary testosterone, produced by the Leydig cells in the testis. Since the germ cells do not possess receptors for FSH and testosterone, the hormonal signals are transduced through the Sertoli cells and peritubular cells by the production of signals that have yet to be defined. Although the hormonal signals are essential for successful spermatogenesis, there is increasing evidence that a multiplicity of growth factors and cytokines are involved in local control mechanisms influencing stem cell renewal by mitosis and the complicated process of the two meiotic cell divisions. The final complex metamorphosis which converts a round cell into the complex structures of the spermatozoa is well defined at a structural level, but the control systems regulating this process still remain to be elucidated.

Key words: Sertoli cell/spermatids/spermatocytes/spermatogenesis/spermatogonia

Introduction

Male fertility requires the production by the testis of large numbers of normal spermatozoa through a complex process known as spermatogenesis. This process can be subdivided into three major steps: (i) the multiplication of spermatogonia by the process of mitosis; (ii) meiosis, which reduces the chromosome number from diploid to haploid and commences with the entry of type B spermatogonia into the prophase of the first meiotic division. These cells, now called primary spermatocytes, divide to form secondary spermatocytes, and then divide again to form round spermatids; (iii) the successful transformation of the round spermatid into the complex structure of the spermatozoon, this phase being called spermiogenesis.

Each of these steps represents a key element in the spermatogenic process. Defects which occur in any of them can result in the failure of the entire process and lead to the production of defective spermatozoa and reduction or absence of sperm production. It is therefore essential that our understanding of these processes is expanded to provide information concerning the regulatory mechanisms. Many of the studies required to achieve this understanding are not possible in the human, and consequently we are dependent on studies in rodents and primates for the experimental paradigms to provide this crucial knowledge.

Regulation of spermatogenesis

Advances in the management of male infertility will only occur when our understanding of the regulation of spermatogenesis has progressed further. There are considerable data emerging to indicate that this regulation occurs at two major levels: (i) hormonal and endocrine and (ii) paracrine/autocrine. It is not possible, in a review of this size, to cover these topics in depth; our intention therefore is to emphasize emerging concepts and to indicate major references in key areas.

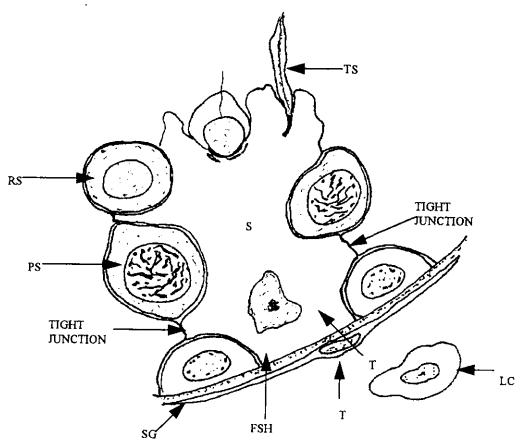

Figure 1. The relationships between the the Sertoli cells (S) and the germ cells: spermatogonia (SG), primary spermatocytes (PS), round spermatids (RS) and elongated spermatids are illustrated. Where two adjacent Sertoli cells abut a tight junction is formed which limits intercellular transport and represents the morphological basis of the blood testis barrier. Testosterone (T), produced by the Leydig cells (LC) under the stimulation of luteinizing hormone, acts through receptors on the Sertoli cells to modulate spermatogenesis. Follicle stimulating hormone (FSH) acts on the Sertoli cells and possibly directly on spermatogonia.

Hormonal control

The past decade has seen an intense investigation of the hormonal mechanisms involved in the control of rat spermatogenesis. These studies have more carefully defined the stages at which testosterone and follicle stimulating hormone (FSH) act and, from these data, the difficulty of creating models to evaluate FSH deficiency has emerged (Figure 1). These problems arise from the ability of high doses of testosterone to stimulate FSH concentrations, even in states of gonadotrophin-releasing hormone (GnRH) deficiency (McLachlan *et al.*, 1994). Nevertheless, these studies have shown that, in the absence of testosterone, there is a loss of round spermatids between steps 7–8 of spermatogenesis (O'Donnell *et al.*, 1994). More recent data indicate that this loss is due to failure of Sertoli cells to produce the adhesion molecule N-cadherin, the production of which appears to require both FSH and testosterone (Perryman *et al.*, 1996). Testosterone also impacts on normal spermatogonial mitosis and the successful completion of meiosis (Sun *et al.*, 1989, 1990).

In the rat, the role of FSH is more difficult to define, but this hormone may modulate the number of germ cells proceeding successfully through the mitotic and meiotic phases of spermatogenesis. Using replacement therapy in the gonadotrophin-deficient (hpg) mouse model in which GnRH is not secreted, Singh *et al.* (1995) suggested that FSH may not be required in this species and that the requirement of testosterone for meiosis maybe even more crucial than its requirement for spermiogenesis. The recent study by Kumar *et al.* (1997) in which the FSH β-subunit gene was knocked out in mice, rendering them FSH deficient, showed that spermatogenesis could proceed to completion, albeit in the presence of smaller testes. These mice were fertile and the results lend support to the work of Singh *et al.* (1995). Other data suggest that FSH may play a role in stimulating mitotic and meiotic DNA synthesis in type B spermatogonia and preleptotene spermatocytes as well as in preventing apoptosis of pachytene spermatocytes and round spermatids (Henriksen *et al.*, 1996; Shetty *et al.*, 1996). McLachlan *et al.* (1995) demonstrated that FSH alone could partially restore spermatogenesis in GnRH-immunized rats and Lerchl *et al.* (1993) observed a similar effect in the photo-inhibited hypogonadotrophic Djungarian hamster. These data, taken together with studies showing the unique testicular localization of the FSH receptor in monkeys (Dankbar *et al.*, 1995) and its stage-specific expression (Kliesh *et al.*, 1992) provide compelling evidence for a specific role.

Whether data in the rat can be extrapolated directly to man is questionable. Several studies have indicated the need for both testosterone and FSH for the successful completion of spermatogenesis and further data in primates are required to confirm these views. It is important to emphasize that, although testosterone and FSH represent hormonal prerequisites for spermatogenesis, germ cells are devoid of receptors for these substances, although FSH binding has been described in spermatogonia. Consequently these hormones exert their effects through Sertoli cells which contain receptors for both FSH and testosterone, through mechanisms that still remain unclear.

Spermatogonial renewal

The spermatogonial population in the mammalian testis arises from the primordial germ cells which

migrate into the developing testis during fetal life. They become associated with the mesenchymal cells which are the forerunners of the Sertoli cells, and seminiferous cords are formed. In this setting, they transform into gonocytes which remain centrally placed, being surrounded by the immature Sertoli cells. Following a period of multiplication, the gonocytes migrate to the basement membrane of the tubule, where they divide to form type A spermatogonia. The number of spermatogonial types that have been identified varies between species, but in the human, A pale, A dark and B spermatogonial forms can be distinguished (Heller and Clermont, 1964). In the rodent testis, multiple type A spermatogonia, intermediate and type B spermatogonia have been identified, and their initial appearance within the developing testis is controlled in a temporal sense. In the rat, spermatogonial proliferation commences at day 4–5, with type B spermatogonia being identified at day 6 postpartum. The completion of a series of mitotic divisions results, through unknown mechanisms, in the entry of type B spermatogonia into the preleptotene stage of the meiotic process.

The past decade has seen some accumulation of evidence concerning the regulation of spermatogonial proliferation. The migration of primordial germ cells into the testicular anlagen is dependent on their surface expression of c-kit protein, which serves as a receptor for stem cell factor (SCF), produced by the immature Sertoli cells. Mutations in the c-kit receptor, encoded at the W-*Locus* in mice, result in failure of spermatogenesis due to the absence of germ cells from the testis (reviewed by Reith and Bernstein, 1991). Several studies have demonstrated that mitotic replication of spermatogonia is dependent on the expression of c-kit and the synthesis of SCF by the Sertoli cells. A neutralising antibody to c-kit, when injected into the mice, leads to the cessation of spermatogonial replication (Yoshinaga *et al.*, 1991). Further, although SCF protein can be identified in the rodent testis throughout the first week of development, there is a marked upregulation of SCF mRNA on day 5, which occurs simultaneously with the commencement of spermatogonial division (Munsie *et al.*, 1997). Thus, the interaction between SCF and c-kit appears to both mediate and modulate spermatogonial multiplication.

Many earlier studies have shown that up to 35% of spermatogonia degenerate, and recent studies have implicated apoptosis as a principal process by which these cells are removed from the epithelium. However, survival of both spermatogonia and spermatocytes appears to be regulated by c-kit/SCF binding (Packer *et al.*, 1995) and, while our understanding of spermatogonial division has been enhanced from the study of c-kit/SCF, little is known of the regulatory mechanisms controlling apoptosis. Contact with the basement membrane has been proposed as a key regulator of this process based on studies of germ cell survival in culture. The addition of FSH to rat tubule cultures also has been shown to influence germ cell apoptosis, affecting both mitotic and meiotic cell populations (Henriksen *et al.*, 1996; Shetty *et al.*, 1996).

Other ligand systems appear to be involved, since Mather *et al.* (1990) have demonstrated that activin A stimulates spermatogonial mitosis in cocultures with Sertoli cells and de Winter *et al.* (1992) have shown the presence of activin receptors on spermatogonia. However, contradictory data have emerged from the studies of Boitani *et al.* (1995), who indicated that there are temporal constraints to the action of activin and a synergism with FSH. Our recent studies have shown the immunolocalization of follistatin, a potent activin binding protein, in spermatogonia and the observation raises the possibility that the regulation of follistatin may modulate the actions of activin (A.Meinhardt, M.K.O'Bryan and J.R.McFarlane *et al.*, unpublished data). Further studies are necessary to clarify the major regulatory molecules controlling stem cell renewal in the testis. The opportunities for advancement in this field have been broadened considerably by the pioneering studies of Brinster and Avarbock (1994), who showed that spermatogenesis can be restored by intratubular injections of spermatogonia into mice that had no spermatogenesis due to the absence of germ cells.

Meiosis

Entry of type B spermatogonia into the prophase of meiosis is represented by the conversion of those cells into primary spermatocytes which divide to form secondary spermatocytes. The latter, after a

very short lifespan of ~6 h in the human, divide to form round spermatids. These two divisions result in the conversion of the diploid to the haploid chromosomal complement, and cells passing through the long phase of the first meiotic division can be subdivided on a cytological basis into preleptotene, leptotene, zygotene, pachytene and diplotene stages. The cellular details of these processes may be found in reviews (de Kretser and Kerr, 1994). Our detailed understanding of the biochemical and molecular regulation of meiosis is limited. However, recent studies are providing data that have enhanced our understanding of the molecules involved and highlighted key steps in these processes. Dix *et al.* (1996) showed that heat-shock protein 70–2 (HSP-70–2), a unique testicular member of the HSP-70 group of proteins, is essential for the completion of meiosis. HSP-70 is expressed in high concentrations in pachytene spermatocytes. Another related protein, heat-shock cognate (HSC-70), also has a testis isoform expressed in post-meiotic spermatids (Maekawa *et al.*, 1989). In genetically modified animals in which the HSP-70–2 gene is deleted, the germ cells fail to complete meiosis and there is a marked increase in spermatocyte apoptosis. Other data suggest that HSP-70–2 is a important component of the synaptonemal complex, which is a crucial element in the pairing of homologous chromosomes during the prophase of meiosis (Allen *et al.*, 1996). Although synaptonemal complexes were found in HSP70–2 -/- mice, they did not exist as unilateral elements in diplotene, as occurs normally. These data suggest that although formation of synaptonemal complexes can occur in the absence of HSP-70–2, the process of desynapsis cannot occur in its absence.

Recently, Byskov *et al.* (1995) identified a series of sterols isolated from bovine testis extracts and from human follicular fluid that induce meiosis in either denuded or cumulus-enclosed mouse oocytes. The meiosis-activating sterol from the testis was found to be 4,4-dimethyl-5α-cholest-8,24-diene-3β-ol, a C_{29} sterol also termed 4,4-dimethyl-zymosterol. Further studies on the action of these compounds in the testis will provide data on their importance in the completion of meiosis.

One of the other marked cytological modifications occurring as cells enter meiosis is the change in mitochondrial morphology. The typical mitochondrion with transversely disposed cristae is transformed following an initial dilatation of the intracristal spaces into the mitochondrion of primary spermatocytes and, later, germ cells which takes on a vacuolated appearance. Studies by Seitz *et al.* (1995) showed that this change in mitochondrial morphology (Figure 2) is dependent on protein secretion by the Sertoli cells, and more recent data have indicated that activin production by the Sertoli cells (de Winter *et al.*, 1993) appears to be an important paracrine modulator of this morphological change (Meinhardt *et al.*, 1996).

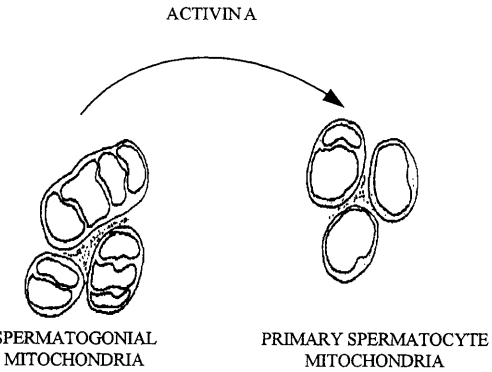

Figure 2. The mitochondria in spermatogonia change their morphology as those cells proceed into the prophase of meiosis (primary spermatocytes). The principal change is the dilatation of the intracristal space to give a vacuolated appearance. Evidence exists to suggest that this change is under the control of activin A.

Spermiogenesis

The round spermatid, which arises from the second meiotic division, undergoes a series of complex cytological events which transform it into the spermatozoon (de Kretser and Kerr, 1994). This process consists of (i) nuclear condensation and movement of the nucleus to the periphery of the cell; (ii) formation of a modified lysosome known as the acrosome, which becomes attached to the surface of the nucleus in apposition to the cell membrane; (iii) flagellar formation which includes the development of a core of microtubules, the axoneme, which arises from one of the centrioles of the round spermatid. Initially, the formation of the axoneme, a series of equally spaced doublet

microtubules surrounding two single central microtubules, occurs in the cytoplasm adjacent to the Golgi complex, but later in spermiogenesis, this structure becomes lodged at the abacrosomal pole of the nucleus through a complex articulation forming the neck of the spermatozoon. Later in the formation of the tail, the axoneme is modified by the development of a series of nine electron-dense fibres, termed the outer dense fibres, in the region of the mid-piece of the spermatozoon and distally by the formation of the fibrous sheath in the region of the principal piece; (iv) finally, following the completion of these events, the spermatid sheds a large part of its cytoplasm as the residual body which is phagocytosed by the Sertoli cell.

The ultrastructural details of these processes have been clearly described in many articles. However, little is known of the regulatory mechanisms involved in this process. There is an increasing understanding of a number of genes which are expressed by the haploid genome which are involved in spermiogenesis. The genes which encode protamines, the proteins which replace the histones bound to DNA, have been subjected to extensive studies. These genes are important since, like many other genes expressed in haploid cells, they are transcribed early in spermiogenesis and the resulting RNA is stored until later in this process for translation into protein (Hecht, 1996). Hecht (1996) has studied the mechanisms by which the mRNA remains stable for up to 7 days in haploid cells. A RNA-binding protein expressed in the testis was identified, which may be involved in maintaining the stability of these mRNAs through recognition of a specific nucleotide sequence (Kwon and Hecht, 1991).

One of the dramatic events that occurs during spermiogenesis is the formation of the tail. The axoneme is clearly of centriolar origin and the generation of motility emerges from the microtubules, composed of protofilaments of tubulin, a heterodimer of α and β tubulin, and the protein components completing the axoneme, including the radial spokes, nexin linkages and dynein arms (see de Kretser and Kerr, 1994, for review). The latter represents a protein with ATPase activity, and genetic defects resulting in absent dynein arms are associated with the immotile cilia syndrome (Eliasson *et al.*, 1977). Further, abnormalities such as the absence of nexin linkages or radial spokes can also result in immotile cilia, indicating the crucial role of these proteins in the control of motility (Afzelius and Eliasson, 1979).

Until recently, the details concerning the proteins composing the outer dense fibres and the fibrous sheath of the sperm tail were unknown. However, recent studies have identified genes which encode for some of the major proteins in the outer dense fibres, e.g. *rts/1* (Burfeind *et al.*, 1993) and in the fibrous sheath (*fsc-1*) (Fulcher *et al.*, 1995).

The outer dense fibres are composed of at least nine major proteins (Kim *et al.*, 1995), and the demonstration that *rts 5/1* encodes a 32 kDa protein component of the outer dense fibres will enable the nature of its role to be explored. The presence of an allele of *rts 5/1* with a 27 bp deletion in exon 2 is not associated with disordered motility (Hofferbert *et al.*, 1993). *fsc-1* encodes an 80 kDa protein of the fibrous sheath that contains a number of serine residues, sites that strongly suggest that phosphorylation may play a major role in its function (Fulcher *et al.*, 1995). Further data will emerge from genetic manipulation which delete these genes from mice and may provide data concerning their role in the structure and function of the sperm tail.

There is a need for many other studies to determine the regulatory processes in this complex process of spermiogenesis. Unfortunately, the inability of in-vitro systems to support spermiogenesis has greatly limited the speed at which this knowledge is acquired.

Regulation of the intratubular environment

There is increasing evidence that the Sertoli cells create a highly specialized environment in which spermatogenesis proceeds. The presence of a blood–testis barrier, which results from the tight junctions formed where adjacent Sertoli cells abut, clearly places the control of the intratubular environment on the Sertoli cell. Only the base of the Sertoli cell and the spermatogonia are in contact with the basement membrane of the tubule, but cells up to the leptotene stage are included in the basal compartment of the testis. Since the Sertoli

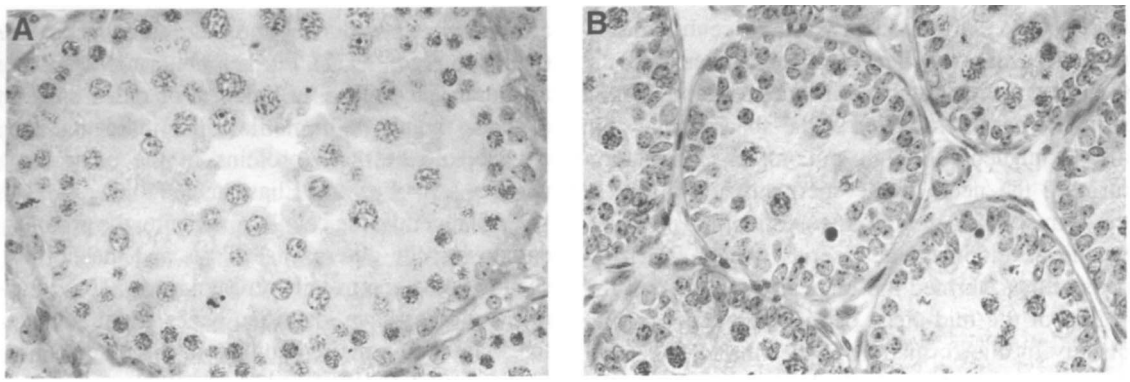

Figure 3. Photomicrographs of sections of testes from (**A**) 30 day old control rats and (**B**) 30 day old rats subjected to hypothyroidism induced by the administration of propyl thiouracil from days 1–25 (see Simorangkir *et al.*, 1997). Note in the hypothyroid state the small size of the tubules due to the absence of a lumen, the decreased numbers, compared to the control, of spermatogonia and primary spermatocytes and the presence of degenerating cells (dense bodies) (magnification ×400).

cells cease dividing in the rodent testis at ~18–20 days of age (Orth, 1982; Wang *et al.*, 1989), the total number of Sertoli cells in the testis is established early during the pubertal process in the rat (Orth *et al.*, 1988). The data that have emerged more recently from the manipulation of the thyroid status of the rat during this developmental phase (van Haaster *et al.*, 1992) strongly support the notion that an increase in the number of Sertoli cells will increase the spermatogenic output of the testis, whereas a decrease will cause the opposite effect (Cooke *et al.*, 1994). The induction of hypothyroidism for the first 30 days of life in the rat results in excessive Sertoli cell proliferation and an increase in the spermatogenic output of the adult testis (Hess *et al.*, 1993). Interestingly, the hypothyroid state is associated with decreased FSH, luteinizing hormone and testosterone concentrations. Increases in FSH as a result of the induction of neonatal hemicastration further augment the number of Sertoli cells in the testis and increase the spermatogenic output of the testis (Simorangkir *et al.*, 1995). These data indicate that, although FSH concentrations are low, the Sertoli cells are still capable of responding to FSH stimulation.

The extended period of proliferation that results from the induction of neonatal hypothyroidism also disrupts the normal spermatogenic process during this time. Our data indicate that there is a marked delay in germ cell maturation (Figure 3) and a large proportion of the germ cells do not progress beyond the primary spermatocyte stage (Simorangkir *et al.*, 1997).

It is clear that many of the interactions between the Sertoli cell and the germ cells are of a paracrine nature (de Kretser, 1987), as indicated above by the role of activin, which is produced by the Sertoli cell, in the maintenance of mitochondrial morphology in spermatocytes (A.Meinhardt, M.K.O'Bryan and J.R.McFarlane *et al.*, unpublished data). There is an extensive amount of literature indicating the dependence of the germ cells on Sertoli cell function, including documentation that the germ cells are dependent on the production of lactate by the Sertoli cells (see review by Jegou and Sharpe, 1993). Further studies by Sylvester and Griswold (1993) showed that the transport of iron within the intratubular environment is dependent on Sertoli cell transferrin production. In turn, the capacity of the germ cells to modulate Sertoli cell activity and biochemical activity is well established, although the ligands involved in these regulatory processes remain unknown. These comments are necessary to emphasize that it is not only Sertoli cell number, but also the function of the Sertoli cell, which is crucial for the successful development and completion of spermatogenesis. To date, no specific tests of Sertoli cell function are available that can be evaluated by measurements using samples of peripheral blood. The development of such techniques would greatly enhance our understanding

of the function of Sertoli cells and their importance in the spermatogenic process.

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