Germ cell–somatic cell interactions during spermatogenesis

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The adult testis has two essential functions, namely the synthesis and secretion of the steroid hormone testosterone and the production of mature spermatozoa. Germ cells undergo mitosis, meiosis and condensation (spermiation) in close association with Sertoli cells and in defined associations with each other within the seminiferous epithelium. Normal spermatogenesis and fertility are dependent upon paracrine interactions between the somatic cells (Sertoli, Leydig and peritubular) and the germ cells, and upon endocrine support from the pituitary gland. Evidence for paracrine interactions between somatic and germ cells has been gained from observations on the patterns of expression of proteins, studies on isolated cell populations, germ cell or Leydig cell ablation with toxicants, mouse mutants and transgenics, and more recently from germ cell transplantation.

Introduction

The process of spermatogenesis involves maturation of diploid spermatogonia into mature haploid spermatozoa and takes place within the seminiferous tubules of the testis. All the germ cells develop within the tubules in close association with Sertoli cells. The most compelling evidence for the importance of the complex cellular associations within the testis in maintaining male fertility is provided by our current inability to recapitulate the process *in vitro*. The purpose of this review is to consider briefly the cellular architecture of the testis as well as some of the evidence relating to the paracrine and endocrine influences and cell–cell interactions that contribute to normal somatic cell and germ cell function. New insights are being gained from studies using germ cell transplantation.

Organization of spermatogenesis

The organization of spermatogenesis within the seminiferous epithelium is essentially the same in all mammals and historically was characterized by histological observations that described defined germ cell associations occupying the circumference of the tubule cross-section (Leblond and Clermont, 1952). These characteristic germ cell associations occupy time and space within the seminiferous epithelium and have been categorized into specific 'stages' (for a review, see Sharpe, 1994). The number of stages identified varies and is a characteristic

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**Table 1.** Organization and duration of spermatogenesis in selected species

Species	Number of stages	Duration of spermatogenesis (days)	Reference
Ram[a]	6 or 8?	49	Hochereau et al. (1964) Wrobel et al. (1995)
Goat	8	48	Franca et al. (1999)
Bull	12	54	Wrobel et al. (1996)
Water buffalo	6?	?	Wrobel and Pawar (1992)
Rat	14	52	Clermont and Harvey (1965)

[a]The ram shows seasonal variation in the efficiency of spermatogenesis (Hochereau-de Reviers et al., 1985).

of that species (Table 1). For example, rats have 14 stages (Leblond and Clermont, 1952); rams have eight stages according to some reports (Hochereau et al., 1964) and six according to others (Wrobel et al., 1995); and bulls have 12 stages (Berndston and Desjardins, 1974). The spermatogenic 'cycle' is the sum of all the stages and the duration of the cycle is the time taken for a single germ cell to pass once through each of the stages; this also varies between species (Table 1). In all ruminants, the stages are arranged sequentially along the length of the seminiferous tubule; as a result, in any tubule cross-section only a single stage, defined by the germ cell associations within it, will be observed (for a review, see Sharpe, 1994). High power views of cross-sections through the seminiferous epithelium of the adult ram (Fig. 1a) and rat (Fig. 1b) are shown. The bulk of the cell nuclei present within the sections are those of the germ cells at different stages of maturation, with the most mature germ cells closest to the luminal surface. Sertoli cell nuclei are located close to the basement membrane. The cytoplasm of the Sertoli cells was immunostained with an antibody directed against sulphated glycoprotein 1 (Fig. 1b), a sphingolipid binding protein, (SGP-1; Collard et al., 1988); fine tracts of Sertoli cell cytoplasm can been seen extending through the whole depth of the epithelium between the different types of germ cell (arrowheads). The outer surface of the tubules is enclosed by a layer of contractile cells known as peritubular myoid cells; cells within the interstitium include the steroidogenically active Leydig cells, cells lining blood vessels, Leydig cell precursors and macrophages (Skinner, 1991).

Germ cell maturation

The testis develops from somatic cells within the genital ridge and germ cells that migrate into this region from the fetal extraembryonic membranes. Differentiation of the somatic cells is dependent upon expression of the testis determining gene *SRY* (sex-determining region of the Y chromosome) (Swain and Lovell-Badge, 1999). Initiation of testis cord formation and detectable expression of anti-Mullerian hormone has been reported at day 42 of pregnancy in the testes of bovine fetuses (Vigier et al., 1983) and day 30 in testes of ovine fetuses (Sweeney et al., 1997). The fetal germ cells are first seen as a 'primordial' alkaline phosphatase-positive population (PGC) in the allantois and undergo migration to reach the genital area where they become associated with Sertoli cells to form testis cords. Once within the testis cords, the cells differentiate to form gonocytes (also called prespermatogonia), which initially proliferate, but then become quiescent (Wrobel, 2000). Mutations in a few genes affect the proliferation and migration of the phosphatase-positive population. For example, the c-kit receptor (see below) is expressed in the phosphatase-positive population, and mice that do not express a functional c-kit receptor have fewer germ cells due to reduced survival of migratory primordial germ cells (Besmer et al., 1993).
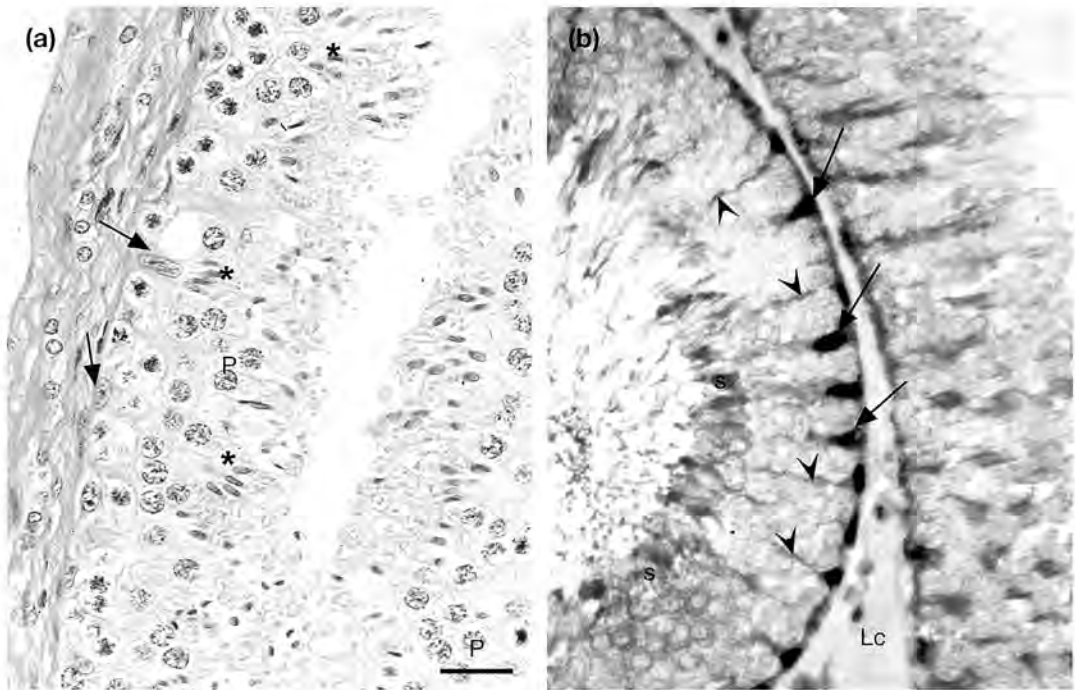
Fig. 1. Organization of the germ cells and Sertoli cells within the seminiferous epithelium. (a) Adult ram testis stained with haematoxylin and eosin. Note that the majority of nuclei are those of germ cells and that Sertoli cell nuclei are located close to the basement membrane (arrows). Multiple developmental stages of germ cells including large pachytene spermatocytes (P) and groups of elongating spermatids (*). (b) Adult rat testis immunostained for androgen receptor (AR, black nuclei, arrows) and sulphated glycoprotein 1 (SGP-1, red staining, cytoplasmic). Note that the cytoplasm of the Sertoli cell (arrowheads) extends from the base to the lumen of the seminiferous epithelium between the germ cells. s: spermatozoa surrounding the lumen of the seminiferous tubule; Lc: adult Leydig cells in interstitium, AR positive. Scale bar represents 50 μm.

In adult mammals, the maturation of germ cells is usually subdivided for convenience into a number of phases that include a spermatogonial–replicative phase, a meiotic phase and spermiogenesis (formation of the acrosome, nuclear condensation and cytoplasmic elimination) culminating in the release of the mature spermatozoa (spermiation) (summarized in Sharpe, 1994).

Spermatogonial stem cells form a self renewing population within the testis which can differentiate to form spermatogonial subtypes that become committed to the process of maturation (De Rooij, 2001). The number of times that the spermatogonial populations divide is different between species: in mice, 9–11 mitotic divisions occur resulting in significant amplification of the numbers of spermatogonia (De Rooij, 2001). In cows, the spermatogonial cells can be divided into three populations and, as in other species, strings of spermatogonia connected by cytoplasmic bridges can be identified in whole mount preparations (Wrobel, 2000). One way in which proliferating cells have been identified within the seminiferous epithelium is by immunostaining for proliferating cell nuclear antigen (PCNA). PCNA is expressed by cells in G1, S and G2M phases of the cell cycle (Wrobel *et al.*, 1996). Immunostaining for PCNA can be used to highlight the distribution of proliferating spermatogonia and the meiotic phase pre-leptotene spermatocytes (see below) (Wrobel, 2000; Wrobel *et al.*, 1996). In rats
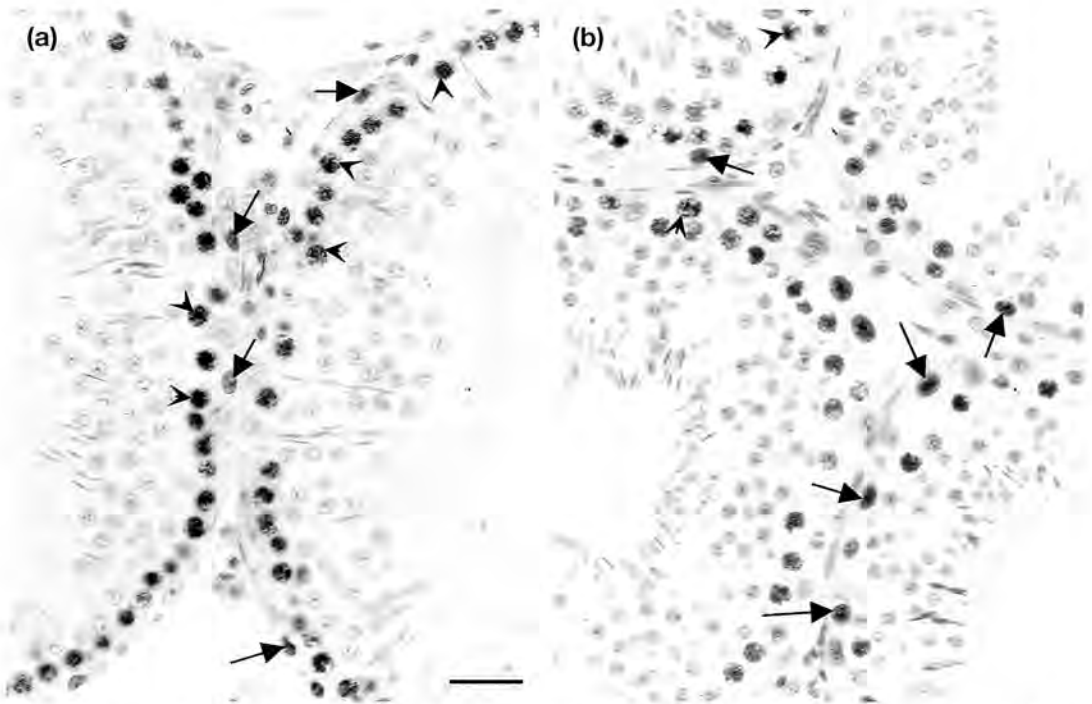
Fig. 2. Immunolocalization of proliferating cell nuclear antigen to germ cells within the seminiferous epithelium. (a) Adult rat testis and (b) adult ram testis. Note that immunopositive staining is found within the cell nuclei of spermatogonia (arrows) as well as more mature spermatocytes (arrowheads). In both species each cross-section is occupied by a single stage of spermatogenesis. Sertoli cell nuclei are not stained as these cells stop dividing as the first wave of germ cells develops. The scale bar represents 50 μm.

and rams (Fig. 2a,b), immunopositive cells at the same stage of development always occupy the entire circumference of the seminiferous epithelium, which is consistent with the 'segmental' organization of spermatogenesis. A different arrangement known as 'helical' occurs in some but not all primates, and in man (Sharpe, 1994).

The final mitotic division of the B type spermatogonia (De Rooij, 2001) gives rise to cells that enter a lengthy meiotic prophase as pre-leptotene spermatocytes. Thereafter, spermatocytes progress through leptotene, zygotene and pachytene phases of meiosis (all the spermatocytes are named according to the phase of meiosis through which they are progressing). At the end of meiotic prophase, spermatocytes undergo the final meiotic divisions to form haploid spermatids.

Thereafter, the spermatids embark on the process of spermiogenesis during which the nucleus of the germ cell is remodelled and compacted into the form found in the mature spermatozoa. The net result of DNA compaction during spermiogenesis is that sperm DNA occupies only 5% of the space of that seen in somatic cells (Ward and Coffey, 1991). Facilitation of this process is achieved in the following way. Somatic histones are replaced sequentially by testis-specific isoforms, transition proteins TP1 (Kremling et al., 1989) and TP2, and finally, by small, highly basic protamines two genes of which have been identified in most species. The importance of the timed expression of protamines and their incorporation into the mature spermatozoon is highlighted by studies in mice in which targeted disruption

of protamines 1 and 2 has shown that a decrease in either protamine disrupts formation of a normal spermatozoa (Cho *et al.*, 2001). The final stage of the process is spermiation when the mature spermatozoa are released from the Sertoli cells into the lumen of the seminiferous epithelium. This is a complex process involving a progressive loss of adhesive junctions and is poorly understood.

Endocrine support for spermatogenesis

Testicular function and normal fertility are dependent upon endocrine (extratesticular) and paracrine (intratesticular) factors. The primary endocrine hormones are the gonadotrophins LH and FSH which are synthesized and secreted by the pituitary gland. Gonadotrophin receptors are expressed only by somatic cells within the testis; the LH receptors are present on Leydig cells and the FSH receptors on Sertoli cells. Synthesis of testosterone by adult Leydig cells is stimulated by LH (Moyle and Ramachandran, 1973). Several studies have demonstrated that loss of pituitary hormones results in loss of germ cells (for a review, see McLachlan *et al.*, 2002) and the hypogonadal mutant mice (*hpg*) which has only very low endogenous circulating concentrations of gonadotrophins (Cattanach *et al.*, 1977) are completely infertile. For example, in hypophysectomized adult rats, germ cell loss occurs first at day 6 after surgery and is associated with a reduction in the diameter of the seminiferous tubules (Ghosh *et al.*, 1992). Some male ruminants, including rams and stags, show a cyclical variation in the efficiency of spermatogenesis that is driven by changes in the stimulation of the testis by gonadotrophins (Blottner *et al.*, 1996; Lincoln *et al.*, 1990). In these species, stimulation of testosterone synthesis from the Leydig cells caused by increases in frequency, amplitude and duration of LH release from the pituitary gland (Lincoln, 1976) results in development of androgen stimulated secondary sexual characteristics.

Separating the roles played by FSH and LH in spermatogenesis has proved challenging and has been confounded by problems with interpretation of cellular changes in some *in vivo* models and concerns over the purity of non-recombinant proteins. Studies on mice with targeted ablation of gonadotrophins, or their receptors, have allowed new and detailed examination of the contributions of LH and FSH to spermatogenesis (Krishnamurthy *et al.*, 2000; Allan *et al.*, 2001). Briefly, the amount of gonadal development in LH receptor knockout mice (LuRKO) seen at birth was similar to that of control mice, but in adulthood these mice had poorly developed seminiferous tubules with sporadic germ cells up to and including round spermatids (Zhang *et al.*, 2001). Leydig cells were poorly differentiated and it is assumed that the limited germ cell development was stimulated by FSH (Zhang *et al.*, 2001). Young male FSH receptor knock-out mice (FORKO; Krishnamurthy *et al.*, 2000) have underdeveloped testis and a 50% reduction in the number of Sertoli cells, consistent with previous observations that FSH stimulates Sertoli cell replication early in life. The FORKO mice experience delayed puberty with postponement in the formation of round spermatids and, surprisingly, adult males also have reduced serum testosterone concentrations despite normal circulating LH concentrations, indicating disturbances in Sertoli–Leydig cell communication. Adult FORKO males have reduced fertility and aberrant spermatozoa with inadequate DNA compaction (Krishnamurthy *et al.*, 2000). Allan *et al.* (2001) developed a new model to examine the impact of FSH on the process of spermatogenesis by combining transgenic expression of FSH in the gonadotrophin deficient background of the *hpg* mouse. The testis size of *hpg* mice with high concentrations of FSH was increased and the testis contained round spermatids and a few elongated spermatids. These results demonstrate that in the absence of testosterone, FSH simulation of Sertoli cells can facilitate completion of meiosis and initiation, but not completion, of spermiogenesis.

Germ cell–soma interactions in adulthood

The number of germ cells that are supported by an individual Sertoli cell varies between species and contributes to the efficiency of the process of spermatogenesis, that is, the number of mature elongate spermatids per Sertoli cell (Sharpe, 1994). Paracrine regulation of spermatogenesis involves interaction between all the major types of cell within the testis (Skinner, 1991) and a few of these are highlighted below.

Leydig cell products

One of the most important interactions is between Leydig cells and Sertoli cells; this has been demonstrated most elegantly in rats by selective ablation of Leydig cells after treatment with the toxicant ethane dimethane sulphonate (Kerr et al., 1985). Removal of Leydig cells by ethane dimethane sulphonate results in degeneration of germ cells in a stage-dependent manner over time (discussed in detail in Sharpe, 1994). The main secretory product of the Leydig cell is testosterone, and studies have shown that the concentrations of testosterone within the testis far exceed concentrations within the peripheral circulation (Sharpe et al., 1988; Sharpe, 1994). Several studies have examined how testosterone might influence germ cell development and survival. Notably, studies using immunohistochemistry have established that the androgen receptors which bind testosterone are expressed in Sertoli cell and peritubular cell nuclei but are not present in germ cells (Fig. 1b) (Saunders et al., 1996; Goyal et al., 1997). This finding has led to a number of studies to establish which Sertoli cell products could be modulated by androgens as these might be key to our understanding of the ways in which the Sertoli cell passes on the androgenic signal coming from the Leydig cells to the germ cells. New data from gene array experiments indicate that testosterone may downregulate twice as many genes as it upregulates within the Sertoli cell (Griswold, 2002), but to date, it is not known how testosterone influences germ cell function.

Leydig cells also contain aromatase cytochrome P450 (Turner et al., 2002), the enzyme that is required for conversion of androgens to oestrogens. Low expression of aromatase has also been detected in round and elongating spermatids and in spermatozoa (Turner et al., 2002). In mice that are unable to synthesize oestrogens due to targeted ablation of aromatase P450, round spermatids undergo apoptosis and, thus, fail to differentiate into mature, elongated spermatids; this lesion appears to arise at between 18 weeks and 1 year of age (Robertson et al., 1999). Two structurally related subtypes of oestrogen receptor (ER), commonly known as α (ERα, NR3A1) and β (ERβ, NR3A2) have been identified in mammals (Nilsson et al., 2001). Differences in the pattern of expression of ERα in the testes occurs between species with detectable ERα protein within Leydig cells of rodents (Fisher et al., 1997), but no ERα in any type of cell in the testes of humans, primates (Saunders et al., 2001) and goats (Goyal et al., 1997). ERβ protein has been immunolocalized to the nuclei of Sertoli cells, Leydig cells and peritubular myoid cells as well as some, but not all, germ cells (Saunders et al., 2001). The presence of ERβ within germ cells raises the possibility that oestrogens could have a direct effect on germ cell function and some support for this idea has come from investigations showing that oestrogen may act as a germ cell survival factor (Pentikainen et al., 2000); germ cell maturation has been induced in hpg mice treated with oestrogens (Ebling et al., 2000). Studies on mice have shown that the loss of fertility in males with targeted disruption of the ERα gene is due to a defect in fluid resorption (Eddy et al., 1996) and is not associated with direct effects on germ cells. Mice in which the functional integrity of the ERβ gene has been disrupted (Dupont et al., 2000) are fertile and, although the fertility of male mice lacking both ERα and ERβ (Dupont et al., 2000) is compromised, their phenotype appears to resemble that

of the ERαKO. Further studies are required to determine whether oestrogens are paracrine, autocrine (or paracrine and autocrine) regulators of germ cell function.

Sertoli cell products

Secretory products of Sertoli cells have been identified by many groups. One deficiency of some of the studies is that they have been conducted on cells isolated from immature rodents that have never been exposed to mature germ cells *in vivo*. A comprehensive list of the factors synthesized by the Sertoli cell is beyond the scope of this review and, therefore, a few examples have been selected to illustrate specific types of interaction and are discussed below. Additional information can be found in reviews by Skinner (1991, 1993), Sharpe (1994) and Griswold (1995).

Sertoli cells synthesize and secrete inhibins, activins and follistatins (Roberts *et al.*, 1989; de Kretser *et al.*, 2001). Inhibin is a glycoprotein that can exist as two heterodimeric forms consisting of an α chain and one of two types of β chain; activins are formed as dimers of the two β chains (de Kretser *et al.*, 2001). Inhibin acts as an endocrine hormone and was originally identified on the basis of its ability to act as a negative regulator of FSH secretion by the pituitary gland. After castration, concentrations of inhibin in the peripheral circulation decrease and those of FSH increase (Ishida *et al.*, 1990). In most species examined so far there is a positive relationship between concentrations of inhibin B produced by the testis and the number of Sertoli cells, and secretion of inhibin by immature Sertoli cells *in vitro* can be stimulated by FSH. Other data show that adult Leydig cells can also produce inhibin and that Leydig cell products may play a role in modulating expression of the inhibin α subunit by Sertoli cells (for a review, see de Kretser *et al.*, 2001). There are conflicting data on the cellular sites of activin subunits but bioassay data support the production of activin A by Sertoli cells (de Winter *et al.*, 1993). Follistatins act as activin-binding proteins and a role for activin supporting germ cell maturation can be inferred from the observation that overexpression of follistatins in mice results in spermatogenic arrest (Guo *et al.*, 1998). Activin has been proposed to have paracrine effects on germ cell maturation at several points including transformation of gonocytes into spermatogonia, stimulation of spermatogonial proliferation and maintenance of mitochrondrial morphology of germ cells beyond the pre-leptotene stage (for a review, see de Kretser *et al.*, 2001).

One of the most well researched aspects of germ cell–Sertoli cell interaction is that of c-kit–kit ligand. In species investigated so far, including rodents, primates and ruminants, c-kit receptors are expressed on adult germ cells (type A spermatogonia up to pachytene spermatocytes) (Loveland and Schlatt, 1997; Roelants *et al.*, 2002). Conversely, the ligand for the receptor, which is known as kit ligand, stem cell factor or Steel factor, is synthesized by the Sertoli cells in both a membrane-bound and soluble form (Besmer *et al.*, 1993). Evidence from studies *in vivo* and *in vitro* indicates that c-kit–kit ligand expression may be important for regulation of spermatogonial cell proliferation and differentiation (De Rooij, 2001) and adhesive interactions between germ cells and Sertoli cells. Studies in which the glial cell-derived neurotrophic factor (GDNF) has been under- or overexpressed in mice have concluded that GDNF, a product of Sertoli cells, plays a role in regulation of self renewal of spermatogonial stem cells; mice with targeted overexpression of GDNF develop testicular germ cell tumours (Meng *et al.*, 2001).

The importance of the structural support provided by the Sertoli cells is often overlooked. The development of basally located tight junctions between Sertoli cells at puberty is essential for the formation of a selective permeability barrier known as the 'blood–testis barrier' which allows the development of a specialized luminal environment within the seminiferous

epithelium (Dym and Fawcett, 1970). Our understanding of the molecular basis of the physical interaction between germ cells and Sertoli cells was advanced recently by studies on mice containing a targeted ablation for the enzyme encoding alpha-mannosidase IIx (Akama *et al.*, 2002). In these mice, germ cells failed to adhere to the Sertoli cells correctly and were released prematurely, and as a result most of the mice were infertile (Akama *et al.*, 2002).

Germ cell products

Studies *in vivo* on rats in which specific germ cell populations are removed by toxicants, such as methoxyacetic acid (Bartlett *et al.*, 1988) have demonstrated that the complement of germ cells in individual seminiferous tubules can modulate Sertoli cell protein synthesis and production of seminiferous tubule fluid (McKinnell and Sharpe, 1992). Specific Sertoli cell proteins regulated by the presence of selected germ cells *in vivo* include inhibin (Allenby *et al.*, 1991), transferrin (Maguire *et al.*, 1997) and cyclic protein 2 (Maguire *et al.*, 1993). The molecules regulating this germ cell–Sertoli cell interaction have yet to be identified.

Germ cell transplantation

Spermatogonial stem cell transplantation is a novel method, originally developed by Ralph Brinster and colleagues, that involves the transfer of mixed or purified populations of germ cells from the testis of a donor animal into that of a recipient (Brinster and Zimmermann, 1994). Several groups have demonstrated that the spermatogonial stem cells from the donor testis can repopulate the testis of germ cell deprived recipients and re-initiate the process of spermatogenesis (for a review, see McLean *et al.*, 2001). For example, using reciprocal transplantation of germ cells from the Steel (kit ligand deficient) and W (c-kit deficient) mice, investigators have shown that kit ligand is essential for maintenance of differentiated spermatogonia and that transplantation of spermatogonial stem cells from an infertile Steel donor to a kit ligand positive testicular environment can restore fertility and result in progeny with the genetic makeup of the infertile donor male (Ogawa *et al.*, 2000; Ohta *et al.*, 2000). One of the most remarkable findings reported using this technique involved transplantation of rat germ cells into the testis of an immunosuppressed mouse. Not only were mature rat spermatozoa produced but also within the testis the rat germ cells developed with timings characteristic of rat spermatogenesis (52 days); in the same testis mouse germ cells developed in 35 days (Franca *et al.*, 1998). These findings show both the remarkable ability of the Sertoli cell to function to support different germ cells and argue against a role for the Sertoli cell in determining the timing of germ cell development. Other xenograft transplantations (dog, hamster and primate) have been less successful (Dobrinski *et al.*, 1999; Nagano *et al.*, 2001). Transplantation has also been attempted in bull, monkey and human testis with limited success (Schlatt *et al.*, 1999).

Conclusions

The complexity of the cellular architecture of the mammalian testis has made it difficult to determine the specific paracrine, autocrine and endocrine interactions that are essential for the maintenance of normal fertility. Most of the progress over the last few years has come from studies in rodent model systems including the use of selective cell ablation, hormonal manipulation, transgenic knockouts and more recently germ cell transplantation. Studies on species other than rodents have been less comprehensive and continue to present new challenges.

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