Efforts to create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment


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Induction of meiotic and post-meiotic alterations of male germ cells in vitro has been the target of several research efforts since 1960. However, to date, the establishment of an ideal culture system in which spermatogonial stem cells can be maintained and directed to proliferate and undergo meiosis and complete spermiogenesis does not exist. This is attributed to the difficulties concerning the isolation and purification of defined subpopulations of germ cells and the establishment of male germ cell lines. In addition, there is no adequate knowledge regarding the optimal biochemical conditions that promote the survival and differentiation of germ cells in long-term cultures. This review focuses on the methodologies that have been proved sufficient to achieve differentiation of cultured male germ cells. Furthermore, the factors regulating spermatogenesis and the technical prerequisites to achieve differentiation of cultured male germ cells are described. Finally, the role of in vitro cultures of immature diploid germ cells in the therapeutic management of men negative for haploid cells in their testes and the subsequent potential genetic and epigenetic risks are discussed.

Key words: artificial testis/in vitro culture system/meiotic maturation/spermatogonial stem cell

Regulation of spermatogenesis: what we have learned from in vitro cultures of spermatogenic cells

The role of the Sertoli cell in the regulation of male germ cell proliferation/differentiation and the apoptotic mechanisms of germ cells

In mammalian embryos, gonads develop in the bilateral gonadal ridges from the interaction of primordial germ cells (PGCs) with local somatic cells represented by two different populations: mesenchymal cells of the mesonephric region and epithelial cells of the overlying coelomic epithelium (Kierszenbaum, 1994).

In mouse, about eight PGCs depart from their site of origin, the yolk sac, and translocate to the epithelium of the hindgut adjacent to the yolk sac. PGCs leave the hindgut by active mechanism to enter the dorsal mesentery and finally settle in the gonadal ridge. Soon after reaching the gonadal ridges, PGCs increase in number and begin their cell–cell interaction with coelomic epithelial and mesenchymal cells to organize testicular cords. Male PGCs become gonocytes, the cell precursor of spermatogonia and enter a mitotic arrest stage (Kierszenbaum, 1994).

Spermatogenesis is a cyclic process, which can be divided into 12 stages (I–XII) in mice (Kierszenbaum, 1994). In stage VIII, As, Apr and a few Aal spermatogonia are present. From stage X onwards, these cells start to proliferate in such a way that the numbers of As and Apr spermatogonia remain relatively constant and more Aal spermatogonia are formed. At about stages II–III (stage XII is followed by stage I), proliferation stops and the cells become arrested in G1–G0 phase. Subsequently, in stages VII–VIII, without division, nearly all Aal spermatogonia formed during the period of active proliferation differentiate into A1 spermatogonia. The A1 spermatogonia enter S phase and in stage IX, divide into A2 spermatogonia,
after which there are five subsequent divisions into A3, A4, In and B spermatogonia and primary spermatocytes, respectively. In total, there are 9–11 mitotic divisions during spermatogonial development. When the numbers of A4, In and B spermatogonia are low, the proliferation period is extended to stage VII. There appears to be a feedback mechanism between A4, In and B spermatogonia and the As, Apr and Aal spermatogonia lying in between these cells. When the numbers of A4–B spermatogonia are about 50% lower than in the normal testis, the proliferation activity of the As, Apr and Aal spermatogonia continues beyond the stage II. Whether the spermatogonial stem cell (SSC) divisions are symmetrical or asymmetrical is a subject of debate. Symmetrical divisions imply divisions by either producing two stem cells or two interconnected cells destined to differentiate (Apr). Another possibility is that SSCs divide asymmetrically into a stem cell and a cell destined to produce Apr spermatogonia and, therefore, not all As spermatogonia are true stem cells. Two differentiation steps appear to occur in the developmental path of spermatogonia. First, there is the step from the As spermatogonia to the Apr spermatogonia. From then on, the germ cells consist of clones of interconnected cells of increasing size, as from Apr onwards all divisions are such that the daughter cells remain connected by bridges. The second differentiation step is that from Aal to A1 spermatogonia and this step brings about a marked change in cell behaviour.

It should be noted that in rat, type A1–A4 spermatogonia are the renewing stem cells and mitosis of these cells constitutes the major phase of germ cell proliferation. The last division of the renewing stem cells (A4) in rat results in the first of two generations of differentiated spermatogonia [intermediate (1N) and B]. In monkey, Ap spermatogonia are the renewing stem cells and the major phase of germ cell proliferation results from mitosis of differentiated type B1–B4 spermatogonia (Plant and Marshall, 2001). The reserve stem cells in primates and rats rarely divide. There appear to be fundamental differences between monkey and rat in the mechanism used for stem cell renewal and germ cell proliferation. Stem cell renewal in rat involves sequential divisions to produce four generations of undifferentiated spermatogonia (A1, A2, A3 and A4) and therefore concomitantly contributes to germ cell proliferation (Kierszenbaum, 1994). On the other hand, in monkey stem cell renewal follows a simpler pattern involving only one type of undifferentiated spermatogonia (type Ap) and thus contributes to proliferation only indirectly by the production of the first of the four generations of differentiated B spermatogonia (Kierszenbaum, 1994).

As mentioned above, several studies have assumed that SSC divisions are symmetrical with divisions by either producing two new stem cells or two interconnected cells destined to differentiate (Apr). Another probability is that stem cells divide asymmetrically into a stem cell and a cell destined to produce Apr spermatogonia. No definite answer can be given yet to the question of whether rodent stem cell divisions are symmetrical or asymmetrical. In Drosophila testes, germ line stem cells normally divide asymmetrically, giving rise to one stem cell and one gonialblast which initiates differentiation starting with the spermatogonial transient-amplifying divisions; Yamashita et al., 2003). The hub, a cluster of somatic cells at the testis apical tip, functions as a stem cell niche: apical hub cells express the signalling ligand unpaired, which activates the Janus kinase-signal transducers and activators of transcription (JAK-STAT) pathway within germ line stem cells to maintain stem cell identity.

Yamashita et al. (2003) have shown that the dividing Drosophila male germ line stem cells use intracellular mechanisms involving centrosome function and cortically localized adenomatous polyposis coli tumour suppressor protein to orient mitotic spindles perpendicular to the niche, ensuring a reliably asymmetric outcome in which one daughter cell remains in the niche and self-renews stem cell identity, whereas the other, displaced away, initiates differentiation. According to Yamashita et al. (2003), the orientation of stem cells towards the niche appears to play a critical role in the mechanism that ensures a reliably asymmetric outcome of Drosophila male germ line stem cellular divisions consistently placing one daughter within the reach of short-range signals from the hub and positioning the other away from the niche. Oriented stem cell division may be a general feature of other stem cell systems, helping to maintain the correct balance between stem cell self-renewal and initiation of differentiation throughout the adult life.

The development of differential display RT–PCR procedures has led to the identification of many genes that are differentially regulated in various cell and tissue types. The above technique to identify genes that are expressed in isolated mouse testicular type A spermatogonia and in more advanced germ cells. The authors identified cDNA fragments for mDEAH9, RanBP5, GC3, GC12 and GC14 genes in the testis and type A spermatogonia from wild-type mice, but not in samples from mutant W/Wv mouse testis. RT–PCR analyses of isolated spermatogonia, pachytene spermatocytes and round spermatids revealed that mDEAH9, RanBP5, GC3, GC12 and GC14 genes were expressed in all three cellular populations. RanBP5 expression appeared to be regulated during the cycle of the seminiferous epithelium with the highest expression in stages III–VII. Expression of GC14 was greatest in the meiotic germ cellular subpopulations. In addition, it was identified that a murine testis cDNA encoding a homologue to human A-kinase anchoring protein-associated sperm protein (ASP). Northern blot and RT–PCR analyses did not detect ASP mRNA in mouse spleen, brain, liver, lung, heart, kidney, skeletal muscle, ovary or Sertoli cells. On the other hand, the above techniques localized ASP mRNA to the germ cell compartment of the seminiferous tubules in the testis.

Mouse models with reproductive defects as a major phenotype have been created and now number over 200 (Matzuk and Lamb, 2002). These models are helping to define mechanisms of reproductive function, as well as to identify potential new genes involved in the pathophysiology of reproductive disorders. Mouse models for the study of reproductive defects have been produced by spontaneous mutations, transgene integrations, retroviral infection of embryonic stem cells, ethynitrosurea mutagenesis and gene targeting technology. Several genes required for vertebrate fertility are highly conserved in evolution with orthologues in Drosophila melanogaster (i.e. DX4), fat facets (DFFRY) and boule (DAZ). Defects in sexual differentiation pathways can cause infertility in mice and humans of both sexes. It has been pointed out by Matzuk and Lamb (2002) that several gene defects or gene-related pathophysiologies leading to defects in sex determination or development (i.e. pseudohermatidism, sex reversal, Denys–Drash syndrome,
pseudovaginal perineoscrotal hypospadias, cryptorchidism or congenital bilateral absence of vas deferens), defects in sperm production and function (i.e. myotonic dystrophy, Noonan syndrome, sickle cell anaemia, b-thalassaemia, Kartagener syndrome, primary ciliary dyskinesia, Fanconi anaemia or ataxia telangiectasia), endocrinopathies and numerical/structural chromosomal abnormalities result in human male infertility. Knockout animal models have provided strong evidence supporting the genetic basis of human male infertility in subpopulations of infertile men.

Factors secreted by Sertoli cells

Sertoli cells are the only somatic cells in the seminiferous tubuli. The essential role of Sertoli cells in testicular function is stressed by the fact that (a) ‘germ cell-only testes’ have never been observed, (b) the great majority of in vitro culture systems for successful differentiation of germ cells requires the presence of Sertoli cells and (c) the number of germ cells sustained by the testes is directly related to the population of Sertoli cells (Griswold, 1998). Sertoli cells limit the expansion of the spermatogonial population, with each Sertoli cell supporting a defined number of germ cells. Sertoli cells form niches for germ cells; these niches allow a certain number of germ cells to reside in or repopulate the seminiferous tubules (Meachem et al., 2001). Moreover, in experiments using animal models in which the size of the testis and/or the spermatogenic output was manipulated by changing the number of Sertoli cells, there was a relatively constant ratio between Sertoli cells and spermatids before and after the manipulation (Orth et al., 1988; Hess et al., 1993; Simorangkir et al., 1995).

Sertoli cells provide critical factors necessary for the successful progression of spermatogenesis into spermatozoa. According to Griswold (1998), glycoproteins secreted by Sertoli cells important for spermatogenesis could be divided into three categories: (a) those that facilitate the transport of ions and hormones or provide bioprotective functions, such as androgen-binding protein (ABP), transferrin and ceruloplasmin, (b) proteases and proteases inhibitors (these proteins have a role in tissue remodeling processes that occur, for instance, during spermiation or movement of preleptotene spermatocytes into the adluminal compartment of the seminiferous tubule) and (c) structural components of the basement membrane between the Sertoli cells and the peritubular cells. A specific transferrin system has evolved to ensure that the tight Sertoli cell junctions are circumvented so that the germ cells can receive a supply of ferritin by Sertoli cells. Recently, vasoactive peptides and tachykinins have been localized in Sertoli cells (Debeljuk et al., 2003). Tachykinins have been shown to stimulate the release of lactate and transferrin by Sertoli cells in vitro and also to stimulate aromatase activity by Sertoli cells (Debeljuk et al., 2003).

Furthermore, the Sertoli cells secrete other glycoproteins that function as growth factors or paracrine factors, such as the Mullerian duct inhibiting substance, c-kit ligand (stem cell factor; SCF), inhibin and glial cell line-derived neurotrophic factor (GDNF). Both survival and proliferation of spermatogonia were found to be stimulated by the administration of SCF (Allard et al., 1996). At the beginning of spermatogenesis there is a dramatic shift in the production (by Sertoli cells) of soluble SCF to membrane-bound SCF (Blanchard et al., 1998), suggesting an important role for the SCF–c-kit system in spermatogonial differentiation. c-kit-receptor and SCF also mediate Sertoli cell–spermatocytes cellular adhesions. This is confirmed by studies indicating that Sertoli cells from mice mutant for the membrane-bound SCF are unable to bind spermatocytes (Marziali et al., 1993). Other molecules, the cadherins, secreted by Sertoli cells also have an important role in the maintenance of the structure of testicular tissue and cell architecture and identity. Expression of mRNA coding for the three ‘classical’ cadherins, E-,- P- and N-cadherin, has been demonstrated in the developing rat testis (Wu et al., 1993).

The bradykinin-B2-receptor (B2-r) has been demonstrated to be present on rat Sertoli cells (Monsees et al., 1996, 1999, 2002, 2003). In situ hybridization (ISH) and immunohistochemistry revealed that B2-r mRNA and protein are solely present on specific cells within the testis. B2-r is located on endothelial cells of blood vessels, Sertoli cells, pachytene spermatocytes and in round and elongated spermatids. Moreover, in the sexually mature rat the expression of B2-r mRNA and protein are dependent on the stage of the spermatogenic cycle (Monsees et al., 2003). This stage-dependent expression of the protease (tissue kallikrein) and the B2-r together with their locations on certain germ cells suggests a potential function of the tissue kallikrein system as a local factor with an effect on spermatogenesis. In organ cultures of immature rat testis a significant stimulation of prespermatogonial cell proliferation after exposure to bradykinin has been observed (Atanassova et al., 1998).

Sertoli cells have receptors for FSH and testosterone, which are the main hormonal regulators of spermatogenesis. Mutations of the FSH receptor have been associated with variably severe reduction in sperm count, but fertility has been maintained (Tapanainen et al., 1997). FSH alone or in synergy with testosterone has been shown to prevent germ cell loss or to restore spermatogenesis quantitatively in hypophysectomized animals (Marshall et al., 1995). In vitro studies in human testicular tissue material demonstrate the role of FSH and testosterone in the prevention of germ cell apoptosis (Erkkila et al., 1997; Tesarik et al., 2001), suggesting that both hormones act as germ cell survival factors. The role of Sertoli cells in the regulation of the apoptotic mechanisms of germ cells is confirmed by the fact that the expression of FasL in the testis is mainly localized in Sertoli cells (Lee et al., 1997).

Support of xenogeneic germ cell maturation in vivo

Experiments have demonstrated that donor spermatogonia transplanted into the seminiferous tubules of syngeneic animals form cell associations with the recipient Sertoli cells that are characteristic of the normal recipient testis (Brinster and Zimmermann, 1994; Russell et al., 1996; Sofikitis et al., 2003). A subpopulation of donor spermatogonia injected into the lumen of recipient seminiferous tubules moves towards the basal compartment of the testis, implying that the adluminal surface of the recipient Sertoli cells can recognize the donor spermatogonia and direct them to their normal location in the basal compartment. Moreover, successful experiments of xenogeneic germ cell transplantation (Clouthier et al., 1996; Russell and Brinster, 1996; Sofikitis et al., 2003) provide strong evidence that the information necessary for the differentiation of male germ cells (i.e. to complete meiosis and spermiogenesis) is inherent in the
germ cells and the role of the Sertoli cells is to facilitate the progression of this process. The maintenance of primate germ cell clusters in mouse testes after the transplantation of primate cells into mouse seminiferous tubules (Nagano et al., 2001b) clearly demonstrates that antigens, growth factors and signalling molecules that participate in the interaction between the donor germ cells and the recipient Sertoli cells have been preserved for 100 million years in these widely divergent species (Nagano et al., 2001b).

Molecular and hormonal mechanisms regulating male germ cell differentiation

Regulation of spermatogonia kinetics and meiosis

Development of a clonogenic assay to evaluate the role of growth factors in gonocyte proliferation and differentiation: The complex process of spermatogenesis has been the target of intensive research efforts for many years. Nowadays, we have a relatively good understanding of the development, proliferation and differentiation of SSCs especially under in vitro culture conditions. In addition, the importance of interaction between somatic cells (Sertoli cells) and SSCs and the role of hormones and intratesticular biochemical factors (growth factors, endorphins) in the various events of the spermatogenic process have been recognized. In vivo, in a normal testis, all the events in SSC development occur at the same time in different regions of the seminiferous tubules and are, therefore, difficult to study. Thus, an in vitro culture system in which SSCs can be maintained, proliferate and proceed through meiosis to the formation of spermatids (in long-term cultures) could be ideal in order to study the complex events of mammalian spermatogenesis. However, no such system exists today because (a) only a limited number of SSCs can be isolated and processed for studies involving in vitro culture systems, (b) germ cells have a limited viability in culture conditions and (c) it is difficult to distinguish SSCs from the more differentiated type A spermatogonia in vitro due to the lack of specific stem cell markers.

The cascade of events that mediate the transition of gonocytes to type A spermatogonia is one of the least understood processes that occur during spermatogenesis. Successful research efforts in germ cell cultures have resulted in the development of a clonogenic method to assay the capacity of gonocytes to proliferate in vitro as described by Hasthorpe et al. (1999). In the latter study a mouse gonocyte was selected taking into consideration its relative large cell size using micromanipulation techniques and placed in a collagen IV-coated microtitre well containing Iscove’s modified Dulbecco’s medium and 20% fetal calf serum (FCS) for 4–5 days. The gonocyte-derived colonies consisted of between four and more than 256 cells per colony which enabled certain spermatogonial subtypes to be identified and collected. In that in vitro culture system, Sertoli cells had an inhibitory activity on gonocyte-derived colony formation when added to the cultures of gonocytes. A Sertoli cell line had an even more pronounced inhibitory effect on the proliferation of gonocytes in vitro (Hasthorpe et al., 2000). Nagano et al. (2003) found that Sertoli cell lines resulted in a great reduction of SSCs after 7 days of culture. These findings suggested that Sertoli cellular exocrine or paracrine factors, which are known to support spermatogonial differentiation, cause a significant reduction in the number of SSCs cultured. It appears that the maintenance of SSCs in culture can be achieved by the suppression of germ cell differentiation. These findings are consistent with other studies demonstrating no stimulatory role of Sertoli cells in the proliferation of gonocytes in co-cultures of Sertoli cells with gonocytes (Orth and Boehm, 1990; de Miguel et al., 1996).

The in vitro clonogenic method has been also used to determine the growth factors that regulate mouse gonocyte proliferation and differentiation (Hasthorpe et al., 2000). In the latter study, it was found that transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) did not exert any inhibitory effect on gonocyte-derived colony formation. Furthermore, Mullerian inhibitory factor and leukaemia inhibitory factor (LIF) had no effects on the proliferation of gonocytes. The authors demonstrated that the growth of gonocytes in vitro was optimal in the presence of FCS. Thus, it has been concluded that no specific growth factors [with a probable exception of platelet-derived growth factor (PDGF) Hasthorpe, 2003] are necessary for the growth of gonocytes in vitro. However, Nagano et al. (2003) found that the addition of GDNF to a culture of mouse SSCs had a positive role in SSC maintenance. Forced expression of GDNF in transgenic mouse testes resulted in the accumulation of undifferentiated spermatogonia in vivo without a change in SSC proliferation kinetics. Thus, it was suggested that GDNF inhibits spermatogonial differentiation (Meng et al., 2000). Hasthorpe (2003) evaluated the role of SCF in the differentiation of spermatogonia. In that study SCF did not exert any effect on mouse type A spermatogonia colony-forming cells, indicating that more highly differentiated spermatogonia represent the target-cellular population for the SCF. The majority of gonocytes express c-kit mRNA, but fail to respond to SCF, indicating that the receptor is not functional. The PDGF appears to stimulate the proliferation of gonocytes, but not the proliferation of type A spermatogonia recovered from 15-day-old animals (Hasthorpe, 2003).

The role of hormones in the regulation of gonocyte proliferation and differentiation: The addition of activin to the gonocyte culture system overrides the antagonistic effect of somatic testicular cells (which produce inhibin β subunit) on gonocyte proliferation. The addition of activin increased gonocyte colony formation; however, in that study, a very little effect on spermatogonia cells was demonstrated (Hasthorpe, 2003). On the other hand, in a study by Nagano et al. (2003) the addition of activin to culture systems significantly reduced the number of SSCs. In the latter study, the authors (Nagano et al., 2003) suggested that the stimulation of spermatogonial proliferation by activin may be exerted on more advanced spermatogonia rather than on SSCs. These inconsistent findings may be attributed to the different methods used for the selection of a spermatogonial population for culture. Hasthorpe (2003) used the in vitro clonogenic method while Nagano et al. (2003) applied a two-step enzymatic digestion method on cryptorchid testes to collect spermatogonia as had been described by Ogawa et al. (1997). The latter method results in the recovery of a heterogeneous population of germ cells and allows the inclusion of testicular somatic cells within the population of the recovered cells. These somatic cells might have negatively affected the proliferation of SSCs in a study by Nagano (2003). Furthermore, the use of feeder layers in a study by Nagano et al. (2003) might also have negatively influenced
the proliferation of the SSC population. In general, *in vitro* attempts to improve the viability and differentiation of cultured gonocytes by adding several growth factors to the basic medium did not yield any clear results till date.

It appears that activin A, follistatin and FSH play a role in germ cell maturation during the period when gonocytes resume mitosis to form the SSCs and differentiating germ cell populations (Meehan et al., 2000). Meehan et al. (2000) have proposed that germ cells have the potential to regulate their own maturation initially through the production of endogenous activin A. Sertoli cells were observed to produce the activin/inhibin bA subunit, the inhibin a subunit and follistatin, demonstrating that these cells have the potential to regulate germ cell maturation as well as their own development. The authors used 1- and 3-day-long cultures of 3-day-old rat testicular fragments and observed that treatment with activin A produced a significantly higher ratio of germ cells to Sertoli cells, whereas treatment with follistatin and FSH increased the number of spermatogonia. Meehan et al. (2000) suggested that locally produced activin can stimulate gonocyte proliferation immediately after birth in the rat testis. Therefore, it is possible that activin and follistatin may play a vital role in the transition of gonocytes to spermatogonia. Toebosch et al. (1988) demonstrated that FSH acts indirectly on the gonocytes by inducing Sertoli cell expression of follistatin and inhibin. It appears that the maturation of gonocytes to form spermatogonia could result from the combined effects of follistatin and inhibin as activin antagonists, with FSH as the stimulus for inhibin production, thus producing effects on both germ cells and Sertoli cells that enable germ cell maturation. In *in vitro* culture systems presented evidence that FSH and activin stimulate Sertoli cell proliferation during early post-natal testis development (Mather et al., 1990; Boitani et al., 1995). The receptor of EGF is functional in spermatogonia and EGF has been proposed to inhibit testicular germ cell differentiation. Haneji et al. (1991) have proven that EGF blocks the proliferation of adult mouse type A spermatogonia stimulated by FSH. In contrast, Wahab-Wahlgren et al. (2003) have shown that EGF stimulates spermatogonial proliferation in adult rat seminiferous tubules in *in vitro* and might have an important role in the paracrine regulation of spermatogenesis.

### Late spermatogonial development: In vitro culture systems

*In vitro* culture systems have provided evidence that spermatogonia in advance stage of differentiation have different regulatory mechanisms (comparatively with the mechanisms regulating the gonocyte proliferation) that control their fate. Thus, SCF and its receptor c-kit play an important role in relatively late spermatogonial development. Dirami et al. (1999) showed that the SCF acts as a mitogen and survival factor for spermatogonia type A cultured in a potassa-

ium-rich medium Potassium Simplex Optimized Medium (KSOM). In the same study, granulocyte macrophage-colony stimulating factor also enhanced the survival of porcine type A spermatogonial cells. Nakayama et al. (1999) demonstrated that insulin-like growth factor-I and -II (IGF-I and IGF-II, respectively) as well as insulin promote spermatogonial differentiation into primary spermatocytes. These findings are consistent with the findings in a previous study by Tajima et al. (1995) who have demonstrated that IGF-I and transforming growth factor-a (TGF-a) stimulate the differentiation of mouse type A spermatogonia in organ culture *in vitro*. In contrast, neither the PDGF nor the fibroblast growth factor (FGF) has the above stimulatory effect. Studies employing a Vero cell conditioned medium rich in growth factors and interleukins showed that in humans FSH inhibits spermatogonia degeneration and stimulates meiosis entry, being further potentiated by testosterone (Sousa et al., 2002).

### Hormonal, molecular and genetic mechanisms regulating spermiogenesis

**Hormones:** Spermiogenesis is a metamorphosis process involving the maturation and differentiation of the early haploid male gamete to a mature spermatozoon. During spermiogenesis, alterations occur in the male gamete nuclear proteins, cellular size, cellular shape, the position and size of pro-acrosomal granules and the localization of the centrioles. This fascinating process that converts a round immotile haploid gamete to an elongated cell with potential for movement is regulated by a complex of factors/mechanisms.

The presence of immunoreactive ABP in Sertoli cell processes that surround the elongated spermatids has suggested a role of ABP in spermiogenesis (Martin du Pan and Campana, 1993). ABP has a high affinity for androgens probably contributing to the generation of high androgen concentrations in the vicinity of certain meiotic germ cells.

O’Donnell et al. (1996) and Sofikitis et al. (1999) have suggested that following withdrawal of intratesticular testosterone in a rat animal model, round spermatids are unable to proceed through the transition between steps 7 and 8 of spermiogenesis and therefore cannot complete the elongation process. This effect may be mediated by the loss of the adhesions of the spermatids with the sustentacular Sertoli cells (Zirkin, 1998). Studies in our laboratory have demonstrated released step 8 round spermatids within the epididymal lumen of rats with low intratesticular testosterone profiles (Sofikitis et al., 1999). It has been proven that lowering of intratesticular testosterone concentration results in the apoptotic death of germ cells (Kim et al., 2001). In addition, a consequence of decrease in intratesticular testosterone is that round spermatids lose their adhesion to the Sertoli cells, slough into the lumen of the seminiferous tubules and are occasionally phagocytized by Sertoli cells. A recent study has suggested that the Bcl-2-modifying factor (Bmf) is likely to play an important role in germ cell death in response to reduced intratesticular testosterone levels (Show et al., 2003). Bmf was found to reside in the subacrosomal space of spermatids with the sustentacular Sertoli cells (Zirkin, 1998). Studies in our laboratory have demonstrated released step 8 round spermatids within the epididymal lumen of rats with low intratesticular testosterone profiles (Sofikitis et al., 1999). It has been proven that lowering of intratesticular testosterone concentration results in the apoptotic death of germ cells (Kim et al., 2001). In addition, a consequence of decrease in intratesticular testosterone is that round spermatids lose their adhesion to the Sertoli cells, slough into the lumen of the seminiferous tubules and are occasionally phagocytized by Sertoli cells. A recent study has suggested that the Bcl-2-modifying factor (Bmf) is likely to play an important role in germ cell death in response to reduced intratesticular testosterone profiles (Show et al., 2003). Bmf was found to reside in the subacrosomal space of spermatids with the sustentacular Sertoli cells (Zirkin, 1998). Studies in our laboratory have demonstrated released step 8 round spermatids within the epididymal lumen of rats with low intratesticular testosterone profiles (Sofikitis et al., 1999). It has been proven that lowering of intratesticular testosterone concentration results in the apoptotic death of germ cells (Kim et al., 2001). In addition, a consequence of decrease in intratesticular testosterone is that round spermatids lose their adhesion to the Sertoli cells, slough into the lumen of the seminiferous tubules and are occasionally phagocytized by Sertoli cells. A recent study has suggested that the Bcl-2-modifying factor (Bmf) is likely to play an important role in germ cell death in response to reduced intratesticular testosterone profiles (Show et al., 2003). Bmf was found to reside in the subacrosomal space of spermatids with the sustentacular Sertoli cells (Zirkin, 1998). Studies in our laboratory have demonstrated released step 8 round spermatids within the epididymal lumen of rats with low intratesticular testosterone profiles (Sofikitis et al., 1999). It has been proven that lowering of intratesticular testosterone concentration results in the apoptotic death of germ cells (Kim et al., 2001).
events in spermatogenesis including spermatogonial proliferation and meiosis. However, testosterone only has been considered to sustain complete spermatid differentiation (McLachlan et al., 1995; Singh and Handelsman, 1996). On the other hand, Tesarik et al. (1998a,b) demonstrated that high concentrations of FSH represent a prerequisite for the completion of meiosis and spermiogenesis in vitro. In the latter studies, cultured round spermatids underwent nuclear changes similar to those occurring during the normal spermiogenesis process, characterized by nuclear condensation, peripheral migration and protrusion after the addition of high concentrations of FSH into the culture medium. It should be emphasized that the high concentration of FSH (25 IU/l) is necessary to obtain alterations in spermatid morphology in an in vitro culture system. Additional studies by Baccetti et al. (1997) showed that exogenous FSH administration in combination with or without human chorionic gonadotrophin in infertile men improved sperm counts. Furthermore, the administration of FSH had a positive role in sperm cytostructural parameters (Baccetti et al., 1997). Studies by Krishnamurthy et al. (2000) have shown that there is an increase in propidium iodide stainability of elongated spermatids and an increased sperm head size in FSH receptor knockout mice. These findings suggest a disturbance in the normal replacement of histones by protamines during spermiogenesis, leading to poor condensation of spermatid nuclei (Krishnamurthy et al., 2000) in FSH receptor knockout mice. FSH turns Sertoli cells competent to bind round spermatids. In addition, studies in humans showed that FSH stimulates meiosis II and round spermatid flagellum extrusion, whereas testosterone potentiates FSH action and stimulates late spermatid differentiation (Sousa et al., 2002).

Studies by Dinulovic and Rodonjic (1990) in diabetic patients tend to suggest a role of insulin in the spermiogenetic process. The identification of insulin gene family members in round spermatids of human and rat testes has provided additional evidence for a role of insulin and IGF in spermiogenesis (Lok et al., 2000). Another hormone having a potential role in spermiogenesis is prolactin. Prolactin receptor expression has been found in rat and human testes (Jabbour and Lincoln, 1999). However, in rat early round spermatids Hondo et al. (1995) did not detect prolactin receptor mRNA expression. The latter findings probably indicate species-dependent differences in the hormones regulating spermiogenesis.

The presence of type I and type II activin receptors in round spermatids indicate that activins may have some actions on early haploid male germ cells (de Winter et al., 1992). Testicular inhibin B in adult men is possibly a joint product of Sertoli cells and germ cells (including the stages from pachytene spermatocytes to early spermatids; Andersson et al., 1998). Marchetti et al. (2003) found that the inhibin B subunit was immunolocalized in germ cells (pachytene spermatocytes to round spermatids) but not in Sertoli cells. Activin actions may be modulated by actions of follistatin, which has been shown to be produced by Sertoli cells, spermatogonia, primary spermatocytes and round spermatids (Meinhardt et al., 1998).

Molecular and genetic mechanisms: Round spermatids express the precursor forms of the nerve growth factor (NGF) gene product, but not the mature form of NGFβ. NGFβ moiety of the NGF precursor proteins exhibits trophic activity in the rescue of Sertoli cell viability, consistent with the paracrine regulation of spermatogenesis.

Spermiogenesis is a very sensitive process to alterations in molecular and genetic factors. Generation of animal models by genetic engineering offers the opportunity to discover genetically regulated molecular factors that are implicated in the spermiogenic process. Alterations in the expression of molecular agents in the testicular tissue due to defects in gene expression (null mutations, gene overexpression, exogenous gene expression and gene misexpression) could lead to deficiency in the completion of different steps of spermiogenesis. Histone replacement by transition proteins (TP) and protamines during spermiogenesis may be affected by disruption of the Tarbp2 gene resulting in infertility and oligospermia (Zhong et al., 1999). A partial or complete failure to synthesize the protamines results in delayed replacement of TP and the spermatids show abnormal nuclear morphogenesis, developmental arrest and degeneration (Zhong et al., 1999). Premature translation of pre-existing protamine-1 (Pram1) mRNA causes precocious condensation of spermatid nuclear DNA and abnormal head morphogenesis (Lee et al., 1995). Successful interaction of mature protamine-2 with chromatin is required for the displacement of TP2 (Wu et al., 2000). Step 15 spermatids in Camk4<sup>−/−</sup> mice demonstrate a loss of protamine-2. These animals are characterized by prolonged retention of TP2. Mice lacking the major TP1 have been obtained following targeted deletion of the Tnp1 gene. Tnp1<sup>−/−</sup> mice demonstrate a normal sperm production quantitatively, but only 23% of the spermatozoa show any movement, and most of these do not show forward progression (Yu et al., 2000). In these animals, sperm heads with a blunted or bent tip are seen in 16% of epididymal spermatozoa possibly generated by the abnormal chromatin condensation that could reduce the rigidity of the fine apex of the spermatozoon (Yu et al., 2000). Tnp1 contains a cAMP-responsive element (CRE) that serves as a binding site for the CRE modulator (CREM). CREM is involved in the regulation of the Tnp1 gene expression, and human CREM protein is synthesized in steps 1–3 round spermatids. This may explain why a reduction in Cre expression and a lack of both CREM and TP1 have been demonstrated in human arrested spermatids at step 3 (Steger et al., 1999). Mice with deletion in Crem presented a spermatogenesis arrest at the round spermatid step (De Cesare et al., 1999). CREM is involved in regulating gene expression in round spermatids. Transcriptional activity of the CREM protein is thought to be regulated by the activator of CREM in the testis (ACT). Steger et al. (2004) applying RT–PCR and ISH demonstrated cell-specific gene expression of ACT in the man, cynomolgus monkey and mouse. Steger et al. (2004) have suggested that there is a conserved function of ACT during the evolution of mammalian spermatogenesis. They also suggested that there is a role for CREM in the ACT transcriptional regulation.

Deficiencies in intratesticular molecular factors due to genetic defects affect the organization and reorganization of the cytoskeleton during spermiogenesis. Thus, homozygous cor knockout mice are sterile and the epididymal spermatozoa exhibit bent tails and compromised flagellar vigour within the uterus (Yeung et al., 2000). Testicular haploid expression gene (THEG) is expressed in round and elongated spermatids. The molecular products of this gene appear to play a role in
the spermiogenesis since abnormal or absent flagella in mice with THEG disruption have been demonstrated and may be due to impairment of the assembly of cytoskeletal proteins such as the tubulins (Yanaka et al., 2000). A specific block in spermiogenesis was observed in homozygous JunD\(^{-/-}\) mice. JunD is one of the three mammalian Jun proteins that contribute to the AP-1 transcription factor complex. Jun proteins can form either homodimers or heterodimers with members of the related Fos family or with the ATF family to create the AP-1 transcription factor. Embryonic JunD expression is initially detected in the developing heart and cardiovascular system. JunD\(^{-/-}\) males exhibit multiple age-dependent defects in reproduction, hormone imbalance and impaired spermatogenesis with abnormalities in head and flagellum sperm structures (Thepot et al., 2000). Lack of molecular factors encoded by the latter gene results in an absence of flagella in spermatids in the lumen of the seminiferous tubules (Thepot et al., 2000; Escalier, 2001). The absence of JunD led to sperm flagellar growth impairment. Additional defects in sperm nuclear and cytoskeletal morphology and in mitochondrial localization can be observed in nectin-null mutant mice. Nectin-2 is a component of cell--cell anchoring junctions playing a role in the connection of the cytoskeletal elements of neighbouring cells. Thus, this molecular system participates in the regulation of cell shape and differentiation through signalling pathways (Bouchard et al., 2000). Further interesting observations on the male gamete cytoskeleton are demonstrated in the null mutant for the zinc-finger transcription factor Egr4. In the latter animals, the flagella is often fragmented, sharply kinked or have tightly coiled distal ends. Spermatozoa with heads that are either separated entirely or bent sharply back on the flagella are observed (Tourtellotte et al., 1999; Escalier, 2001).

In null mice for Slal2a2 gene (normally expressing the Na\(^{+}\)–K\(^{+}\)–2Cl\(^{-}\) co-transporter) few spermatids are present, but defects are striking when the male gametes gradually acquire the features of spermatozoa (Escalier, 2001). Defects in the molecular system of Na\(^{+}\)–K\(^{+}\)–2Cl\(^{-}\) co-transporter result in morphological abnormalities of spermatids. Spermatids show abnormalities in acrosomal vesicle during the cap phase and abnormalities in the nuclear shape (Pace et al., 2000). Other morphological abnormalities of the male gamete accompany the lack of factors that are normally expressed by the CsnK2a2 gene. CsnK2a2 could be a candidate globozoospermia gene. Mice deficient for CsnK2a2 show abnormalities of spermatid nuclear morphogenesis. Further abnormalities are observed in the nuclear and acrosomal shape (Xu et al., 1999).

Robertson et al. (1999) have demonstrated that deficiency in the production of aromatase enzyme cyp19 due to targeted disruption of the cyp19 gene in ArKO mice results in maturation arrest at early stages of spermiogenesis. Round spermatids do not complete elongation and spermiation. Furthermore, morphological defects in round spermatids are seen in tubules exhibiting spermiogenic arrest. Moreover, abnormalities of spermatid cap phase, acrosomal vesicle and nuclear shape are observed. These findings may suggest that estrogens have a role in spermatid differentiation (Robertson et al., 1999).

Production of polyploid spermatids and male gamete DNA fragmentation are demonstrated after the disruption of the protein phosphatase catalytic subunit Pp1cy (Varmuza et al., 1999; Jurisikova et al., 1999; Escalier, 2001).

Deficiency in the production of an epithelial, microtubule-associated protein due to defects in the expression of the E-MAP-115 gene results in abnormal shape and progressive degeneration in all condensed spermatids. Abnormalities of the microtubular manchette and nuclear shape are also observed (Komada et al., 2000; Escalier, 2001). Subnormal expression of the molecular products of the gene Tg737, which encodes one of the components of the raft protein complex, designated Polaris in the mouse and IFT88 in both Chlamydomonas and mouse, results in defective ciliogenesis and abnormalities in flagellar development in spermatids as well as asymmetry in left–right axis determination (Kierszenbaum, 2002). Polaris/IFT88 is detected in the manchette of mouse and rat spermatids. Intraflagellate transport has the features of intraflagellar transport machinery but, in addition, facilitates nucleocytoplasmic exchange activities during spermiogenesis (Kierszenbaum, 2002).

During spermiogenesis, histone-to-proteamine exchange causes chromatin condensation. Spermatozoa from infertile men are known to exhibit an increased protamine-1 (PRM1) to protamine-2 (PRM2) protein ratio. Patients undergoing testicular sperm extraction followed by ICSI procedures reveal low fertilization rates. Steger et al. (2001) investigated whether the outcome of ICSI could be related to the presence of round spermatids expressing PRM1 mRNA and PRM2 mRNA. The above investigators showed that the PRM1 mRNA to PRM2 mRNA ratio in round spermatids may serve as a possible predictive factor for the outcome of ICSI (Steger et al., 2001). In another study Steger et al. (2002) have shown that PRM1 mRNA and PRM2 mRNA in round spermatids are associated with RNA-binding proteins. In addition, Steger et al. (2003) have demonstrated that there is a decreased PRM1 transcript level in the testes from infertile men. The result is an aberrant Prm1/Prm2 mRNA ratio that plays an important role for the development of male infertility and may serve as a possible predictive factor for the outcome of ICSI (Steger et al., 2003).

**A dynamic balance of germ cell regeneration and death**

**Spontaneous apoptosis in the human testis**

The maintenance of normal architecture of the seminiferous tubuli is achieved by a dynamic balance of germ cellular regeneration and elimination. Sinha Hikim et al. (1998) have provided strong evidence that germ cell death during normal spermatogenesis in men occurs via apoptosis and have indicated the presence of ethnic differences in the inherent susceptibility of germ cells to the apoptotic cell death. In addition, apoptosis has been reported to be a possible mechanism of spermatogonial death in pre-pubertal boys (Sinha Hikim et al., 1998) or of 2-methoxy acetic acid-induced spermatocyte death in cultured seminiferous tubules of middle-aged human donors (Sinha Hikim et al., 1998). The above investigators provided strong evidence for the presence of germ cell apoptotic process in the human. The exact incidence of adult male germ cell apoptosis remains unclear, since not all degenerating germ cells display the classical morphology of apoptosis. Spermatogonia and round spermatids almost certainly die by apoptosis, since they demonstrate many of the apoptosis classical morphological and biochemical features, such as compaction of DNA at the nuclear margin and labelling of nuclei by terminal deoxynucleotidyl transferase...
(Print and Loveland, 2000). Apoptotic round spermatids often degenerate en masse as multinucleated symplasts. Apoptotic germ cells are either sloughed into the tubule lumen or phagocytosed by Sertoli cells. The extent of spermatocyte and elongated spermatid apoptosis is less clear; some can be labelled with terminal deoxynucleotidal transferase and annexing V, but they do not show the characteristic nuclear changes usually associated with apoptosis possibly due to the unusual morphology and DNA configuration of these cells (Print and Loveland, 2000).

Whether male germ cells survive or die is determined by a complex network of signals. These include paracrine signals such as SCF, LIF and Desert Hedgehog (Dhh) (Gnassi et al., 1997), as well as endocrine signals such as pituitary gonadotrophins, estrogens and testosterone, among others (Schlatt et al., 1997; O’Donnell et al., 2001). In addition, male germ cells respond to external signals, and to their internal milieu, by activating intracellular signalling pathways that ultimately determine their fate.

**The role of the Bcl-2 signalling pathway in governing the mitochondria-dependent apoptotic pathway in human**

Proteins of the Bcl-2 family provide one signalling pathway which appears to be essential for male germ cell homeostasis. Some members of this family promote cell survival (i.e. Bcl-2, Bcl-xL and Bcl-w, among others) while others antagonize it (e.g. Bax, Bak and Bim, among others). Up-regulation of Bax expression is a feature of germ cell apoptosis in vitro. The pro-survival protein Bcl-xL may play an important role in determining germ cell fate. Bcl-xL potentially promotes germ cell survival during embryogenesis. Bcl-xL may also regulate germ cell survival during the first wave of spermatogenesis since it is expressed at high levels in testis at this time. In the adult testis, Bcl-xL is less abundant than in immature testis and appears to be restricted to spermatocytes and spermatids. The pro-survival protein Bcl-w plays an important role in the regulation of testicular germ cell number. The incidence of germ cell apoptosis in Bcl-w knockout mice becomes dramatically elevated between 2 and 4 weeks of age (Ross et al., 1998). Other members of the Bcl-2 family are expressed in the testis, but their role in spermatogenesis has not been clarified (i.e. Bad, Bok, Bem and Boo9, among others). Oldereid et al. (2001) have shown that spontaneous apoptosis occurs in male germ cell subpopulations in the human and that Bcl-2 family proteins are distributed preferentially within distinct germ cell compartments. They provided evidence for a specific role for these proteins in the processes of cellular differentiation and maturation during the human spermatogenesis process. Bcl-2 and Bak are preferentially expressed in the compartments of human spermatocytes and differentiating human spermatids (Oldereid et al., 2001). Bcl-x is preferentially expressed in human spermatogonia. Bax demonstrates a preferential expression in the nuclei of human round spermatids. Bad can be detected by immunocytochemistry in the acrosome region of various stages of human spermatids. On the other hand, Mcl-1 staining does not demonstrate a particular pattern in the human testis. In the human testis, Bcl-w, p53 and p21 cannot be detected. Since Bax is an apoptotic promoter, the Bax preferential expression in human round spermatids may suggest that round spermatids may be particularly prone to apoptosis when DNA is damaged. The apoptotic rate in spermatogonia is significantly lower in aged men compared with controls (Kimura et al., 2003). However, in that study (Kimura et al., 2003), the balance of spermatogonial proliferation and apoptosis showed no significant difference between the group of aged men and the control group. This is believed to be one of the reasons explaining why spermatogonial numbers in aged men are similar to that of controls. On the other hand, the apoptotic rate of primary spermatocytes in aged men is significantly elevated compared with younger controls resulting in a decrease of the number of human primary spermatocytes per Sertoli cell in aged men. Furthermore, Kimura et al. (2003) have demonstrated that the expression of Bcl-xL is inversely related with the apoptotic rate in human primary spermatocytes, suggesting that Bcl-xL may contribute to the regulation of human primary spermatocyte apoptosis. In another study, Erkkila et al. (2002) showed that the human germ cell death in vitro is inhibited effectively and dose-dependently by lactate, indicating that lactate has an important effect on the regulation of cellular death in human male germ cells. Thus, Erkkila et al. (2002) have demonstrated that human testicular germ cell death is effectively regulated by lactate, which may be regarded as a potential compound for optimizing in vitro methods for the maintenance of the function of male germ cells for assisted reproduction technique (ART) purposes. Regarding the mechanisms of the anti-apoptotic role of lactate in the human, it appears that the death suppressing mechanism of lactate is not related with changes in intracellular AMP, ADP and ATP levels. Erkkila et al. (2002) have hypothesized that the action of lactate is downstream along the cell-death pathway activated by the Fas receptor of the germ cells.

**Contribution of transcription factors to the mechanism responsible for the elimination of human damaged meiotic germ cells**

Transcription factors may provide additional fate-determining signals; c-myc may regulate the apoptosis of pre-meiotic germ cells. The E2f family of transcription factors can induce both apoptosis and proliferation in somatic tissues (Holberg et al., 1998). Mechanisms responsible for the detection and elimination of damaged meiotic germ cells are present in the human male as evidenced by the high incidence of spermatocyte apoptosis and round spermatid apoptosis described in infertile men. In addition, men with ataxia telangiectasia and mice lacking the ATM gene are infertile as a result of extensive spermatocyte apoptosis (Burgoyne and Baker, 1984). The CREM is a transcription factor required for the expression of post-meiotic germ cell-specific genes. Spermatids of mice lacking the CREM are arrested in the first step of spermiogenesis and appear to be subsequently removed by apoptosis. Several mechanisms may mediate the selective apoptosis of damaged germ cells. For example, the cell cycle regulator p53 appears to be necessary for the radiation-induced apoptosis of spermatogonia (Hasegawa et al., 1998). There are also p53-independent mechanisms. Failed synapsis appears to induce spermatocyte apoptosis through a p53-independent pathway.

**Paracrine mechanisms and growth factors in apoptotic pathways in the testis**

Grataroli et al. (2004) demonstrated the tumour necrosis factor alpha-related apoptosis-inducing ligand (TRAIL) and its
receptors in different human testicular germ cell types. In addition, TRAIL, DR5/TRAIL-R2 (receptor) and DcR2/TRAIL-R4 (receptor) are localized in Leydig cells. DR4/TRAIL-R1 (receptor) is seen in human peritubular and Sertoli cells. It appears that the TRAIL pathway may have a role in the induction of apoptosis in the human testis (Gratralori et al., 2004).

Several paracrine signals are regulators of germ cell fate. LIF promotes the survival of PGC in culture. Other factors that promote PGC survival in vitro include interleukin-4 (IL-4), basic FGF (bFGF), a soluble form of SCF and the bone morphogenetic protein (BMP)-4. In contrast, TGF-beta has been reported to promote gonocyte apoptosis in vitro. The survival of gonocytes co-cultured with Sertoli cells is promoted by bFGF, LIF and ciliary neurotrophic factor (Dolci et al., 1991; Matsui et al., 1992; Cooke et al., 1996). In adults, members of the BMP family promote germ cell survival in vivo. BMP-8A and BMP-8P appear to provide survival signals to spermatocytes. Dhh secreted by Sertoli cells is another paracrine signal known to promote germ cell survival indirectly.

GDNF, neurturin, persephin and artemin are related members of the TGF-b superfamily (Lin et al., 1993; Kotzbauer et al., 1996; Baloh et al., 1998; Milbrandt et al., 1998). GDNF mRNA was found to be expressed in many tissues in addition to the brain and kidney, including intestine, stomach, muscle, cartilage, lung and testis (Trupp et al., 1995). Expression of GDNF mRNA in testis is related with the expansion of the Sertoli cell population. GDNF contributes to the paracrine regulation of spermatogonial self-renewal and differentiation (Meng et al., 2000).

The PDGF-A and PDGF-B genes encode A and B chains of the PDGF and are located on human chromosome 7p and 22q, respectively (Antoniades and Hunkapiller, 1983). PDGF is produced by many cells and exerts its effects on cells by receptor phosphorylation, leading to cellular responses including migration, proliferation, contraction and alteration of cellular metabolic activities such as matrix synthesis, cytokine production and lipoprotein uptake (Heldin and Wernemark, 1999). PDGF-A gene, PDGF-B gene and the genes encoding the PDGF receptor alpha and beta subunits are expressed in the human fetal testis and this expression increases in the adult testis, suggesting a connection between the PDGF system and the initiation of spermatogenesis (Basciani et al., 2002). Human Leydig cells express both the ligands and receptors of the PDGF system. This allows us to hypothesize that the ontogeny of this cell type is profoundly influenced by PDGF (Basciani et al., 2002).

**Regulation of the fate of testicular germ cells by factors derived from Sertoli cells by direct membrane contact**

In addition to paracrine signals, germ cells also depend upon signals derived from Sertoli by direct membrane contact. Membrane-bound SCF is expressed on Sertoli cell precursors in the embryonic genital ridge and its receptor, the c-kit tyrosine kinase, is expressed on the surface of adjacent PGCs. SCF is also required during the first wave of spermatogenesis. In adults, membrane-bound SCF is expressed on the basal regions of Sertoli cells, while c-kit is expressed on the corresponding surface of spermatagonia. When SCF/c-kit interaction in adults is blocked in vivo, the incidence of apoptosis in spermatogonia and spermatocytes is increased. The c-kit gene is the cellular homologue of the feline sarcoma oncogene v-kit (Besmer et al., 1986). It is located at the White spotting (W) locus in the mouse and on chromosome 4 in the human (Giebel et al., 1992; Vandenbark et al., 1992). The ligand SCF has been identified as an analogue of the murine Steel (Sl) gene and is located on chromosome 12 in the human encoded by nine exons (Flanagan et al., 1991). In the post-natal and adult testis, the c-kit is detected in the proliferating spermatogonia A1–A4 and is also present in interstitial somatic Leydig cells (Manova et al., 1990; Orth et al., 1996). In contrast, Sertoli cells are the unique source of SCF in the testis (Rossi et al., 1991). SCF/c-kit system is involved in different functions in the testis, including germ cell (GC) migration, cell adhesion, cellular proliferation and anti-apoptotic actions. W and Sl homozygous mutations, resulting in the absence of functional production of c-kit or SCF, respectively, are associated with the absence of germ cells in the post-natal testis. These alterations of spermatogenesis are related to defects in PGC migration and/or induction of apoptosis (Mauduit et al., 1999). Therefore, the SCF/c-kit complex appears to represent one of the key regulators of spermatogenesis.

Pentikainen et al. (1999) have shown that the Fas–FasL system regulates germ cell apoptosis in the human testis. Expression of FasL has been observed in the human testis. Antagonistic antibodies to the FasL block human germ cell apoptosis in vitro (Pentikainen et al., 1999). Among the apoptotic receptors comprising the tumour necrosis factor receptor superfamily, CD95/APO-1 (Fas) is the best characterized. FasL, a cell surface molecule binds to its receptor Fas, thus inducing apoptosis of Fas-bearing cells (Nagata and Golstein, 1995).

Fas is abundantly expressed in various tissues, particularly in activated T and B cells, thymocytes, hepatocytes and the heart tissue (Watanabe-Fukunaga et al., 1992). Of particular interest is the observation that FasL is constitutively expressed by cells in immune privileged sites such as the testis (Suda et al., 1993) and the anterior chamber of the eye (Bellgrau et al., 1995; Sofikitis et al., 2003). Fas is expressed at low levels in the mouse testis (French et al., 1996; Lee et al., 1997) and appears to be restricted to some germ cells (Lee et al., 1997). In addition, Sertoli cells may employ the Fas system to regulate the germ cell fate. Furthermore, with regard to the human testis, the expression and the function of Fas and FasL are a matter of debate. Besides the immunoregulative role of FasL in the testis, the Fas system has also been proposed as a key regulator of physiological germ cell apoptosis (Korbett et al., 1997; Lee et al., 1997; Pentikainen et al., 1999; Riccioli et al., 2003).

Caspase inhibitors inhibit programmed human germ cell death, suggesting that Fas-associated human germ cell apoptosis is mediated via the caspase pathway. It appears that human germ cell death can be inhibited (Pentikainen et al., 1999) by blocking the interaction between Fas and FasL. The FasL is constitutively expressed by the human Sertoli cells and is suggested to bind to the Fas molecule of the germ cells. Thus, it causes death of these Fas-bearing germ cells. In rat testes, up-regulation of Fas was observed in germ cells undergoing apoptosis after in vivo administration of Sertoli cell toxicants (Lee et al., 1997). However, in human testes the expression of Fas does not seem to be up-regulated during an enhanced apoptotic process after the withdrawal of survival factors. Therefore, additional pathways
leading to increased apoptosis in the human testis may occur. Alternatively, Pentikainen et al. (1999) hypothesized that Fas activation may be more effective in unfavourable conditions, thus enhancing the ability of the Fas–FasL system to mediate apoptotic human germ cell death.

A role of hormones in the regulation of apoptosis in human germ cells

Hormones such as testosterone, FSH and LH are known to influence the germ cell fate. Their removal induces germ cell apoptosis. Estrone treatment, which is thought to mimic a gradual withdrawal of gonadotrophins, also induces apoptosis of all germ cells including elongated spermatids (Blanco-Rodriguez and Martinez-Garcia, 1996). In addition, Pentikainen et al. (2000) have demonstrated that estradiol acts as a germ cell survival factor in the human testis in vitro (Pentikainen et al., 2000).

In human seminiferous tubuli, apoptosis is induced under serum-free conditions in vitro (Erkkila et al., 1997). The fact that this apoptosis is suppressed by testosterone indicates that testosterone in the human male is a critical germ cell survival factor. The mechanism by which androgen withdrawal induces germ cell death remains unclear. It is tempting to speculate that androgen withdrawal alters the expression of the Bcl family proteins in germ cells, since Bcl-xl and Bcl-2 in the testis is altered following long-term anti-androgen treatment for prostate cancer (MacGregor et al., 1999).

Somatostatin (SRIF) is a regulatory peptide hormone playing a role in the regulation of the proliferation of the male gametes. Its biological actions are mediated by five receptors (sst1–sst5) (Reubi, 1997). The injection of an SRIF analogue (SMS201995) in healthy adult males is followed by a rapid (2 h after the injection) rise in serum testosterone level. Such an increase in testosterone secretion occurs without a simultaneous increase in LH secretion, suggesting that SRIF can modulate testosterone secretion at the testicular level (Vasankari et al., 1995). The presence of SRIF and its receptors in human testes (Bau et al., 2000) supports the existence of autocrine loops controlling local testosterone secretion. Indeed, sst3, sst4 and sst5 are expressed in human normal testicular tissue, while sst1 and sst2 are usually not detected. Goddard and co-workers have provided evidence for an inhibitory role of SRIF in the control of spermatogonial proliferation. In the perinatal porcine testis, SRIF might exert its actions both directly on spermatogonia by preventing SCF-induced proliferation and indirectly by inhibiting SCF mRNA expression by Sertoli cells (Goddard et al., 2001).

Apoptosis in spermatogonia

In human, the presence of nuclear DNA damage in ejaculated spermatogonia has pointed to a possible role of apoptosis during spermatogenesis. Sakas et al. (1999) have suggested that apoptosis is a major mechanism in regulating spermatogenesis in the human and that there are clear differences in molecular markers of apoptosis between males with normal and abnormal sperm parameters. Sakas et al. (1999) have proposed that the presence of Fas-labelled spermatogonia in the ejaculate of men with abnormal semen parameters is indicative of ‘abortive apoptosis’ having taken place, whereby the normal apoptotic mechanisms have malfunctioned (Sakkas et al., 2003), have been overridden or have not been completed.

Technical prerequisites and outcome of successful in vitro culture systems

Recovery and purification of spermatogonia cells prior to culture

Methods to generate animal models with relatively large proportions of undifferentiated spermatogonia and SSCs

Rapid and effective preparation of pure populations of spermatogonia is the basis to achieve induction of in vitro spermatogenesis. However, obtaining a pure population of spermatogonia or SSCs is considered difficult due to the problems associated with the limited number of spermatogonia in the testis (Meachem et al., 2001) and the technical difficulties concerning the identification of specific biochemical or surface antigen markers characterizing the spermatogonia cells and their subpopulations (de Rooij and Grootegoed, 1998). Until now, the most common way to prepare a relatively pure population of SSCs for culture is to collect SSCs from neonatal or pre-pubertal testes. In the latter testes spermatogenesis is arrested at an early stage and thus fewer contaminating differentiating/differentiated germ cells are present and a larger number of SSCs is present (Creemers et al., 2002).

In experimental animals, testicular pathologies can be induced in which undifferentiated spermatogonia A are virtually the single germ cell present (Table I). Induction of vitamin A deficiency in rodents results in deterioration of spermatogenesis until virtually only type A spermatogonia remain present (Mitrandon et al., 1979). Most, if not all, of these remaining spermatogonia A (in vitamin A deficiency models) are quiescent and unable to differentiate (Van Pelt et al., 1995). Van Pelt et al. (1996) used a Percoll gradient system in order to isolate spermatogonia cells from vitamin A-deficient 10-week-old rats. The administration of the Sertoli cell toxicant 2,5-hexadiene (Boekelheide, 1988; Allard et al., 1995) or exposure to X-irradiation (Kangsniemi et al., 1996; Table I) in animal models results in testicular histologies characterized by actively proliferating but not differentiating type A spermatogonia. The latter animal models can serve as a source of SSCs without contaminating differentiating germ cells. The cryptorchid model provides a rich alternative source of SSCs that can be further processed for in vitro cultures; one cell in 200 testicular cells in prepared testicular cellular suspensions in cryptorchid animals is an SSC (Shinohara et al., 2000a; Sofikitis et al., 2003). Shinohara et al. (2000a) found that testicular cells recovered from cryptorchid mice were highly enriched in SSCs. SSCs isolated from cryptorchid testes can be processed for in vitro cultures since it has been demonstrated that the functional properties of SSCs are not adversely affected by the elevated temperature of a cryptorchid testis (Shinohara et al., 2000a; Sofikitis et al., 2003). This has been proven by achieving induction of hamster spermatogenesis within rat testes (Sofikitis et al., 2003) after the transplantation of hamster spermatogonia recovered from cryptorchid hamsters within the seminiferous tubuli of immunodeficient rats.

Methods to isolate spermatogonia subpopulations

Procedures for isolation of cellular subfractions of germ cells employing elutriation (Bucci et al., 1986) or velocity sedimentation (Bellve et al., 1977; Dym et al., 1995; Dirami et al., 2000a; Sofikitis et al., 2003). In experimental animals, testicular pathologies can be induced in which undifferentiated spermatogonia A are virtually the single germ cell present (Table I). Induction of vitamin A deficiency in rodents results in deterioration of spermatogenesis until virtually only type A spermatogonia remain present (Mitrandon et al., 1979). Most, if not all, of these remaining spermatogonia A (in vitamin A deficiency models) are quiescent and unable to differentiate (Van Pelt et al., 1995). Van Pelt et al. (1996) used a Percoll gradient system in order to isolate spermatogonia cells from vitamin A-deficient 10-week-old rats. The administration of the Sertoli cell toxicant 2,5-hexadiene (Boekelheide, 1988; Allard et al., 1995) or exposure to X-irradiation (Kangsniemi et al., 1996; Table I) in animal models results in testicular histologies characterized by actively proliferating but not differentiating type A spermatogonia. The latter animal models can serve as a source of SSCs without contaminating differentiating germ cells. The cryptorchid model provides a rich alternative source of SSCs that can be further processed for in vitro cultures; one cell in 200 testicular cells in prepared testicular cellular suspensions in cryptorchid animals is an SSC (Shinohara et al., 2000a; Sofikitis et al., 2003). Shinohara et al. (2000a) found that testicular cells recovered from cryptorchid mice were highly enriched in SSCs. SSCs isolated from cryptorchid testes can be processed for in vitro cultures since it has been demonstrated that the functional properties of SSCs are not adversely affected by the elevated temperature of a cryptorchid testis (Shinohara et al., 2000a; Sofikitis et al., 2003). This has been proven by achieving induction of hamster spermatogenesis within rat testes (Sofikitis et al., 2003) after the transplantation of hamster spermatogonia recovered from cryptorchid hamsters within the seminiferous tubuli of immunodeficient rats.
Establishment of spermatogonial cell lines

Isolation procedures

Boekelheide (1988) Administration of 2,5-hexanedione results in testicular histologies characterized by proliferating but not differentiating type A spermatogonia.

Kangasniemi et al. (1996) X-irradiation results in testicular histologies characterized by proliferating but not differentiating type A spermatogonia.

Shinohara et al. (2000a) The cryptorchid condition does not affect SSC function/activity. It provides a rich source of SSCs.

Shinohara et al. (2000b) Sl infertile mutant mice provide an enriched source of SSCs.

Isolation procedures

Bellve et al. (1977) Velocity sedimentation method is based on the size and shape of germ cells.

Bucci et al. (1986) Elutriation method is based on the size and shape of germ cells.


Von Schonfeldt et al. (1999) Magnetic cell sorting using c-kit antibody to detect the c-kit receptor of differentiating spermatogonia.

Shinohara et al. (2000a) Selection of SSCs using a system of magnetic beads and antibodies against α-6 and β-1 integrins.

Van der Wee (2001) Immunomagnetic bead techniques using c-kit antibody.

Establishment of spermatogonial cell lines

Hofmann et al. (1992) Generation of immortalized germ cells using the SV40 large T antigen.

Tascou et al. (2000) Development of the GC-4spc cell line with characteristics between preleptotene and pachytene spermatocytes.

Van Pelt et al. (2002) Generation of immortalized germ cells expressing Hsp90a and oct-4 specific markers for SSCs.

Feng et al. (2002) Generation of mTERT cells.

In vitro spermatogenesis

Immortalized spermatogonial cell lines may serve as a powerful tool in elucidating the molecular mechanisms of spermatogenesis under in vitro conditions. Efforts have been directed to establish male germ cell lines from neonatal and older mice to study the early stages of spermatogonial differentiation. Immortalized male germ cell lines have been generated using the Simian Virus 40 (SV40) large T antigen. The first cell line established included GC-1spg cells that had characteristics of B spermatogonia and early spermatocytes. However, the latter cells were unable to differentiate further in vitro in co-cultures with immortalized Sertoli cells (Hofmann et al., 1992). Other immortalized germ cell lines have characteristics of more advanced germ cell types. GC-2spd(ts) and GC-3spc(ts) cells under culture conditions express the markers lactate dehydrogenase C4 and cytochrome C, which are specific for meiotic and post-meiotic germ cells. The latter cell lines can undergo meiosis to generate round spermatids in vitro (Hofmann et al., 1994). The generated round spermatids present with an acrosomal granule and with development of a flagellar axoneme. Tascou et al. (2000) developed the GC-4spc cell line with characteristics between preleptotene and early pachytene spermatocytes (Table I).
More recently, Van Pelt et al. (2002) established two immortalized cell lines by transfecting a mixed population of purified rat A$_s$ (stem cells), A$_{rep}$ and early A$_{al}$ spermatogonia with the SV40 large T antigen. These cell lines were found to express Hsp90a and oct-4 specific markers for SSCs but not the c-kit receptor (which is known to be expressed at the stage of late A$_{al}$ spermatogonia). The latter cells, post-transplantation, were able to colonize recipient seminiferous tubules, but did not differentiate after the spermatogonial stage (replicating without differentiation in vivo). SV40 virus has been widely used to immortalize cells and to produce tumours in transgenic animals. SV40 T antigen immortalizes efficiently and can induce efficiently, as well, a tumorigenic state (Rassoulzadegan and Cuzin, 1998).

Feng et al. (2002) reported the in vitro generation of spermatocytes and spermatids from telomerase-immortalized mouse type A spermatogonial cells (mTERT cells) in the presence of SCF. This differentiation could occur in the absence of supportive cells. mTERT-immortalized mouse spermatogonia cells have the properties of type A SSCs because they can proliferate to renew themselves and can also give rise to differentiated cells upon ligand stimulation.

Immortalized cell lines greatly facilitate research on the behaviour of SSCs under in vitro culture conditions, enabling analysis of gene expression and elucidating mechanisms that regulate spermatogonia proliferation and differentiation in vitro.

**Biochemical conditions in vitro facilitating survival and proliferation of cultured male germ cells**

**Difficulties concerning the interpretation of the outcome of in vitro culture systems**

Culture of isolated male germ cells, in general, is difficult for a number of reasons: (a) there is a remarkable decline in the viability of the cultured cells within 1 week (Morena et al., 1996; Dirami et al., 1999) and (b) the cell proliferation rate is usually low, minimal or absent under culture conditions. Tritiated thymidine incorporation has been demonstrated in spermatogonia and spermatocytes in cultures of pre-pubertal rat germ cells (Tres and Kierszenbaum, 1983: 11 days after the time of $[^3]$Hthymidine labelling, a large number of labelled cells had become mid/late pachytene spermatocytes displaying the condensed XY bivalent). In neonatal mouse germ cell cultures, proliferation during the first 3 days has been demonstrated to occur only in a small fraction of the germ cells (Maekawa and Nishimune, 1991). Another factor adding difficulties to the performance of cell culture studies is that the cell biochemical and morphological characteristics are altered during in vitro culture. Thus, the identification of the various germ cell subpopulations during/at the end of in vitro culture experiments becomes difficult. During culture, identification of the types of subpopulations of germ cells has been achieved using either specific morphological characteristics (Tres and Kierszenbaum, 1983) or specific biochemical markers (e.g. c-kit; van der Wee et al., 2001). In humans, co-cultures of Sertoli cells and diploid germ cells in a Vero cell conditioned medium supplemented with FSH and testosterone resulted in the production of late spermatids, thus first showing in mammals that meiosis and spermiogenesis can be achieved in vitro; this technology offers a new system to investigate factors needed to support human spermatogenesis in vitro (Sousa et al., 2002).

**Factors extending/improving the viability, proliferation and differentiation of germ cells in vitro**

Methods to improve the outcome of in vitro culture systems include (Table II): (a) the addition of FCS to the culture medium (it has been discussed in previous sections of this study; Haasthorpe et al., 1999, 2000; Izadyar et al., 2003); (b) the use of a specific culture medium (Dirami et al., 1999; KSOM medium developed according to the simplex optimization method); (c) the maintenance of germ cells on feeder layers (Van Dissel-Emiliati et al., 1993; Nagano et al., 1998); (d) the co-culture of germ cells with Sertoli cells (it will be discussed extensively in next sections of this study; Izadyar et al., 2003); (e) the addition of growth factors or other factors that are normally secreted by the Sertoli cells to the culture medium (Dirami et al., 1999; Feng et al., 2002; Wahab-Wahlgren et al., 2003); (f) the addition of hormones to the culture medium (Tesarik et al., 1998a–c; it will be discussed in next sections of this study) (Table II).

Several growth factors such as GDNF, IGF-I, LIF and EGF support the in vitro expansion of SSCs and increase the number of SSCs. A recent study by Kubota et al. (2004) emphasizes the positive influence of several growth factors on SSC proliferation. Feeder layers of SIM mouse embryo-derived thioguanine and ouabain resistant (STO) cells, Vero cell feeder layer or Vero cell conditioned mediums have a beneficial role in SSC maintenance and proliferation. It has been demonstrated (see above sections) that FSH may stimulate early events in spermatogenesis including spermatogonial proliferation and meiosis. In addition, it has been shown that high concentrations of FSH represent a prerequisite for the completion of meiosis and spermiogenesis in vitro (Tesarik et al., 2001; Sousa et al., 2002). Cultured round spermatids undergo nuclear changes similar to those occurring during the normal spermiogenesis, characterized by nuclear condensation, peripheral migration and protrusion (Tesarik et al., 2001; Sousa et al., 2002) after the addition of high concentrations of FSH into the culture medium. Adding cholesterol,

<table>
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<th>Table II. Methods to extend/improve the viability of germ cells in <em>in vitro</em> cultures (in chronological order)</th>
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<tr>
<td>Nagano et al. (1998)</td>
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<td>Tesarik et al. (1998a)</td>
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<td>Cremades et al. (1999)</td>
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<td>Izadyar et al. (2003)</td>
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<td>Kanatsu-Shinohara <em>et al.</em> (2003)</td>
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<td>Nagano et al. (2003)</td>
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probably because of active proliferation of pup SSCs. Pup cells (compared with adult germ cells) after the first 2 days in culture, significantly larger number of pup germ cells remained alive SSCs disappeared under the culture conditions. However, a significant pattern of decrease in SSC number was also observed with pup cells, indicating that regardless of the animal age the majority of SSCs disappeared under the culture conditions. In addition, a distinct proliferation of testicular somatic cells was noted after the addition of FCS.

Izadyar et al. (2003) developed a method for long-term culture of bovine type A spermatagonia. Testes from 5-month-old calves were used. Populations of type A spermatogonia were isolated employing an enzymatic digestion method described by Izadyar et al. (2002) and cultured in MEM or KSOM medium (in the presence of Sertoli cells). Culture in MEM medium containing 2.5% FCS at 37°C provided the most optimal conditions for survival, proliferation and even differentiation of bovine type A spermatogonia. After 1 month of culture, bovine type A spermatogonial colonies were formed (Izadyar et al., 2003). The majority of these colonies consisted of cells that underwent differentiation (c-kit positive cells), but occasionally there were large round colonies consisting of c-kit negative type A spermatogonia (presumably SSCs). The formation of two types of colonies means that bovine type A spermatogonia can remain alive for a long period in a culture system and that these cells can proliferate and produce new type A spermatogonia or they can undergo further differentiation (Izadyar et al., 2003). On the other hand, in a study conducted by Dirami et al. (1999), culture of porcine type A spermatogonia in KSOM medium (additional mediums were also used) did not improve the viability of porcine spermatogonia. It appears that there are differences in the potential of germ cells of different species to proliferate under culture conditions.

The role of various types of feeder cells in SSC maintenance

Long-term culture of germ cells is most feasible when these cells are co-cultured with feeder cells, suggesting that the feeder cells may have a significant impact on the maintenance of germ cells in vitro (Nagano et al., 1998). The influence of feeder cells on SSC maintenance has been evaluated after co-culture of adult or pup mouse germ cells for 7 days with various types of cells as feeder cells (Nagano et al., 2003). Both types of cells, when co-cultured with STO feeder cells with no growth factor supplements, rapidly decreased in number; more than half of adult SSCs disappeared after 2 days of culture; 12% of the adult SSCs remained alive in vitro after 7 days of culture. A similar pattern of decrease in SSC number was also observed with pup cells, indicating that regardless of the animal age the majority of SSCs disappeared under the culture conditions. However, a significantly larger number of pup germ cells remained alive (compared with adult germ cells) after the first 2 days in culture, probably because of active proliferation of pup SSCs. Pup spermatogonia cells rapidly incorporate "H-thymidine (Maekawa and Nishimune, 1991) and self-renew more actively and rapidly in vitro than adult spermatogonia do in vitro (Nagano et al., 2001a).

When SSCs were co-cultured with OP9 and L cells as feeder layers, the efficiency of spermatogonial maintenance was increased 2-fold; thus, using the above feeder layers 24% of the SSCs remained alive after 7 days of culture (Nagano et al., 2003). In that study, the authors also examined the effects of the basic medium type on the survival of germ cells in co-culture with the feeder cells. It was suggested that using modified Eagle’s medium-a (MEM-a), the survival rate of the cultured cells was 1.7-fold higher than using Dulbecco’s modified Eagle’s medium (DMEM). MEM-a was prepared after supplementation of the basic DMEM with glutamine (2 mM), sodium bicarbonate (1.25 g/l), pyruvic acid (0.4 mM), lactic acid (6 mM) and 2-mercaptoethanol (0.1 mM). It appears that alterations in the biochemical conditions of the culture system have an effect on the overall outcome of the in vitro spermatogonial cell culture.

The role of feeder cells in the maturation of primary spermatocytes and/or round spermatids is discussed in the next sections of this study.

Culture mediums supplemented with growth factors

Proliferation of mouse gonocytes isolated from neonatal testes was achieved over a 5 month culture period in a culture system positive for various growth factors (GDNF, EGF, FGF-b and LIF) (Kanatsu-Shinohara et al., 2003). The gonocytes had the ability post-culture following their transplantation into the seminiferous tubules of congenitally infertile recipients to restore the fertility potential of these infertile recipients (Kanatsu-Shinohara et al., 2003). It appears that the addition of specific growth factors in culture mediums facilitates the proliferation of gonocytes in vitro. Other studies referring to the role of the growth factors on gonocyte proliferation in vivo or in vitro have been mentioned in previous sections of this study.

In vitro culture systems allowing infection of spermatogonia with a virus-mediating gene delivery

Nagano et al. (2001a) have demonstrated that appropriate biochemical conditions in an in vitro culture system (including mitomycin C-treated Single-Minded homolog SIM mouse embryo-derived thioguanine- and ouabain-resistant fibroblast cell line feeder cells) allow infection of spermatogonia with a virus-mediating gene delivery. In vitro retroviral-mediated gene delivery into SSCs of cryptorchid adult or immature mice (under culture conditions) resulted in a stable integration and expression of a transgene in 2–20% of SSCs (Nagano et al., 2001a). After transplantation of the in vitro transduced SSCs into the testes of infertile recipient male mice, 4.5% of offspring derived from the recipient males were found to be transgenic. In addition, the transgene was transmitted to, and expressed in, the subsequent generations. However, in general, it is very difficult to transfer exogenous genes into primary cultures of spermatogonia and the efficiency and definition of integration of exogenous genes into the male genome in vitro have to be improved. It appears that under appropriate culture conditions, spermatogonia can be infected with a virus-mediating gene delivery. This technology may have applications in the management of male infertility in the future if a virus-mediating gene delivery technique is created that allows (in vitro) (a) proliferation or (b) induction/ completion of meiosis of spermatogonia cell fractions that are not able to proliferate or undergo
complete meiotic process, respectively, under normal (in vivo) conditions due to an inherent genetic defect.

Methods to achieve meiotic or post-meiotic differentiation of cultured male germ cells

Co-culture systems of male germ cells with Sertoli cells

In vitro co-culture systems of animal male germ cells with Sertoli cells

Attempts to induce the first and/or the second male meiotic division: Attempts to achieve the differentiation events characterizing mammalian spermatogenesis during in vitro culture of immature germ cells have been reported since the early 1960s. Steinberger et al. (1964) and Steinberger and Steinberger (1965) reported optimal biochemical conditions for animal organ culture resulting in a limited differentiation of rat spermatogenic cells in the presence of Sertoli cells. These pioneer studies demonstrated that at least partial spermatogenesis can be induced in vitro in the presence of Sertoli cells. Cultures of fragments of testicular tissue provide an important tool in studying male germ cell development since both the normal structural relationships among the various testicular compartments and the associations of germ cells with Sertoli cells are maintained during the culture period (Orth et al., 1998). In other studies culture of testicular fragments did not result in the differentiation of rat male germ cells beyond the pachytene stage of the first meiotic prophase (Steinberger, 1975).

Parvinen et al. (1983) have demonstrated that rat primary spermatocytes are able to complete two meiotic divisions, in vitro, after 2 days of culture of defined segments of seminiferous tubules containing late pachytene and diakinet primary spermatocytes. The generated round spermatids had acrosomes. In additional similar experiments it was found that the viability of primary spermatocytes drops to 20% after a culture period of 6 days. This study demonstrated that primary spermatocytes in the presence of Sertoli cells can complete in vitro meiosis. Tres and Kierszenbaum (1983) provided strong evidence that the maintenance of viability of rat spermatogenic cells in vitro is facilitated by their co-culture with Sertoli cells in FSH/growth factor-supplemented (serum-free) medium. In that study spermatogenic cells recovered from 20- to 35-day-old rats were cultured in vitro in the presence of Sertoli cells. Under these in vitro culture conditions, proliferation of spermatogonia and differentiation of meiotic prophase spermatocytes into secondary spermatocytes were demonstrated.

Marh et al. (2003) have demonstrated the production of normal, fertile offspring by ooplasmic nuclear injections of haploid spermatids derived from mouse primary spermatocyte precursors co-cultured with Sertoli cells. Several groups using either midpachytene rat spermatocytes co-cultured with Sertoli cells (Le Magueresse-Battistoni et al., 1991; Weiss et al., 1997) or immortalized mouse testicular germ cells (Hofmann et al., 1994) reported the occurrence of animal (rat or mouse) first and second meiotic division and generation of round spermatids under in vitro conditions. Rassoulzadegan et al. (1993) reported that mouse immature germ cells differentiated up to the spermatid stage in vitro when they had been co-cultured with a cell line, 15P-1, exhibiting features of Sertoli cells. In that study in vitro-derived spermatids never proceeded to form mature spermatozoa (under culture conditions). Hue et al. (1998) reported survival of differentiating rat spermatogenic cells and generation of round spermatids during 3-week culture period in the presence of Sertoli cells within a chemically defined medium supplemented with vitamin and hormones (FSH and testosterone). In that study, tubular fragments had been gently manipulated avoiding the disruption of the interactions between Sertoli cells and spermatogenic cells. The viability of the derived round spermatids was assessed by monitoring their ability to transcribe spermatid-specific genes until the end of the culture period.

Goto et al. (1996) showed that bovine spermatids developed in vitro (as a result of the second meiotic division) and injected into the cytoplasm of bovine oocytes could trigger embryonic development up to the blastocyst stage. Testicular cell suspensions had been filtered through a mesh (of pore size equal to 50 μm) and the filtrate was placed on a culture dish. The authors isolated secondary spermatocytes taking into consideration their size (12–15 μm). They subsequently cultured the isolated cells for 18 h in tissue culture medium with HEPES and calf serum supplemented with epinephrine and norepinephrine at 35°C in 5% CO₂. It was shown that in the above-defined hormone-supplemented medium, the second meiotic division could be induced in the absence of Sertoli cells.

The differentiation of a purified bovine type A spermatogonial population to cells with molecular characteristics of spermatocytes and elongated spermatids was reported by Izadyar et al. (2003) (Table III) after 100 days of culture. Highly purified type A spermatogonia including SSCs and contaminating Sertoli cells and myoid cells were cultured in MEM or the KSOM with different concentrations of FCS at 37°C. Under these culture conditions, Sertoli cells proliferated and formed a monolayer that improved spermatogonial survival and proliferation. The authors suggested that the generation of an actively proliferating Sertoli cellular monolayer might be essential for the survival and differentiation of the spermatogonia cells in vitro. After 2 months of culture, cells in which the nuclei contained three nucleoli resembling those of round spermatids had been formed (Izadyar et al., 2003). The authors used Western blot analysis and immunohistochemical techniques to evaluate the expression of SCP3 (spermatocyte marker) and the ODF-2 protein (first expression in elongated spermatids; Petersen et al., 1999) in the cells post-culture. Cells with the molecular characteristics of spermatocytes and elongated spermatids were demonstrated post-culture. However, abnormalities in nuclear condensation process, cytoplasmic elongation and acrosomal formation were observed in the spermatids.

Feng et al. (2002) have demonstrated that the employment of factors produced by Sertoli cells can induce differentiation of immortalized mouse type A spermatogonia without the presence of Sertoli cells in the culture system. The authors reported the generation and in vitro differentiation of a type A spermatogonial cell line from telomerase-immortalized mouse germ cells. After 1-year culture period, spermatogonial cells were treated with SCF to stimulate spermatogenesis in vitro. SCF induced the generation of spermatocytes within 1 week. After two meiotic divisions, the derived spermatocytes gave rise to haploid step 1 or step 2 round spermatids. Some of the latter cells differentiated into more mature spermatids.
Novel methods for induction of meiosis

Meiotic divisions of male germ cells into ooplasm

Co-cultures of germ cells on feeder layers

- Steinberger et al. (1964): Testis organ culture resulted in a limited differentiation of rat spermatogenic cells
- Matte and Sasaki (1971): Achievement of meiotic maturation of human male germ cells
- Parvinen et al. (1983): Rat primary spermatocytes differentiated up to the round spermatid stage
- Rassoulzadegan et al. (1993): Mouse diploid germ cells differentiated up to the round spermatid stage
- Weiss et al. (1997): Rat primary spermatocytes differentiated up to the round spermatid stage
- Hue et al. (1998): Rat primary spermatocytes differentiated up to the round spermatid stage
- Tesarik et al. (1999): Birth of a healthy child after fertilization of oocytes with elongated spermatids obtained after in vitro meiosis of primary spermatocytes
- Sousa et al. (2002): First in vitro human meiotic maturation with differentiation up to the late spermatid stage
- Izadyar et al. (2003): Bovine type A spermatogonia differentiated up to the elongated spermatid stage
- Marh et al. (2003): Mouse primary spermatocytes underwent meiosis

Co-cultures of germ cells on feeder layers

- Van Dissel-Emiliiani et al. (1993): Failure to culture gonocytes on STO- or BRL-cell lines
- Nagano et al. (1998): Culture of mouse germ cells on STO feeder layers for 4 months
- Cremades et al. (1999): Culture of human round spermatids on a Vero cell monolayer. Maturation was achieved up to the elongating/elongated stage and even to the stage of mature spermatoozon
- Cremades et al. (2001): Differentiation of human round spermatids into late spermatids in Vero cell conditioned medium
- Tanaka et al. (2003): Primary spermatocytes differentiated up to the round spermatid stage after co-culture with Vero cells

Meiotic divisions of male germ cells into ooplasm

- Kimura and Yanagimachi (1995a,b): Development of normal mice after injection of secondary spermatocyte nuclei into M-II oocytes
- Ogura et al. (1998): Ooplasmic injection of mouse primary spermatocyte resulted in the generation of offspring
- Sofikitis et al. (1998a,b): Birth of a child after induction of the male second meiotic division within an oocyte

Novel methods for induction of meiosis in vitro

- Kawamura et al. (2003): In vitro meiosis of human male germ cells in co-culture with xenogeneic Sertoli cells
- Geijsen et al. (2003): Fertilization of oocytes by male haploid germ cells derived from embryonic stem cells in vitro

In vitro spermatogenesis

Table III. Historical steps in the development of methods for maturation of spermatogenic cells in in vitro culture systems

<table>
<thead>
<tr>
<th>Method</th>
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<tr>
<td>Testis organ culture</td>
<td>Resulted in a limited differentiation of rat spermatogenic cells</td>
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<tr>
<td>Culture of mouse germ cells</td>
<td>Differentiated up to the round spermatid stage</td>
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<tr>
<td>Culture of human round spermatids</td>
<td>Differentiated up to the elongated spermatid stage</td>
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<tr>
<td>Culture of human round spermatids</td>
<td>Achieved up to the stage of mature spermatoozon</td>
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<tr>
<td>Culture of human round spermatids</td>
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<td>Culture of human round spermatids</td>
<td>Achieved maturation up to the stage of mature spermatoozon</td>
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</table>

In humans, co-cultures of Sertoli cells and diploid germ cells showed that FSH as potentiated by testosterone acts as a survival factor; it induces meiosis entry, conversion of late St1 to St2 and full spermatid differentiation (Sousa et al., 2002).

Attempts to induce spermiogenesis in vitro: Differentiation of round spermatids has been demonstrated in an elegant study by Gerton and Millette (1984). Seminiferous cellular suspensions, purified pachytene spermatocytes or purified round spermatids of adult mice were cultured in Eagle’s MEM supplemented with fetal bovine serum. After overnight culture some of the isolated round spermatids (20%) and some round spermatids contained in the seminiferous cellular suspensions (20%) elaborated flagella-like structures. The fact that isolated subpopulations of mouse round spermatids are able to undergo generation of flagella within a few hours of culture indicated that the presence of Sertoli cells or factors secreted by Sertoli cells is not necessary to support the later stages of mouse spermatogenic differentiation. The inclusion of lactate in the culture medium of spermatids results in a great stimulation of the cellular metabolism of spermatids and facilitates their differentiation (Jutte et al., 1981). In addition to the above studies, Tres et al. (1991) reported the development of motile flagella of rat spermatids (in vitro co-cultured with Sertoli cells). In similar studies, mouse round spermatids co-cultured with Sertoli cells could generate 1–4 flagella, all emerging from the same cellular pole and displaying actively propagating bending waves (Kierszenbaum and Tres, 2002). It appears that round spermatids can undergo flagella development both in the presence or absence of Sertoli cells.

The above-mentioned studies, in conclusion, indicate that Sertoli cell endocrine or paracrine factors play an important role in maintaining the viability of spermatogonia cells and stimulating animal meiosis in vitro. In the absence of animal Sertoli cells, factors known to be produced by Sertoli cells (i.e. SCF) have been proven to stimulate meiosis and early stages of spermiogenesis. On the other hand, studies evaluating the outcome of the culture of early haploid male gametes in the absence of Sertoli cells in chemically defined media have shown that the elongation of animal round spermatids can be achieved in vitro (in the absence of Sertoli cells and Sertoli cell factors). In addition, the bovine second male meiotic division can be completed in culture systems (negative for Sertoli cells and Sertoli cell factors) containing chemically defined media supplemented with hormones.
In vitro maturation of human male germ cells supported by Sertoli cells

In vitro culture of human diploid or haploid round germ cells: The first studies for the achievement of meiotic maturation of human male germ cells during in vitro culture of testicular crude suspensions were reported by Matte and Sasaki (1971) and Ghatnekar et al. (1974) (Table III). In those studies whole segments of sectioned seminiferous tubules were cultured without any attempt for cellular dissociation. However, the lack of employment of specific markers for accurate confirmation of the identity of the cellular populations pre- and post-culture led to the intense criticism of their findings. Then, several research efforts were directed to the maturation of human round spermatids in vitro. Taking into consideration animal studies showing that the presence of Sertoli cells does not represent a prerequisite for the completion in vitro of some steps of spermiogenesis (Gerton and Millette, 1984), different groups of scientists attempted to achieve maturation of human round spermatids in vitro without the presence of Sertoli cells. However, Bernabeu et al. (1998) failed to induce in vitro morphological maturation of ejaculated round spermatids of azoospermic patients in culture systems negative for Sertoli cells or Sertoli cell factors. The patients were negative for testicular foci of elongated spermatids or spermatozoa. After 1–3 days of culture no morphological changes in the cultured round spermatids were observed. Although ooplasmic injections of these early haploid male gametes, post-culture, elicited high fertilization rate, no pregnancies could be obtained post-embryonic transfer.

On the other hand, rapid flagellar growth of human spermatids in vitro without the support of Sertoli cells was reported by Tanaka et al. (1997) and confirmed in a study by Aslam and Fishel (1998). Few cultured round spermatids (22%) generated flagella after 1–2 days of culture (Aslam and Fishel, 1998). However, the in vitro generated flagella could not move and no other concomitant changes characterizing spermiogenesis occurred. Furthermore, the cells lost their viability gradually during the first 24 h of culture (Aslam and Fishel, 1998). The above two groups employed different culture mediums. Tanaka et al. (1997) used Ham’s F10 medium supplemented with epinephrine and norepinephrine, while Aslam and Fishel (1998) used modified Eagle’s MEM without any hormonal supplementation. In both studies, the cultured spermatogenic cells were incubated in a low temperature (32°C) since it has been suggested that higher temperatures may not be optimal for round spermatid protein synthesis (Nakamura et al., 1978) or have an adverse effect on round spermatid fertilizing capacity (Sofikitis et al., 1996). In studies dealing with the in vitro generation of round or mature spermatids, it is of great importance to use appropriate mediums for spermatid maintenance prior to the application of ooplasmic injections of spermatids. Studies in our laboratory led to the development of the SOF medium for the maintenance of viability and function of spermatogonia, spermatocytes and spermatids for relatively long periods (Sofikitis et al., 1998a,b) in the absence of Sertoli cells. Protection of round spermatids against environmental shock, as well as stabilization of the spermatid membrane, was achieved by adding cholesterol to the SOF medium in low concentrations. Citrate was included in the SOF medium because it regulates the osmotic equilibrium. Vitamins and ferric nitrate were also selected as components of the SOF medium due to their positive influence on spermatid viability (Sofikitis et al., 1998a,b).

Tesarik and co-workers were unable to detect any signs of ongoing human spermigenesis in pure round spermatid cultures without the presence of Sertoli cells (Tesarik et al., 1998a). It was demonstrated that high concentrations of FSH have a positive role in the induction of both male germ cell meiosis and spermigenesis during in vitro culture of human male germ cells in GAMETE-100 medium in the presence of Sertoli cells (Tesarik et al., 1998a). In addition, testosterone was found to potentiate the effect of FSH on meiosis and spermigenesis; this was most likely due to preventing apoptosis in Sertoli cells (Tesarik et al., 1998b,c). In the latter studies, it was suggested that human spermogenesis can proceed in vitro at an unusually fast speed in the presence of Sertoli cells and high concentrations of FSH and testosterone, though the haploid gametes generated are often morphologically abnormal. The authors also suggested that the proximity of Sertoli cells, and not a direct contact, with the cultured spermatogenic cells is necessary for the progression of spermatid cytoplasmic maturation, reflected by acceleration of acrosomal development (Tesarik et al., 1998b). However, in the absence of adequate Sertoli cell-mediated stimuli, the cultured spermatogenic cells are prone to undergo apoptosis. A birth was achieved after fertilization of oocytes with elongated spermatids obtained by in vitro meiosis of primary spermatoocytes and further differentiation (Tesarik et al., 1999). Intracytoplasmic injection of in vitro derived or cultured spermatids resulted in the delivery of five newborns (Tesarik et al., 1999, 2000).

Studies in humans showed that in the absence of Sertoli cells, round spermatids can be normally differentiated in late spermatids using either Vero cell monolayers (Cremades et al., 1999) or Vero cell conditioned medium (Cremades et al., 2001). In highly controlled conditions it has been demonstrated that a 2- to 3-week period of culture is necessary to obtain late spermatids from St1 stage (Cremades et al., 2001).

In vitro culture of testicular spermatozoa: In vitro culture of testicular spermatozoa obtained from testicular biopsies of patients with non-obstructive azoospermia at 30°C in a medium containing Sertoli cells supplemented with recombinant FSH (rFSH) enhanced testicular sperm motility and resulted in higher fertilization rate, implantation rate and clinical pregnancy rate after ICSI procedures compared with ICSI techniques using spermatozoa maintained in standard simple media (without supplementation with rFSH) (Balaban et al., 1999). In addition, the percentage of testicular spermatozoa with DNA strand breakage was significantly lower in testicular biopsy samples post-culture (in the presence of FSH and testosterone) than in testicular biopsy samples pre-culture obtained from patients with non-obstructive azoospermia (Tesarik et al., 2001). When post-culture testicular spermatozoa were used for ICSI procedures resulted in a relatively high fertilization rate (FR) [FR post-culture, 71.6% versus FR pre-culture (previous ICSI attempts), 55–75%]. The authors concluded (Tesarik et al., 2001) that culturing testicular tissue in vitro in the presence of Sertoli cellular material, FSH and testosterone may be recommended even when mature spermatozoa can already be retrieved from the fresh testicular biopsy samples.
In vitro culture of testicular tissue from pre-pubertal patients: Larsen et al. (2002) established a long-term culture system for testicular biopsy material recovered from boys with undescended testes. In that culture system some spermatogonia survived for 3 weeks during culture at 34°C. The addition of rFSH (50 mIU/ml) in the culture medium did not result in any morphological differences between the spermatogonia in the group of testicular fragments that were cultured for 1 week and the spermatogonia in the uncultured fragments (Larsen et al., 2002). However, the addition of LH (5 mIU/ml) either alone or in combination with FSH caused a significant reduction in the ratio of spermatogonia and gonocytes per cross-sectioned testicular tubule (S/T ratio). It appears that the number of spermatogonia is significantly reduced when LH is added to a culture system of testicular biopsy material recovered from boys with undescended testes. This observation may question the role of HCG given often to children with cryptorchidism.

The above-mentioned studies indicate that human male meiosis and elongation of spermatids can be achieved in vitro in the presence of Sertoli cells and FSH and testosterone. On the other hand, there are studies supporting and investigations negating the probability to achieve human spermatid elongation when Sertoli cells are absent in the culture system.

Interaction of spermatogenic cells with somatic feeder cells in co-culture systems

Studies on animal spermatogenesis in vitro

Feeder cell lines are known to have a beneficial effect on cultures of embryonic stem cells and PGCs. This beneficial effect during culture conditions may be due to the cellular associations between the germ cells and the feeder cells or may be attributable to the growth factors and cytokines secreted by the feeder layer (Smith et al., 1988; Matsui et al., 1991). Thus, feeder cells, such as STO cells, have been shown to promote the survival of PGCs in vitro (Dolci et al., 1991; Resnick et al., 1992). However, the first attempts to co-culture gonocytes with somatic cells other than Sertoli cells resulted in a lack of spontaneous proliferation. In that study, Van Dissel-Emiliiani et al. (1993) observed that gonocytes could not adhere to STO or to buffalo rat liver cell lines and subsequently could not be maintained in vitro. In the latter co-culture system gonocytes had formed aggregates with each other and finally after 4 days of culture disappeared.

In contrast, Nagano et al. (1998) showed that mouse SSCs could be cultured in DMEM containing 10% FCS on a feeder layer of STO cells for 4 months. Pre-meiotic germ cells survived under relatively simple culture conditions without the addition of external growth factors and/or hormones into the culture media. Cultured mouse SSCs survived and retained their ability to generate spermatogenesis following transplantation into the testes of recipient mice (Nagano et al., 1998). In general, it appears that culture of spermatogonial cells on feeder layer of STO cells increasing their number provides a larger number of spermatogonia cells and SSCs for transplantation techniques.

Studies on human spermatogenesis in vitro

Induction of meiosis: The Vero cell lineage obtained from kidney cells of the African green monkey (Cercopithecus aethiops) has a characteristic growth pattern in culture (Bianchi and Ayres, 1971). The cells of this lineage growing in monolayers are elongated in shape and are similar to fibroblast cells, with little cytoplasmic granulation (Genari et al., 1998). Secreted factors from Vero cells, including small weight metabolites, interleukins and growth factors (Huang et al., 1997; Desai and Goldfarb, 1998) probably have a beneficial effect on germ cell survival and maturation process. In addition, removing toxic compounds from the culture medium improves the survival and the capacity for differentiation of the cultured germ cells.

Sousa et al. (2002) prepared a mixed cellular population, containing Sertoli cells, spermatogonia and spermatocytes (obtained from testicular biopsies of patients with non-obstructive azoospermia) for culture with Vero cell conditioned medium for up to 21 days. In some experiments the culture system was supplemented with rFSH or rFSH and testosterone (Sousa et al., 2002). Best results were achieved when both hormones had been added to the culture system. This culture system resulted in a meiotic index equal to 6.9%. Differentiation into morphologically normal late spermatids was also achieved after 2 or 3 weeks of culture. In vitro matured round spermatids and normal elongating or elongated spermatids elicited (after ooplasmic injections) a relatively low fertilization rate (30–37%). Some of the fertilized oocytes developed up to blastocysts. However, many of the generated embryos displayed an abnormal sex chromosomal constitution. According to the authors, the abnormal chromosomal profiles of the generated embryos may be attributed to the immaturity of the spermatids. This study provided strong evidence that substances secreted by Vero cells may have a beneficial role in the induction of meiosis and part of spermiogenesis in mixed cultures of male diploid germ cells and Sertoli cells.

Recently, Tanaka et al. (2003) isolated primary spermatocytes obtained from testicular biopsies of patients with non-obstructive azoospermia. These cells were cultured under various conditions on Vero cell monolayers and some of them exposed the capacity to differentiate up to the round spermatid stage. The highest rate of meiosis induction was achieved within MEM medium supplemented with 50% boar rete testicular fluid or within human synthetic oviduct fluid containing 10% human serum. The authors emphasized that when Vero cells had not been included in the culture system none of the cultured primary spermatocytes exposed the capacity to undergo meiosis. The latter study provides strong evidence for the positive role of feeding cell lines or Vero cells in the induction of meiotic divisions in vitro.

Completion of steps of spermiogenesis: Cremades et al. (1999) co-cultured human round spermatids with Vero cell monolayers and demonstrated that it is possible to induce maturation of these early haploid male gametes up to the elongating/elongated spermatid stage and even to mature spermatozoa. In the latter study round spermatids had been recovered from men with non-obstructive azoospermia or globozoospermia. Mature spermatozoa with normal morphology were detected after 5 days of culture of spermatids in two cases. The authors suggested that co-culture of round spermatids with Vero cell monolayers could support full maturation of human round spermatids. In additional similar studies, Cremades et al. (2001) provided further evidence that round spermatids can differentiate in vitro up to the elongated spermatid stage. Round spermatid differentiation up to the elongated spermatid or spermatozoon stage was achieved.
under culture conditions after a 7- to 12-day co-culture with Vero cells (Cremades et al., 2001). The elongating and elongated spermatids obtained in vitro had the capacity to fertilize human oocytes and normal embryonic development was demonstrated. The latter two investigations (Cremades et al., 1999, 2001) support vividly the beneficial role of Vero cell factors on the induction of the spermiogenetic process in vitro.

It appears that factors secreted by Vero cells have a positive influence on the induction of male meiosis or the completion of a part of the spermiogenetic process in vitro. This is of clinical importance for the therapeutic management of non-obstructed azoospermic men with early maturation arrest or complete late maturation arrest (i.e. arrest at the round spermatid stage).

**Induction of meiosis of male germ cells into the cytoplasm of oocytes**

**Completion of the male second meiotic division within the cytoplasm of an oocyte**

Technological improvements in microfertilization techniques have enabled the employment of immature male germ cells as substitute gametes. Generation of normal offspring has been reported after microfertilization of oocytes with round spermatids in the mouse (Kimura and Yanagimachi, 1995a), rabbit (Sofikitis et al., 1994), rat (Sofikitis et al., 1999) and human (Hannay, 1995; Tesarik et al., 1995).

Kimura and Yanagimachi (1995b) have shown that the injection of secondary spermatocyte nuclei into metaphase II (MII) oocytes followed by electrical activation of the mouse oocytes results not only in the completion of the female second meiotic division and extrusion of the female second polar body but also in the completion of the male second meiotic division within the cytoplasm of the oocyte. The overall result is the generation of one round spermatid nucleus (that is subsequently transformed into male pronucleus) within the cytoplasm of the oocyte and the extrusion of a second round spermatid nucleus into the perivitelline space of the oocyte as a male polar body. In that study it was demonstrated that normal mice could develop from oocytes injected with secondary spermatocyte nuclei, indicating that (a) the problem of DNA ploidy associated with the use of such early stages of spermatogenic cells that have not yet completed the second meiotic division could be resolved by the use of this technique and (b) the male second meiotic division can be completed within the ooplasm of an oocyte appropriately activated after the ooplasmic injection. The oocytes had been artificially electroactivated 2 h after the secondary spermatocyte nucleus injection (Kimura and Yanagimachi, 1995b). Following oocyte activation, the chromosomes of both oocyte and secondary spermatocyte completed their second meiotic division (female and male, respectively), culminating in the extrusion of two separate polar bodies and the formation of one female and one male pronucleus in about 75% of the injected oocytes. The 2- or 4-cell embryos arising from the injected oocytes had been transferred to foster mother and 24% developed into normal offspring.

Sofikitis et al. (1998c) reported the birth of a healthy child after injections of human mature oocytes with secondary spermatocyte nuclei obtained from non-obstructed azoospermic men. An activation stimulus was applied to the oocytes 60–120 min post-injections. Fluorescence in-situ hybridization (FISH) techniques in isolated male polar bodies demonstrated haploid (X) or (Y)-n-DNA signal indicating that the chromosomally haploid 2n-DNA of the injected human secondary spermatocyte nucleus had completed the male second meiotic division within the cytoplasm of the oocyte (Sofikitis et al., 1998c; Table III).

Genetically, the fertilization of mature oocytes with spermatids or secondary spermatocytes is reasonable because both cell types are chromosomally in the haploid stage. However, secondary spermatocytes are 2n-DNA cells and spermatids are 1n-DNA cells.

**Induction of both male meiotic divisions within the cytoplasm of oocytes**

Ogura et al. (1997) demonstrated that the chromosomes of primary spermatocytes could undergo meiotic divisions after incorporation into maturing oocytes. Primary spermatocytes were electrofused with immature oocytes shortly before or after germinal vesicle breakdown. After culture for 15 h most of the oocytes containing spermatocyte chromosomes underwent maturation and arrested at MII. It was found that 74% of these oocytes had one group of chromosomes and one polar body, indicating that male chromosomes had intermingled with the female chromosomes and completed the first male meiotic division. Chromosome analyses of these MII oocytes demonstrated their diploidy. Then, the metaphase chromosomes were transferred to enucleated MII oocytes (Ogura et al., 1997); following artificial activation, the reconstructed MII oocytes resumed meiosis and developed till morula or the blastocyst stage. These findings indicate that the chromosomes of primary spermatocytes can undergo meiotic divisions in maturing oocytes and participate in the formation of diploid embryos. However, none of the blastocysts generated by this methodology implanted after embryo transfer. This may be attributed to asynchrony of the primary spermatocyte and oocyte cell cycles. Primary spermatocytes are in the G2 cell cycle phase before the first meiotic division and should be introduced into oocytes of either the G2 or M phase (Ogura et al., 1998). To test this theory Ogura et al. (1998) introduced the nuclei of pachytene/diplotene spermatocytes into oocytes that had been arrested in prophase I, metaphase I (MI) or MII. The authors found that MI arrested oocytes were the best recipients for the nuclei of primary spermatocytes since normal segregation of paternal chromosomes occurred only when the primary spermatocytes were introduced into MI arrested oocytes. Pachytene/diplotene spermatocytes appear to be the youngest spermatogenic cells that can be used to fertilize oocytes and produce normal diploid embryos (three full-term offspring were produced in the latter study; Ogura et al., 1998) since the chromosomes of leptotene/zygotene spermatocytes are not ready to enter MI. This has been demonstrated using chemical treatment with the phosphatase inhibitor okadaic acid (Handel, 1998) and is probably because the synapses between homologous chromosomes in leptotene/zygotene spermatocytes are immature (Handel, 1998). In addition, the birth of healthy offspring in a study by Ogura et al. (1998) provides direct evidence that genomic imprinting in mouse male germ cells is completed before the end of prophase I, as occurs in the oocytes.
In similar research efforts, Sasagawa et al. (1998) achieved the birth of two mouse pups after microfertilization of mouse oocytes with primary spermatocytes (Sasagawa et al., 1998). When single spermatocyte nuclei were injected into MII oocytes, the spermatocyte nuclei transformed into MI configuration, resulting in the formation of oocytes with both maternal (MII) and paternal (MI) chromosomal components. After activation of these oocytes, half of each chromosome set was separated into polar bodies. The male nuclei from polar bodies of paternal origin were transferred into other MII oocytes. This procedure resulted in the formation of oocytes with two sets of Met-II chromosomes. When these oocytes were activated, two pronuclei and two polar bodies were formed and zygotes began development. The successful meiotic divisions of immature spermatogenic cells within the cytoplasm of oocytes confirmed that common factors drive cell cycle progression in both oocytes and spermatocytes. However, in the latter study (Sasagawa et al., 1998) the live birth rate (post-embryonic transfer) was low (0.7%). According to the authors, technical difficulties, incomplete genomic imprinting and/or incomplete DNA repair might be responsible for the poor zygotic development. Another factor contributing to the poor zygotic development after ooplasmic injections of primary spermatocyte genetic material in a study by Sasagawa et al. (1998) may be the fact that sister chromatid pairs of primary spermatocytes segregate prematurely within oocytes undergoing meiosis II. Kimura et al. (1998) reviewed the above-mentioned technical difficulties, genomic imprinting issues and optimal meiotic stages (i.e. synchronization of the male and female gamete) when injections of primary spermatocyte nuclei into oocytes are scheduled. In that study the authors observed a high frequency of chromosomal breakage/rearrangements in oocytes injected with primary spermatocyte nuclei (only one among the 12 injected eggs examined had structurally and numerically normal chromosomes in both paternal and maternal chromosomal groups). Such abnormalities in chromosomal behaviour may be the major cause for the poor preimplantation development of zygotes injected with primary spermatocyte nuclei.

Studies evaluating the developmental potential of oocytes post-injection of secondary or primary spermatocyte nuclei provide a means to obtain knowledge on the developmental competence of male germ cells and the mechanisms of male meiosis. It appears that both male meiotic divisions can be completed within the cytoplasm of female gametes. Thus, the cytoplasm of the female gamete can serve as a biochemical ‘medium’ for the induction of the first or second male meiotic division. The delivery of a healthy human newborn after injections of secondary spermatocyte nuclei recovered from a non-obstructed azoospermic man into oocytes and the experimental studies on the induction in vitro of both male meiotic divisions into the cytoplasm of oocytes are of great clinical importance for the therapeutic management of men with complete early maturation arrest. However, it should be emphasized that the above procedures are susceptible to genetic risks.

**In vitro maturation of human male germ cells in co-culture with xenogeneic Sertoli cells**

The generation of donor spermatogenesis into the testicles of xenogeneic recipient animals after transplantation of donor germ cells into the seminiferous tubuli of the recipient animals suggests that the xenogeneic recipient Sertoli cells have the capacity to support the survival and subsequently the differentiation of donor chromosomally diploid germ cells into haploid cells (Russell and Brinster, 1996; Sofikitis et al., 2003). It appears that growth factors, proteins and hormones secreted by recipient Sertoli cells have the capacity to stimulate meiosis and spermiogenesis of donor xenogeneic germ cells. Recent research efforts in our laboratory have been directed to the induction of male meiosis of human spermatagonia/primary spermatocytes collected from non-obstructed azoospermic men in a co-culture system with xenogeneic Sertoli cells. Thus, we developed two culture systems containing rat germ cells, rat Sertoli cells and human testicular germ cells (Kawamura et al., 2003). The human testicular germ cells had been recovered from 12 non-obstructed azoospermic men with complete spermatogenic arrest at the primary spermatocyte stage (12 experiments were performed). FISH techniques and transmission electron microscopy techniques in testicular tissue from the latter men confirmed the absence of both spermatids and spermatocytes.

Type 1 system contained rat Sertoli cells (number of nuclei, $2 \times 10^5$/ml), rat round germ cells (60–70 $\times 10^5$/ml; rat round germ cells and Sertoli cells had been separated from rat elongated spermatids and spermatozoa by enzymatic treatment with pronase as we previously described for the mouse species; Yamamoto et al., 1999) and human spermatogonia/primary spermatocytes (60–70 $\times 10^5$/ml). Human spermatogonia/primary spermatocytes were collected after mincing and filtering via a 19–20 $\mu$m pore size filter and then via a 13 $\mu$m pore size filter (quantitative morphometric parameters of human spermatocytes have been previously published; Sofikitis et al., 1998c) testicular biopsy specimens recovered from the above 12 men. The sediment in each 13 $\mu$m pore size filter (containing human spermatogonia/primary spermatocytes) was kept and the filtrate was discarded (Kawamura et al., 2003). These purification procedures of human spermatogonia/primary spermatocytes were similar to those we previously published for mouse spermatogonia/primary spermatocytes purification (Yamamoto et al., 1999). Prior to culture, FISH techniques (with probes for human chromosomes 18, Y and X) were applied in subfractions of cells to distinguish between: (a) human and rat germ cell nuclei and (b) confirm the absence of human Sertoli cells and spermatids. Thus, it was possible to count the number of each cell type pre-culture.

Type 2 system contained rat round germ cells (60–70 $\times 10^5$/ml; prepared as previously described for mouse round germ cells; Yamamoto et al., 1999) and human spermatogonia/primary spermatocytes (60–70 $\times 10^5$/ml). Type 2 system contained less than 100 000 rat Sertoli cell nuclei/ml. In type 1 system, the rat minced testicular material had not been filtered, prior to its treatment with pronase; therefore, the type 1 system was positive for a significant number of rat Sertoli cells (Yamamoto et al., 1999). In contrast, in type 2 system, the rat minced testicular material had been filtered repeatedly prior to its treatment with pronase (as we previously described for mouse testicular tissue preparation; Yamamoto et al., 1999) and the vast majority of rat Sertoli cell nuclei were removed (they did not pass through the filters but they remained in the sediments of the respective filters; Yamamoto et al., 1999). Rat FSH had been added to both culture systems (final concentration of 15 ng/ml).
Human rFSH had also been added to both systems (final concentration of 50 IU/ml) as well. Water-soluble testosterone had also been added to both systems (final concentration of 1 mmol/l). Seventy-two hours post-culture 1.8–4.6 (× 10^6/ml) and 0 human round spermatids were found in the type 1 system and type 2 system, respectively, by FISH techniques and confocal scanning laser microscopy. Human elongated spermatids or human spermatozoa could not be demonstrated in type 1 systems or type 2 systems (Kawamura et al., 2003).

It appears that animal Sertoli cells can stimulate human meiosis in vitro, in other words, animal Sertoli cellular paracrine or endocrine factors can affect positively human meiosis in vitro. Although it should be emphasized that co-culture of animal Sertoli cells with human germ cells may result in virus transmission to the human cells or contamination of the human germ cells by animal antigens or animal cellular membrane-binding molecules, the probability to induce human meiosis in germ cells collected from non-obstructed azoospermic men after co-culture with animal Sertoli cells may be of clinical significance in the future and justifies further experimental efforts on this direction. It is known that some types of oligospermia and azoospermia do not have a genetic aetiology and are due to Sertoli and Leydig cell secretory deficiency resulting in a non-permissive testicular microenvironment for the completion of spermatogenesis (Sofikitis et al., 1998a, 2003). Thus, men with non-obstructive azoospermia due to a non-permissive testicular microenvironment attributable to secretory deficiency of testicular somatic cells may become candidates for ART after successful culture of their chromosomally diploid germ cells with animal Sertoli cells.

**Male germ cells derived from embryonic stem cells or PGCs in vitro**

Stem cells are unique in their ability to self-renew, proliferate indefinitely and differentiate into specialized tissues according to the source of the stem cells (Lo et al., 2003). Blastomeres from 2- to 8-cell-stage embryos are considered totipotent because they can differentiate and generate a complete organism. Pluripotent stem cells retain the ability of (a) self-renewal and (b) to differentiate into cells and tissues from all three germ layers (Lo et al., 2003). These multipotent stem cells persist into adulthood and are responsible for the regenerative capacity of several tissues. Tissues with regenerative potential include the gastrointestinal, integumentary, haematopoietic and spermatogenic systems (Lo et al., 2003).

SSCs originate from PGCs. PGCs require several members of the BMP ligand family for their development, which proliferate and migrate to the genital ridges. SSCs are responsible for maintaining spermatogenesis throughout life in males by proliferation and differentiation; these characteristics depend on their ability to self-renew and produce daughter cells that differentiate into spermatozoa. It has been shown (Brinster and Zimmermann, 1994) that SSCs isolated from testes of donor male mice would repopulate immunologically compatible sterile testes when injected into the seminiferous tubules of these recipient animals. Donor spermatogenesis in recipient testes showed normal morphological features characteristic of the donor species. In another study (Brinster and Avarbock, 1994), it was shown that mouse SSCs transplanted into the testes of infertile mice colonized the recipient seminiferous tubuli and initiated donor spermatogenesis in more than 70% of recipients. The most striking result of these experiments was the production of healthy offspring (by matings) from spermatozoa generated within the recipient testes by donor SSCs. Another group (Jiang and Short, 1995) transferred PGCs or gonocytes recovered from male rat fetuses or neonates into the rete testes of adult recipients that had been treated with busulfan. The authors indicated the achievement of normal donor spermatogenesis in 62% of recipients. The authors concluded that the pattern of donor cell colonization and donor spermatogenesis following transplantation in terms of their spatial location and connection with the recipient seminiferous epithelium depends on the developmental stages of the donor cells at transfer. In a similar study (Ogawa et al., 2003) SSCs were transplanted from an infertile mouse strain (SI mouse as donor of germ cells) to an infertile recipient mouse [dominant white spotting (W)] to determine if SSCs from that infertile male mouse are capable of generating spermatogenesis. Post-transplantation, the recipient mice were demonstrated to be fertile. Thus, fertility was restored after transplantation of SSCs from an infertile donor into an infertile recipient. Xenogeneic transplantation of mammalian SSCs was successful as well. One group (Clouthier et al., 1996) transplanted rat germ cells into the seminiferous tubuli of immunodeficient mice and generated rat spermatogenesis in all 10 recipient mice. Some recipients were found positive for donor spermatozoa into their epididymis. Sofikitis et al. (2003) demonstrated, at the level of the transmission electron microscope, the development of hamster round spermatids in the seminiferous tubuli of immunodeficient animals (mice and rats) after transplantation of SSCs recovered from cryptorchid hamsters into the tubuli of the immunodeficient animals. Although SSCs arise from PGCs, it is not clear whether fetal male germ cells function as SSCs able to produce functional spermatozoa. Transplantation of fetal germ cells into the seminiferous tubules of an adult testis demonstrated that donor germ cells (recovered at 14.5 days post-coitum) are able to initiate spermatogenesis in the adult recipient seminiferous tubuli (Ohta et al., 2004). Recently, spontaneous differentiation of male germ cells from human embryonic stem cells in vitro has been reported (Clark et al., 2004). The results of the latter study showed that the differentiation of human embryonic stem cells into embryoid bodies in vitro results in formation of cells that express markers specific to gonocytes. Suemori et al. (2001) established embryonic stem cell lines from monkey blastocysts produced by IVF or ICSI. If the results of the latter study can be reproduced in the human, this might be of great clinical importance in the future since additional human resources for stem cells will be available. The latter sources may be valuable for producing embryonic stem cell-derived spare tissues to treat diseases. In vitro studies have shown that both male and female PGCs demonstrate cell-autonomous entry into meiosis and differentiation into oocytes if they are set apart from the male gonadal environment. Transition from the mitotic PGC stage into the leptotene stage of the first meiotic division has been achieved in vitro (Nakatsuji and Chuma, 2001). Such entry into meiosis appears to be programmed in PGCs before reaching the genital ridges and unless it is inhibited by putative signals from the testicular somatic cells. In conclusion, differentiation of
mouse PGCs into female or male germ cells has been achieved in vitro (Nakatsuji and Chuma, 2001). Naito et al. (1999) have shown that PGCs isolated from embryonic blood can differentiate into functional gametocytes. Chuma et al. (2005) demonstrated that epiblast cells and PGCs can establish colonies of spermatogenesis after transfer into post-natal seminiferous tubules of surrogate infertile mice. Furthermore, they obtained normal fertile offspring by microinsemination using spermatozoa or spermatids derived from PGCs harvested from fetuses as early as 8.5 days post-coitum.

Recently, scientists were able to isolate and cultivate mouse embryonic stem cells and produce functional PGCs in vitro (Toyooka et al., 2003). The generated PGCs were transplanted under the testicular capsules of host nude mice. Transplanted cells progressed to the final stages of male gamete differentiation (elongated spermatids). In addition, Geijsen et al. (2003) achieved fertilization of mouse oocytes after ooplasmic injections of haploid male gametes derived from in vitro maturation of PGCs. Embryoid bodies supported the maturation of the PGCs into haploid male gametes. The injected oocytes restored the somatic diploid chromosome complement and developed into blastocysts. The derivation of male gametes from embryonic stem cells provides an in vitro model system for studies on germ line epigenetic modification and mammalian gametogenesis.

Hubner et al. (2003) have demonstrated that mouse embryonic stem cells in culture can also develop into oogonia that have the capacity to enter meiosis, recruit adjacent cells to form follicle-like structures and later develop into blastocysts. The derivation of oocytes can be accomplished by both female and male embryonic stem cells. However, the generation of haploid male and female gametes from pluripotent embryonic stem cells is susceptible to ethical issues. Application of this technique to humans raises the probability that two men could have a biologically related child carried by a surrogate mother. On the other hand, the demonstration of haploid female and male gamete generation from embryonic stem cells provides the opportunity for investigating germ cell development, epigenetic reprogramming and germ line gene modification. Although the above studies raise the spectre of children derived from artificial sex cells, cautions against attempting to apply this methodology to human infertility management should be considered. At first, there is no way to test whether the embryonic stem cells have acquired mutations during the cell in vitro maintenance (Vogel, 2003). In addition, stem cell consequences of embryo epigenetic defects should be kept in mind (Allegrucci et al., 2004). Increasing evidence from a range of mammals shows a propensity for epigenetic errors with embryo technologies or manipulations. If paralleled in human embryos, the effects of embryo manipulation technologies on tumorigenic and differentiation properties of embryonic stem cells need to be established (Allegrucci et al., 2004).

There is much debate and ethical barriers regarding the employment of embryonic stem cell technology for infertility treatment, testis/ovary regeneration or any other therapeutic purposes. Indeed, there is an increasing amount of evidence suggesting that adult stem cells from various organs can contribute to the regeneration of other often dissimilar organs (Lo et al., 2003). For example, stem cells from bone marrow, which arises from mesoderm, have been shown to contribute to the regeneration of liver which arises from endoderm (Uchida et al., 1998).

A new promising type of adult stem cells, multipotent adult progenitor cells (MAPCs), derived from murine bone marrow have been cultured and differentiated into cells of all three germ layer origin in vitro. Transgenic studies have demonstrated the presence of MAPC origin colonization in multiple organs of chimeric progeny (Lo et al., 2003). Although opponents of embryonic stem cell research are already heralding MAPCs as proof that study of human embryonic stem cells is no longer needed, many scientists support that there is still lack of evidence demonstrating that adult stem cells match the versatility of embryonic stem cells derived from embryos (Lo et al., 2003) and that further research efforts on embryonic stem cells are necessary.

Reprogramming of genome function through manipulation of factors that affect epigenetic mechanisms, in the future, may allow reprogramming of differentiated cells back to totipotency, which in turn may result in redifferntiation of the full repertoire of adult cells from a single original cell of any kind (Surani, 2001). A better understanding of mechanisms that regulate this exceptional genomic plasticity may allow manipulation of stem cells for male infertility-related therapeutic purposes.

Scientific and clinical applications of culturing male germ cells

Applications in basic research

In vitro culture of SSCs promises to become a powerful tool in studies concerning the control of spermatogonial proliferation and differentiation increasing our understanding of the molecular regulation of spermatogonial multiplication and stem cell renewal. Experimental models in animals (Table I) enable the development of testicular pathophysiologies in which seminiferous tubules contain only Sertoli cells and undifferentiated spermatogonia cells (Sofikitis et al., 2003). These animal models provide a relatively rich source for purification of stem cells. In addition, long-term culture of SSCs enables generation of sufficient number of cells for germ line gene modification.

In vitro culture of testicular mixed spermatogonial populations represents an attractive way to increase the SSC subpopulation for transplantation purposes. Thus, in studies having transplantation of spermatogonia into recipient host testes as an objective, in vitro culture of the spermatogonial fractions prior to transplantation will provide a larger number of SSCs available for transplantation. This is of great importance given the facts that (a) the SSCs, having the potential to renew themselves, play an important role in the colonization of the recipient seminiferous tubuli and (b) the number of available stem cells in any testicle is limited.

Recently, the introduction of a foreign gene into the genome of mouse or rat SSCs in vitro by retrovirus-mediated infection resulted in the production of immortalized germ cell lines (Hofmann et al., 1994; Nagano et al., 2001a; Hamra et al., 2002). The availability of immortalized germ cell lines together with testicular somatic cell lines offers an opportunity to study the molecular mechanisms regulating meiosis as well as the interactions between germ cells and Sertoli cells.

A precise manipulation of SSC genome by gene transfer is not efficient till now since these exogenous genes are integrated randomly into the genome. On the other hand, these experiments
represent the initial step to explore new genetic engineering methods and the development of new transgenic technologies. Ultimately, these in vitro culture techniques of SSCs followed by gene integration by retrovirus-mediated infection and/or cellular transplantation techniques into recipient animals might provide new modes for the therapeutic management of genetic diseases or male infertility-related pathophysiologies (of genetic origin) in humans (Sofikitis et al., 2003).

Clinical applications

Therapeutic management of non-obstructive azoospermia

Employment of the technique of in vitro maturation of human male germ cells may have an important role in ART. It is known that a significant percentage of non-obstructed azoospermic men have testicular foci of active spermatogenesis up to the spermatid or spermatozoon stage (Silbert, 1996; Amer et al., 1997; Antinori et al., 1997; Sofikitis et al., 1998a,b). Ooplasmic injections of spermatozoa (Silbert et al., 1995; Palermo et al., 1998), spermatids (Amer et al., 1997; Antinori et al., 1997; Van der zwalmen et al., 1997; Sofikitis et al., 1998b) or secondary spermatocytes (Sofikitis et al., 1998c) recovered from testicular foci of spermatogenesis of non-obstructed azoospermic men have resulted in alleviation of infertility associated with non-obstructive azoospermia. In contrast, non-obstructed azoospermic men with complete pre-meiotic block in spermatogenesis (i.e. meiotic arrest at the primary spermatocyte stage is the most common type of maturation arrest found in men with non-obstructive azoospermia; Martin du Pan and Campana, 1993) do not have foci of haploid cells in their testicular tissue and subsequently cannot be candidates for participating in ART programmes, nowadays. In vitro culture of spermatogonia or primary spermatocytes recovered from men with early maturation arrest (i.e. arrest at the primary spermatocyte stage) having the potential to overwhelm the in vivo pre-meiotic block opens a new perspective to the management of infertility for these men. In addition, candidates for the performance of male germ cell culture techniques in vitro could be non-obstructed azoospermic men negative for testicular spermatozoa but positive for round spermatids in the testicular biopsy material (Cremades et al., 1999, 2001). Thus, non-obstructed azoospermic men with complete late maturation arrest (complete arrest at the round spermatid stage; Tesarik, 1998) may have a benefit from the clinical application of male germ cell culture procedures in vitro. For the latter men, performance of testicular round spermatid injections (ROSI) or testicular round spermatid nuclear injection (ROSNI) techniques offers them a probability to father their own children. However, round spermatid material injection techniques have been proven to be of seldom beneficial clinical interest and the pregnancy rate after the application of these techniques is very low. In vitro maturation of testicular round spermatids and production of elongating and/or elongated spermatids or even spermatocytes is of clinical significance for men with complete spermatogenetic arrest at the round spermatid stage because the outcome of ooplasmic injections of elongating/elongated spermatids is much higher than the outcome of ROSI or ROSNI procedures (Sofikitis et al., 1998a). The development of flagella by round spermatids cultured in vitro provides an excellent way to identify their viability. Intracytoplasmic injections of in vitro derived or cultured spermatids resulted in the delivery of five newborns (Tesarik et al., 1999, 2000).

Other groups have similarly demonstrated that in vitro matured spermatids are able to fertilize human oocytes and elicit normal embryonic development. Cremades et al. (2001) showed that injections of in vitro generated elongating and elongated spermatids into oocytes resulted in a fertilization rate of 40% and a blastocyst formation rate of 60%. Sousa et al. (2002) demonstrated that injections of in vitro derived round spermatids elicited 37.5% fertilization rate and 28.6% blastocyst formation rate. In vitro generated elongating and elongated spermatids with abnormal morphological characteristics enabled 8.3 and 27.3% fertilization rates, respectively. However, no fertilized oocyte developed up to the blastocyst stage. Morphologically normal elongating and elongated (in vitro generated) spermatids elicited 30.5% fertilization rate and 42.9% blastocyst development rate. However, most of the generated embryos showed sex chromosomal abnormalities (Sousa et al., 2002).

Culture of ejaculated round spermatids obtained from men with non-obstructive azoospermia for 24h, although did not influence cell morphology, elicited a higher fertilization rate after injection into oocytes compared with non-cultured round spermatids (Bernabeu et al., 1998). Given that it is not possible, nowadays, to induce maturation of ejaculated round spermatids in vitro up to the stage of elongated spermatids, it appears that testicular biopsy should be performed in non-obstructed azoospermic men who ejaculate round spermatids and participate in ART programmes. This recommendation is supported by studies suggesting that a significant percentage of non-obstructed azoospermic men who ejaculate round spermatids are positive for testicular spermatozoa (Sofikitis et al., 1998a).

Tesarik et al. (2001) demonstrated that culture of testicular biopsy samples yields a significantly larger percentage of spermatozoa without apoptosis-related DNA damage than the respective percentage in the original spermatozoal population (prior to culture). This is important because apoptotic process in early haploid male gametes appears to be one of the factors contributing to the low outcome of ROSI/ROSNI procedures in cases of complete spermiogenetic failure (Tesarik et al., 1998c). Similarly, Balaban et al. (1999) suggested that culturing testicular spermatozoa for approximately 24 h in rFSH supplemented medium containing Sertoli cells yields an increased number of motile spermatozoa available for ICSI. Thus, culture of testicular biopsy samples, recovered from non-obstructed azoospermic men, may be recommended even if these biopsy samples are positive for testicular spermatozoa.

Salvaging reproductive potential in patients with oncological disease

Application or employment of toxic agents in humans (Sofikitis et al., 1995) may affect the secretory function of Sertoli cells or Leydig cells resulting in a non-permissive intraseminiferous tubular environment with suboptimal profiles (within the seminiferous tubuli) of ABP or testosterone, respectively. The overall result may be development of quantitative and/or qualitative defects in spermatogenesis.

The detection, isolation and maintenance (in vitro) of SSCs from pre-pubertal boys with oncological disease who are scheduled to undergo chemotherapy are not practical due to
the very low number of stem cells in pre-pubertal testes (10⁴ germ cells are thought to contain about two stem cells; Meistrich and van Beek, 1993). The development of an in vitro culture system to increase via mitotic divisions the number of available (for maintenance in vitro) SSCs of boys or men with oncological disease is of paramount importance. Such an in vitro culture system allows spermatogonial populations (containing SSCs) that have been isolated from recovered testicular tissue (prior to the application of chemotherapy) to be cultured (to increase the SSC number) and subsequently processed for freezing procedures (Sofikitis et al., 2003). Several months after the end of chemotherapy, the cultured/frozen/thawed spermatogonia cells that contain SSCs subpopulations can be cultured again and then transferred back to the seminiferous tubuli or rete testis (i.e. autotransplantation procedures) (Kaponis et al., 2003) of the patients aiming to recolonize the patients’ seminiferous tubuli with SSCs that have not been affected/exposed to chemotherapeutic agents (Sofikitis et al., 2003). These cultured/frozen/thawed/re-cultured SSCs retain their ability to undergo mitoses, meiosis and spermiogenesis post-autotransplantation (Sofikitis et al., 2003). Recently, Hasthorpe (2003) developed an in vitro clonogenic system in that neonatal mouse spermogonia can be replicated successfully in vitro. Development of a similar clonogenic system in vitro in boys or men with oncological disease who are going to undergo chemotherapy may allow the preservation of their fertility after autotransplantation of these in vitro proliferating/proliferated SSCs back to the patients’ testes (several months after the end of chemotherapy; Sofikitis et al., 2003).

It should be emphasized that freezing testicular SSCs from patients with oncological disease has two advantages compared with the cryopreservation of spermatozoa: (a) SSCs can undergo mitoses during culture in vitro both before and after the freezing/thawing techniques; thus, at the end of the first (prefreezing) or the second (post-freezing) culture period new SSCs will have developed and finally all these proliferating/proliferated SSCs can be either transferred back to the patients’ testes (Sofikitis et al., 2003) or cultured in vitro to produce haploid germ cells and (b) spermatozoa are finally differentiated haploid cells that cannot undergo mitosis and thus freezing any spermatogonial population represents maintaining in vitro a limited part of the genotype of the patient; in contrast, by culturing, freezing, thawing and then re-culturing a relatively large number of SSCs, a larger amount of genetic information of the patient can be maintained (Sofikitis et al., 2003) since, under culture conditions, SSCs can renew themselves, and additionally their chromosomes can undergo the process of chiasma formation (allowing exchange of chromosomal genetic material) during the first meiotic division (at the stage of primary spermatocyte).

**Preservation of endangered species**

In vitro culture of spermatogonia populations (containing SSCs) of endangered species increasing the number of SSCs enables, subsequently, the cryopreservation of spermatogonia cells containing a larger subpopulation of SSCs. The latter frozen cells after thawing techniques and additional culture procedures may be transplanted into the testes of immunodeficient animals having the establishment of the endangered species spermatogenesis within a recipient animal testis as an objective (Sofikitis et al., 2003). The recipient animal may become able to produce both its own gametes and xenogeneic gametes as well (Sofikitis et al., 2003).

**Genetic and epigenetic risks after ART using in vitro generated male haploid germ cells**

Although induction of human meiosis and spermiogenesis in an in vitro culture system represents an attractive alternative solution for the therapeutic management of men who are positive for spermatogonia/spermatocytes but negative for haploid cells in their testes, the application of this approach may be limited by ethical considerations or safety-related factors. For instance, application of ooplastic injections of human haploid cells generated in vitro culture systems containing xenogeneic Sertoli cells (Kawamura et al., 2003) is susceptible to ethical considerations and risks concerning contamination of the human germ cells by animal viruses or animal molecules. Similarly, a major drawback for the application of ooplastic injections of haploid male gametes derived in vitro culture systems of human diploid germ cells with supporting animal feeder somatic cells, such as Vero or STO cells, concerns the risks of transmitting infectious agents to the human germ cells. The growth phase of Vero cells is usually achieved in the presence of newborn calf serum, which still poses the risk of virus or animal molecule transmission to the cultured human cells (Cremades et al., 2001). In addition, performance of ART procedures using immature haploid germ cells derived or cultured in vitro is susceptible to genetic and epigenetic risks.

**Genetic considerations**

Kimura et al. (1998) observed high frequency of abnormalities in male meiotic chromosomal behaviour when they injected mouse primary spermatocytes into the ooplasm of MII oocytes. It seems that most primary spermatocytes have not acquired the competence for normal chromosomal segregation within the ooplasm and/or that the ooplasm does not provide adequate factors required to segregate the spermatocyte chromosomes that are still synapsed.

In the human, Sousa et al. (2002) reported that most of the embryos, produced after ooplastic injections of spermatids that had been generated in vitro, demonstrated sex chromosomal abnormalities. The high abnormal genetic constitution of the derived human embryos may have been due to (a) a deficient male meiotic process in vitro or (b) the immature status of the in vitro generated haploid cells. Tesarik et al. (1998a) showed a very rapid progression of meiosis and/or spermiogenesis during in vitro culture of human primary spermatocytes and/or round spermatids, respectively. It is possible that the action of multiple checking mechanisms, whose role is to control/coordinate the male gamete morphogenetic and molecular transformations during spermatogenesis in vivo, cannot be completed (totally or partially) during the in vitro culture of spermatogenic cells. The overall result may be a high percentage of abnormal products of meiosis and/or spermiogenesis. This is consistent with the fact that an increase in DNA degradation of round spermatids during in vitro culture has been observed (Tesarik et al., 1998c). Thus, it appears that the clinical employment of ooplastic injections of in vitro derived haploid germ cells may be
associated with genetic risks attributable to the completion of meiosis or a part of the spermiogenetic process under in vitro conditions. In addition, the clinical employment of ooplasmic injections of in vitro generated haploid germ cells may be susceptible to genetic risks that are related with the population of the men to whom ART is applied. Thus, ooplasmic injections of immature haploid spermatogenic cells derived in vitro from diploid germ cells (that had been recovered from men with non-obstructive azoospermia) may carry a substantial risk for transmitting chromosomal or gene defects. The latter men may have an intrinsic genetic problem, such as increased DNA fragmentation and/or mutations/deletions affecting specific regions (AZFa, AZFb and AZFc) of the Y chromosome (Sofikitis et al., 1998a). Successful in vitro culture techniques of diploid germ cells from these men may have as a result that the above genetic alterations will be transmitted to the generated embryos and offspring after ooplasmic injections of the generated in vitro haploid gametes.

Epigenetic risks

In addition to the above-described genetic factors, defects in epigenetic factors may contribute to the abnormal characteristics of embryos produced by ooplasmic injections of in vitro derived male gametes (Sofikitis et al., 1998a; Sousa et al., 2002). Abnormalities/defects in the expression of oocyte-activating factor in in vitro produced male haploid cells may result in defects in the capacity of the male gamete (after its entrance into the ooplasm) to activate the cascade of ooplasmic events that result in fertilization and normal embryonic development. Furthermore, deficiency in the functionality of the reproducing element of the primary spermatocyte (Schatten, 1994) or the presence of an abnormal number of centrioles in in vitro derived haploid male gametes may cause aberrant spindle formation, after male gamete ooplasmic injections, resulting in aberrant embryonic development. In addition, Luetjens et al. (1999) demonstrated that abnormalities in the male gamete nucleus condensation could retard the sperm X chromosome decondensation resulting in embryonic aneuploidy through zygotic mitotic errors. Thus, we cannot rule out the probability that (a) abnormalities in the nuclear condensation status of in vitro derived haploid gametes or (b) abnormalities in the capacity of in vitro derived spermatocytes to decondense at an appropriate chronological order within the ooplasm (post-ICSI) may cause chromosomal abnormalities in the embryos.

Achievement of the induction of meiosis of male diploid germ cells and partial completion of spermiogenes genetic factors may contribute to the abnormal characteristics of embryos produced by ooplasmic injections of in vitro derived male gametes (Sofikitis et al., 1998a; Sousa et al., 2002). Abnormalities/defects in the expression of oocyte-activating factor in in vitro produced male haploid cells may result in defects in the capacity of the male gamete (after its entrance into the ooplasm) to activate the cascade of ooplasmic events that result in fertilization and normal embryonic development. Furthermore, deficiency in the functionality of the reproducing element of the primary spermatocyte (Schatten, 1994) or the presence of an abnormal number of centrioles in in vitro derived haploid male gametes may cause aberrant spindle formation, after male gamete ooplasmic injections, resulting in aberrant embryonic development. In addition, Luetjens et al. (1999) demonstrated that abnormalities in the male gamete nucleus condensation could retard the sperm X chromosome decondensation resulting in embryonic aneuploidy through zygotic mitotic errors. Thus, we cannot rule out the probability that (a) abnormalities in the nuclear condensation status of in vitro derived haploid gametes or (b) abnormalities in the capacity of in vitro derived spermatocytes to decondense at an appropriate chronological order within the ooplasm (post-ICSI) may cause chromosomal abnormalities in the embryos.

An important issue is whether genomic imprinting establishment has been completed in immature male haploid gametes. Kerjean et al. (2000) demonstrated that the methylation patterns of H19 and MEST/PEG1 genes are established as early as spermatogonial differentiation in humans. On the other hand, Ariel et al. (1994) demonstrated that spermatogenesis-specific genes undergo late epigenetic reprogramming at the level of epigenetics. Studies in animals suggest that at least in the mouse, genomic imprinting is complete in the testis at/prior to the primary spermatocyte stage (Fishel et al., 1996; Ogura et al., 1998). Studies in our laboratory have suggested that in the rabbit the genomic imprinting is complete at/prior to the round spermatid stage (Sofikitis et al., 1994). Hajkova et al. (2002) have demonstrated that mouse PGCs exhibit dynamic changes in epigenetic modifications between days 10.5 and 12.5 postcoitum. PGCs acquire genome-wide de novo methylation during early development and migration into the genital ridge. However, following their entry into the genital ridge there is a rapid erasure of DNA methylation of regions within imprinted and non-imprinted loci. Thus, there is an active demethylation process initiated upon the entry of PGCs into the gonadal anlagen. The time of reprogramming of PGCs is of paramount importance because it ensures that germ cells in the male sex acquire a certain epigenetic state prior to the differentiation of the definitive male germ cells in which new parental imprints are then established (Hajkova et al., 2002). Defects in the epigenetic reprogramming in any cultured (in vitro) immature diploid germ cell population may result in the inheritance of epimutations in the haploid cells generated from the culture of the immature germ cells. The fact that DNA methyltransferase is present in spermatids may be an argument against the thesis that genomic imprinting is complete at the round spermatid stage. Another hypothesis is that even if the genomic imprinting has not been completed at the round spermatid stage, the male gamete genomic imprinting may be completed after the transfer of immature haploid spermatogenic cells within the ooplasm (Kimura and Yanagimachi, 1995a,b) or even during the early embryonic development (Fishel et al., 1996; Sofikitis et al., 1998a). This hypothesis is supported by the fact that waves of DNA methylation have been demonstrated during early embryonic development, the blastocyst stage and the time of implantation (Fishel et al., 1996). There is strong evidence supporting the presence of activity of the DNA methyltransferase during early embryonic development (Fishel et al., 1996; Sofikitis et al., 1998a). In addition, from the limited data available, it appears that the imprint establishment has been completed in humans by the time the spermatid stage is reached (Shamanski et al., 1999; De Rycke et al., 2002). Although most of the above studies tend to suggest that the genomic imprinting process in the human has been completed prior to the spermatid stage in vivo, it is unknown whether the rapidly proceeding meiosis and early spermiogenesis occurring under in vitro culture conditions allow the completion of genomic imprinting process within these relatively short periods. This is a question of clinical importance because abnormalities in the completion of genomic imprinting during in vitro gameteogenesis may be manifested (post-fertilization) as tumour susceptibility and/or tumorigenesis. Follow-up studies of children born after ICSI with epididymal and testicular spermatozoa have shown no additional risks as compared with children born after
ICSI with ejaculated spermatozoa (Bonduelle et al., 2002). However, there is a case report of two major malformations among four pregnancies obtained after ICSI with elongated spermatids (Zech et al., 2000). Another study on larger series did not detect an increased incidence of malformations (Sousa et al., 2000) after ooplasmic injections of spermatids. However, considering that the number of human pregnancies achieved after ooplasmic injections of in vitro generated spermatids is limited, no definite conclusions can be drawn on the safety of ooplasmic injection of in vitro generated early haploid male gametes.

Another epigenetic mechanism the male gamete undergoes during spermiogenesis in vivo is the replacement of the nuclear histones (low disulphide bond proteins) by protamines (high disulphide bond proteins). Histones protect the early haploid male gamete DNA (within the cytoplasm of the oocyte) after ooplasmic injections. The presence of low disulphide bond proteins around the round spermatid DNA post-ROSI or post-ROSI has been considered to be a factor responsible for the low outcome of these techniques. In contrast, protamines protect the spermatozoal DNA within the ooplasm (post-ICSI techniques). In the case of ooplasmic injections of in vitro derived spermatozoa, the survival of the spermatozoal DNA within the ooplasm may be detrimentally affected by defects in the male gamete nuclear protein matrix that have been developed due to the short in vitro culture period that does not allow the haploid male gamete nuclear protein matrix to undergo the normal alterations characterizing the spermiogenesis process. There are epigenetic differences between the parental genomes during the evolution of genomic imprinting in mammals. These epigenetic differences between the parental genomes are enhanced in the zygote by means of DNA demethylation of the paternal genome shortly after fertilization, whereas the maternal genome demonstrates de novo methylation (Mayer et al., 2000). Such opposite effects on the parental genomes within the same oocyte cytoplasm might be achieved by the differential binding of stored cytoplasmic factors to the paternal genomes (Arney et al., 2002). Arney et al. (2002) have demonstrated a preferential interaction of HP1beta protein with the maternal genome immediately after sperm entrance into the mouse oocyte. Paternal genome binding of HP1beta is only detected at the pronuclear stage. Considering that it is unknown whether oocytes at the two pronuclei plus second polar body stage that have been fertilized by in vitro-generated human haploid male gametes (generated from the culture of human primary spermatocytes of men with primary testicular damage) demonstrate normal paternal genome binding of HP1beta, it appears that the probability that ooplasmic injections of in vitro-derived early haploid male gametes are accompanied by epigenetic risks related to lack or abnormalities in the pattern of binding of HP1beta protein with the paternal genome cannot be ruled out.

Although (a) in vitro culture systems of human primary spermatocytes (isolated with laboratory techniques from surgically recovered specimens), or spermatogonia, or even SSCs and (b) ooplasmic injections of spermatocytes (see previous sections) may have a theoretical role in the alleviation of infertility in non-obstructed azoospermic men with the absence of testicular spermatoozoa and spermatids, it should be emphasized that utilization of immature male germ cells with a probable abnormal imprint status for in vitro culture techniques or ooplasmic injections is susceptible to the risks of creating embryos with (a) oncological disease or (b) pathophysiology related to genomic imprinting abnormalities (Sofikitis et al., 1998a,b). On the other hand, in the theoretical case of injecting an imprint-free immature male germ cell nucleus into an oocyte, fertilization may be anticipated but it should lead to embryonic lethality. Transplantation of imprint-free PGC nuclei into oocytes has resulted in embryonic lethality, partly due to abnormal extraembryonic tissues resulting from the inappropriate silence or activation of imprinted genes (Ferguson-Smith and Surani, 2001). So far, imprinting, during passage through at least some stages of spermiogenesis, is essential since a male genome devoid of imprints cannot acquire all of them within a mature oocyte (Ferguson-Smith and Surani, 2001).

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In vitro spermatogenesis


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