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Review

The Sertoli cell as the orchestra conductor of spermatogenesis: spermatogenic cells dance to the tune of testosterone

Fotios Dimitriadis,[1] Chara Tsiampali,[2] Nikolaos Chaliasos,[1] Panagiota Tsounapi,[3] Atsushi Takenaka,[3] Nikolaos Sofikitis[1]

[1]Laboratory of Molecular Urology and Genetics of Human Reproduction, Department of Urology, Ioannina University School of Medicine, Ioannina, Greece; [2]Department of Ophthalmology, Kilkis General Hospital, Kilkis, Greece; [3]Department of Urology, Tottori University School of Medicine, Yonago, Japan

ABSTRACT

Spermatogenesis is contingent upon hormones and growth factors acting through endocrine and paracrine pathways either *in vivo* or *in vitro.* Sertoli cells (SCs) furnish essential factors for the successful advancement of spermatogenesis and spermiogenesis. Moreover, receptors for follicle stimulating hormone (FSH) and testosterone, which are the main hormonal regulators of spermatogenesis, are identified on SCs. Testosterone, FSH and luteinizing hormone are known to determine the destiny of germ cells and in their absence germ cells undergo apoptosis. Bcl-2 family proteins determine one signaling pathway which seems to be crucial for the homeostasis of male gametes. In addition to paracrine signals, germ cell development also relies on signals generated by SCs via direct membrane contact. The regulatory peptide somatostatin has an important role in the regulation of the proliferation of the male germ cells. Activin A, follistatin and FSH control germ cell development. *In vitro* culture systems have provided initial evidence supporting the achievement of the completion of the first and second male meiotic division *in vitro.* This review article provides an overview of the literature regarding the hormonal pathways governing spermatogenesis and spermiogenesis.

Key words: Gonadotrophin, Growth factors, Hormones, Spermatogenesis, Spermatogonial stem cells, Spermiogenesis

INSIGHTS INTO SPERMATOGONIAL DIFFERENTIATION AND PROLIFERATION

Spermatogonia are settled on the basal membrane of the seminiferous tubule.[1,2] A small fraction of their population operates as unipotent stem cells to bring forth exclusively mature spermatozoa. These unique stem cells, in contrast to other adult stem cells, present some characteristic features. As immediate descendants of primordial germ cells and principal components of the germline they are theoretically immortal. In studies using murine serial germ cell transplantation, spermatogonia were demonstrated as having an extremely long lifetime.[3,4] A large number of spermatozoa are required to preserve male fertility

Address for correspondence:
Fotios Dimitriadis, Laboratory of Molecular Urology and Genetics of Human Reproduction, Department of Urology, Ioannina University School of Medicine, Ioannina, Greece;
Tel.: +30 2310411121; e-mail: helabio@yahoo.gr
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and the key role of spermatogonia is to colonize the basal membrane of the seminiferous tubule at a critical stage when germ cells need to expand through mitosis prior to meiosis. In addition, spermatogonia must preserve the integrity of male DNA against internal damages such as accumulation of replication faults and external risks such as exposure to environmental mutagens. It is thus clear that spermatogonial stem cells (SSCs) represent key regulators of the control of the equilibrated evolutionary changes of the genetic material.

The rise of A_0 spermatogonia as the result of the resumed proliferation of gonocytes marks the start of spermatogenesis.[5] The spermatogenic process is a cyclic procedure, which in mice can be divided into 12 stages (I-XII).[6] In stage VIII, only a few A-aligned (A_{al}) spermatogonia are present, whereas A-single (A_s) and A-paired (A_{pr}) spermatogonia are predominant. As stage X starts over, A_s and A_{pr} spermatogonia begin to expand in such a way that the populations of A_s and A_{pr} spermatogonia remain relatively constant, whereas the population of A_{al} spermatogonia increases. Stage XII is followed by stage I, while during stages II and III germ cell expansion is arrested and the cells enter the G_1-G_0 phase. During the subsequent stages VII and VIII, without any further division, nearly all A_{al} spermatogonia that have been produced during the active proliferation period differentiate into A_1 spermatogonia. Next there follows the S phase and in stage IX A_1 spermatogonia differentiate into A_2 spermatogonia, after which five subsequent divisions give rise to A_3, A_4, I_n (intermediate spermatogonia), B spermatogonia and primary spermatocytes, respectively.

In total, nine to eleven mitotic divisions occur during spermatogonial development. Once the populations of A_4, I_n and B spermatogonia have decreased, the proliferation period continues to stage VII. Between A_4, I_n and B spermatogonia and A_s, A_{pr} and A_{al} spermatogonia there appears to be a feedback mechanism. This is evident from the fact that when the populations of A_4-B spermatogonia decrease to 50% of those in the normal testis, A_s, A_{pr} and A_{al} spermatogonia continue to proliferate beyond stage II.

An issue still under debate is whether the SSC divisions are symmetrical or asymmetrical. During symmetrical divisions one stem cell produces either two stem cells or two interconnected cells destined to differentiate (A_{pr}). On the other hand, when SSCs divide asymmetrically it results in a stem cell and a cell destined to produce A_{pr} spermatogonia. Thus, not all A_s spermatogonia are true stem cells. During the development of spermatogonia two differentiation steps appear to occur. First, A_s spermatogonia differentiate into A_{pr} spermatogonia. From then on, the germ cells consist of clones of cells interconnected through bridges which increase in size. The second step consists in the differentiation of A_{al} to A_1 spermatogonia which includes marked changes in cell behavior. In Drosophila, germ stem cells normally divide asymmetrically, generating one stem cell and one gonialblast. The latter starts the differentiation with spermatogonial transient-amplifying divisions.[7] A cluster of somatic cells at the testis apical tip, which is called a hub, represents the stem cell niche. The apical hub cells are responsible for the maintenance of stem cell identity through the expression of the unpaired signaling ligand, which activates the Janus kinase-signal transducers and activators of transcription (JAK/STAT) pathway within germline stem cells.[7] In the adult Drosophila, the JAK/STAT pathway is involved in stem cell renewal in the male germline[8,9] In the early stages of embryonic development, JAK/STAT signaling plays an important role in sex determination[10] and modulates embryonic segmentation through the control of the expression of the pair-rule genes even-skipped, runt and fushi tarazu.[11,12]

In humans, the germ cells can be classified as follows:[13-18] progenitor A_{dark}-spermatogonia (stem cells or reserve spermatogonia); progenitor A_{pale}-spermatogonia; committed A_{pale}-spermatogonia; B-spermatogonia; preleptotene (young primary spermatocytes), leptotene, zygotene, pachytene and diplotene spermatocytes; secondary spermatocytes; spermatids; and spermatozoa. The progenitor A_{dark}-spermatogonia divide sporadically, but with as yet unidentified stimulation, and generate progenitor A_{pale}-spermatogonia. Progenitor and committed A_{pale}-spermatogonia are of similar shape and morphology, but the latter give rise to B-spermatogonia.

Four consecutive processes characterize human spermatogenesis:[18-20]

1. Proliferation and differentiation of A_{dark}- and A_{pale}-

spermatogonia, production of B-spermatogonia from the division of committed A_{pale}-spermatogonia and formation of preleptotene spermatocytes from the division of B-spermatogonia.

2. The spermatocytes enter meiosis with the last synthesis of DNA in preleptotene spermatocytes and two meiotic divisions which gives rise to spermatids.
3. Spermiogenesis which involves the transformation of the spherical spermatid to a sperm-like mature spermatid.
4. Spermiation which involves the release of spermatozoon into the tubule lumen.
   All the previous processes have distinct requirements of modulatory molecules derived from the nearby Sertoli, peritubular and Leydig cells, as well as the vascular network.

Spermatogenesis in humans is different from the process in other common mammals such as bulls, mice, rabbits, rats or stallions. Obvious differences include the 3-dimensional organization of the seminiferous epithelium and the low number of sperm produced daily per gram of testis.[18]

Spermatogenesis in several species, and particularly in the human, has been the target of several research efforts worldwide. There are many unanswered questions in the spermatogenesis process. For example, there is no information concerning the exact time in human spermatogenesis that the A_{pale}-spermatogonia start cellular divisions; also unknown is the exact number of spermatogonia mitotic divisions resulting in the production of primary spermatocytes.

HORMONAL CONTROL AND SECRETORY ACTIVITY OF SERTOLI CELLS

Within the seminiferous tubules, Sertoli cells (SCs) represent the only somatic cells. Their role in exocrine testicular function appears to be crucial and this is emphasized by the fact that (a) 'germ cell-only testes' have never been reported, (b) the presence of SCs is required in the vast majority of *in vitro* culture systems for successful differentiation of germ cells and (c) the number of SCs is directly related to the population of germ cells sustained by the testes.[21] Each SC can support a defined number of germ cells, limiting in this way the expansion of the spermatogonial population. This is evidenced by the fact that SCs form germ cell niches which allow a certain number of germ cells to reside in or repopulate the seminiferous tubules.[22] Additionally, in experimental studies in which the size of the testis and/or the spermatogenic output was manipulated by changing the number of SCs, there was a relatively constant ratio between SCs and spermatids before and after the manipulation.[23-25]

The contribution of SCs to spermatogenesis is based on the production of critical factors necessary for the successful development of spermatogonia up to the stage of the spermatozoon. Griswold[21] reported that glycoproteins secreted by SCs are crucial for spermatogenesis and could be divided into three main categories. First, SCs secret glycoproteins which facilitate the transport of ions and hormones or provide bioprotective functions, such as androgen-binding protein (ABP), transferrin and ceruloplasmin. Second, SCs provide both proteases and protease inhibitors with 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. Third, SCs furnish glycoproteins that function as structural components of the basement membrane between the SCs and the peritubular cells. A specific transferrin system has developed to ensure that the tight SC junctions are circumvented so that the germ cells can receive a supply of ferritin produced by SCs. Moreover, SCs secrete vasoactive peptides and tachykinins which have been shown, *in vitro*, to induce the release of lactate and transferrin by SCs and also to stimulate aromatase activity by SCs.[26]

Furthermore, SCs secrete other glycoproteins that serve either as growth factors or paracrine factors, such as c-kit ligand (stem cell factor; SCF), the Mullerian duct inhibiting substance, inhibin and glial cell line-derived neurotrophic factor (GDNF). Both survival and development of spermatogonia were found to be stimulated by the action of SCF.[27] When spermatogenesis starts, SCs dramatically change the production of soluble SCF to membrane-bound SCF,[28] pointing to an important role for the SCF/c-kit system in spermatogonial differentiation. The c-kit-receptor and SCF also mediate SC-spermatocytes cellular adhesions, which

activity has been corroborated by studies showing that SCs from mice mutant for membrane-bound SCF are not able to bind spermatocytes.[29] Cadherins are also secreted by SCs; their function is the maintenance of the structure of testicular tissue and cell architecture and identity. Studies in the rat model demonstrated expression of mRNA coding for the three 'classical' cadherins, E-, P- and N-cadherin, in the developing rat testis.[30] Additionally, SCs produce peptides including prodynorphin and nutrients or metabolic intermediates.[21] The transfer of metabolic products, such as carbohydrates, amino acids, vitamins, lipids and metal ions,[31] from the SCs to the proliferating germ cells is only possible due to the tight relationship between SCs and germ cells; it is therefore essential that germ cells receive enough energy substrates.[32-34] Moreover, SCs deposit extracellular matrix components such as collagen and laminin, form specialized junctions and demonstrate a well-organized cytoskeleton significant for maintenance of the seminiferous epithelium.[31]

A good knowledge of the metabolism of SCs and the production of lactate, acetate and other metabolic factors that are essential for the normal occurrence of spermatogenesis is imperative.[35] It has long been postulated that close metabolic cooperation exists between SCs and developing germ cells, since developing germ cells rely on the lactate produced by SCs as metabolic fuel.[35] Indeed, in germ cells lactate is not only used as fuel but also presents antiapoptotic properties.[32] Meanwhile, SCs demonstrate some unique peculiarities in regard to carbohydrate metabolism. In fact, the majority of glucose is converted into lactate and not oxidized via the citric acid cycle,[34,36] a phenomenon known as "the Warburg effect", when cells (such as cancer cells) convert glucose to lactate even in the presence of sufficient and physiological oxygen levels to support mitochondrial oxidative phosphorylation.[37] This peculiar metabolic behavior of SCs is consistent with the ability of the more-developed germ cells, such as spermatids, to use lactate as an energy substrate rather than glucose.[34] Although the reasons why SCs preferentially export lactate for germ cells are not entirely elucidated,[38] it has been demonstrated that lactate production increases as the SCs differentiate during pubertal development.[39] The SC uptakes glucose through the action of glucose transporters, particularly glucose transporter-1 and glucose transporter-3.[40] Once glucose enters the cells, it is catabolized by a series of reactions (glycolysis) to pyruvate.[40] Pyruvate is converted to lactate by the lactate dehydrogenase. SCs also actively produce acetate. Both substrates, lactate and acetate, are exported to the adluminal fluid by monocarboxylate transporters to support the development of germ cells.[40] The lactate produced by SCs is then used as fuel by the developing germ cells, this implying that lactate is more than a metabolic substrate and can control spermatogenic cell development *via* a complex pathway.[35] Thus, lactate production by SCs appears to be an excellent target for male fertility control.[35] In-depth scientific research into SCs metabolism has demonstrated hormonal regulation of the latter. Alterations in SCs metabolism result in impaired lactate delivery to developing germ cells that may compromise spermatogenesis.[41]

In order to ensure satisfactory lactate concentration in the microenvironment where germ cells develop, SCs have the ability to adapt their metabolism according to the needs of the spermatic cells.[42] Several studies have focused on the hormonal control of SCs metabolism and the mechanisms behind such hormone-related effects.[42-44] Rato et al showed that 5α-dihydrotestosterone (DHT) and 17β-estradiol (E_2) are key modulators of lactate production/export by SCs and that DHT is more capable of inhibiting the lactate production/export under their experimental conditions.[41] In that study, it was determined that sex steroid hormones have key roles that reach far beyond reproductive processes. It was also demonstrated that DHT modulates glucose consumption and lactate production in cultured rat SCs. Moreover, both DHT and E_2 decreased lactate dehydrogenase-chain A and monocarboxylate transporter-isoform 4 mRNA expression levels after 50h treatment.[41] Similarly, studies using primary cultures of human SCs reported that following E_2 and DHT treatment, lactate production by SCs was altered after 50h of culture.[43] Although the exact mechanism is not yet fully elucidated, recent evidence suggests that sex steroid hormones action in SCs metabolism is mediated through modulation in glycolysis-related transporters and enzymes, particularly at the transcriptional level.[45] DHT decreased glucose transporter-1 protein levels and increased LDH activity after 25h, providing evidence for a

role of this hormonal action in the regulation of SC metabolism.[45]

Furthermore, the androgen receptors (ARs) also play an important role in the regulation of testosterone levels. They are localized in the testicular somatic cells, including Leydig cells, peritubular myoid and blood vessel smooth muscle cells and also in SCs. By contrast, germ cells of the mature testis do not seem to require functional ARs. This fact indicates that androgens affect spermatogenesis indirectly through SCs, as these cells interact directly with developing germ cells and express functional ARs.[46,47] Moreover, when ARs are ablated in SCs, germ cell development stops at the spermatocyte stages[46] or early spermatid,[48] meaning that ARs present in the SCs play a crucial role in maintaining SCs ability to support normal spermatogenesis and that that SCs are the primary site that mediates androgen support of regular spermatogenesis.

The contribution of estrogens in the initiation and maintenance of testicular function and spermatogenesis is quite perplexing as estrogens participate in numerous physiologic functions of the male reproductive system including the hypothalamus-pituitary-testis axis, Leydig cells, SCs, germ cells and epididymis.[44] The concentration of estrogen is high in rete testis fluid,[49] while in the rat epididymis it is about 25 times higher than the estrogen levels measured in the plasma,[50] suggesting that estrogens have a key role in the regulation of spermatogenesis and in epididymal function. Furthermore, some studies confirmed that SCs are the major source of estrogens in immature individuals, whereas Leydig cells produce estrogens in adults.[51] Estrogens have the ability to disrupt the development of fetal Leydig cells and to enhance spermatogenesis by inhibiting apoptosis of the post-meiotic spermatogenic cells.[52,53] Also, estrogens affect proliferation and differentiation of gonocytes and spermatogonia and inhibit testosterone production by Leydig cells.[52,53] Moreover, estrogens regulate the function of mature spermatozoa and the incubation of human spermatozoa, while estrogens are known to stimulate sperm functions such as motility,[54] lactate production[55] and the metabolization of several substrates.[56] Conversely, estrogens can also induce apoptosis in the spermatogenic cell via the Fas ligand (FASL) pathway and the release of cytochrome c from mitochondria.[57]

Adjacent SCs form specialized interconnections in the form of junctions, creating the Sertoli/blood-testis barrier.[58] This barrier causes a physiological compartmentalization of the seminiferous epithelium into basal and apical (or adluminal) compartments and its proper functioning is required for the normal occurrence of spermatogenesis.[59] The physical and nutritional support of spermatogenesis is only one of the purposes of the blood-testis barrier. One other fundamental function of this barrier is the creation of an immune privilege status within the testis, repressing the immunological response to autoantigens residing within germ cells undergoing meiosis and also in developing spermatids during spermiogenesis.[60] Moreover, SCs transport water from the interstitial fluid to the lumen in order to use it as a vehicle for sperm transport from the testis to the epididymis.[61] Another important role of the blood-testis barrier is the regulation of the exchange of substances between the rete testis fluid and the lymph or plasma,[62] controlling the luminal fluid content where germ cells develop. Thus, it is understandable that a) several diseases, (such as diabetes mellitus)[63] which deregulate the dynamics and functioning of the blood-testis barrier or b) other extreme metabolic disorders (such as obesity) which deregulate the energy demands of the reproductive system,[64] result in male subfertility/infertility.[63]

The SC contributes to the development of the male reproductive potential via additional mechanisms. It has been reported that SCs play an essential role in the maintenance of peritubular myoid cells phenotype in fetal and neofetal life.[65] The proliferation of the adult population of Leydig cells and the maintenance of Leydig cells numbers in the adult testis was also reported to be stimulated and regulated by SCs action.[65] Leydig cells control testosterone levels and are also known to play a crucial role in spermatogenesis. Furthermore, recent reports emphasize that the interaction between SCs and Leydic cells may additionally serve as a molecular target to control male fertility. However, further research efforts are mandatory to elucidate these mechanisms and their relevance for male reproductive health.[35]

SCs possess receptors for both follicle stimulating hormone (FSH) and testosterone, which are the principal hormonal regulators of spermatogenesis. FSHβ knock-out mice are fertile in spite of a decrease

in testicular size and a 75% reduction in sperm numbers.[66] The combination of small testes and fertility in FSHβ deficient mice tends to suggest that FSH is necessary for testicular volume but may not be absolutely essential for spermatogenesis and normal fertility.[66] Similarly, mutations of the FSH receptor have been associated with variably severe reduction in sperm count, though fertility has been maintained.[67] In contrast to FSHβ knock-out male mice which have very low FSH levels, male FSHR knock-out mice have 3-fold elevated FSH levels.[68] Moreover, FSH alone or in synergic action with testosterone prevents germ cell loss or restores spermatogenesis quantitatively in hypophysectomized animals.[69] *Ex vivo* studies in human testicular tissue demonstrate the role of FSH and testosterone in the prevention of germ cell apoptotic death.[70,71] This evidence implies that both hormones function as germ cell survival regulators. The expression of Fas ligand in the testis is mainly localized in SCs, which underlines the key role of SCs in the regulation of the apoptotic mechanisms of germ cells.[72]

Normal SC secretory function is of paramount importance for the regulation of the spermatogenic process. A few studies during the last eight years have indicated that specific phosphodiesterase families are expressed in the testis and play a role in the regulation of SC secretory function in azoospermic men and in the regulation of Leydig cell secretory function in oligoasthenospermic men.[73] Inhibitors of phosphodiesterase 5 have been shown to improve focal advanced spermatogenesis in azoospermic men[74] and sperm counts in oligospermic men.[73] Furthermore, the phosphodiesterase 11 knock-out mouse is infertile.[75] The expression of different phosphodiesterase families in the SCs, Leydig cells, peritubular myoid cells and spermatogenic cells is highly selective, this depicted in Table 1.

An understanding of the molecular mechanisms and the biochemical pathways responsible for the control of the metabolic cooperation between SCs and developing germ cells may open up new insights into the subfertility/infertility conditions associated with several systemic diseases affecting male fertility, such as diabetes mellitus,[40] and may offer a basis for investigation into the development of new therapeutic strategies.

MECHANISMS REGULATING GERM CELL SURVIVAL AND DEATH

Apoptotic regulation in the human testis

The maintenance of normal architecture of the seminiferous tubules is accomplished by a dynamic equilibrium of germ cellular regeneration and elimination. There are eleven different types of cell survival mechanisms that have been reported. Ten cellular survival mechanisms are genetically programmed, while necrosis is the only cell death mechanism that is non-genetically programmed and occurs in pathological conditions.[76] However, only three of the genetically programmed cell death mechanisms, known to include caspase-independent apoptosis,[77] caspase-dependent apoptosis[78,79] and anoikis,[80] are described in the testis. Caspase-dependent apoptosis is the most common cellular death mechanism in the testis and is distinct from other forms of cell death because of its participation in caspase activation via pathways utilizing death receptors and/or BCL-2 family regulators.

Apoptosis has earned keen attention due to its involvement in a range of human testicular pathologies, such as cancer and other diseases.[81] Sinha Hikim et al[82] have not only provided strong evidence that germ cell death during normal human spermatogenesis occurs via apoptosis but they have also indicated the presence of ethnic differences in the inherent susceptibility of germ cells to apoptotic cell death. In fact, testicular samples obtained at autopsy from five Chinese and nine non-Hispanic Caucasian men were analyzed using a modified TUNEL technique and showed that the incidence of spermatogonial as well as spermatid apoptosis was higher in Chinese than in Caucasian men. This data could also explain the greater efficacy of testosterone-induced spermatogenic suppression in azoospermia observed in Asian compared to non-Asian men.[82] Moreover, apoptosis has been reported to be a possible mechanism of 2-methoxy acetic acid-induced spermatocyte death in cultured seminiferous tubules of middle-aged human donors or of spermatogonial death in pre-pubertal boys.[82] The researchers also furnished strong evidence implicating apoptosis in human germ cell processes. During normal spermatogenesis, apoptosis regulates the homeostasis of SCs to germ cell number by inducing programmed cell

Table 1. Expression of different phosphodiesterase families in the Sertoli cells, Leydig cells, peritubular myoid cells and spermatogenic cells

Type of cell			PDE	Species	Reference
Sertoli cells			PDE3	Rat	Swinnen et al[205]
			PDE4		Swinnen et al[205]
			PDE4B		Farooqui et al[206]
			PDE4D		Levallet et al[207]
Leydig cells			PDE11A	Human	D'Andrea et al[208]
			PDE4B	Rat	Farooqui et al[206]
			PDE5		Scipioni et al[209]
			PDE8A	Mouse	Vasta et al[210]
			PDE11		Baxendale et al[211]
Peritubular myoid cells			PDE5	Rat	Scipioni et al[209]
Germ cells	Spermatogonia		PDE11	Human	Baxendale et al[211]
			PDE11	Mouse	Baxendale et al[211]
	Spermatocytes		PDE11	Human	Baxendale et al[211]
	Developing spermatocytes		PDE3B	Rat	Degerman et al[212]
	Pachytene spermatocytes		PDE4A	Rat	Salanova et al[213]
			PDE1C		Yan et al[214]
			PDE4D		
	Spermatids		PDE11	Human	Baxendale et al[211]
			PDE11	Mouse	Baxendale et al[211]
			PDE4A	Rat	Farooqui et al[206]
		Round	PDE4A	Rat	Salanova et al[213]
			PDE4D		Farooqui et al[206]
		Round/Elongating	PDE1A	Mouse	Yan et al[214]
			PDE1C		
		Elongating	PDE4D	Rat	Salanova et al[213]
	Spermatozoa		PDE1	Human	Fisch et al[215]
			PDE1A		Aversa et al[216]
			PDE3A		Lefievre et al[217]
			PDE4		
			PDE5		
			PDE6	Mouse	Baxendale et al[218]
			PDE3B		
		Acrosomal region/ head	PDE4D	Mouse	Baxendale et al[218]
		Flagellum	PDE1A	Mouse	Baxendale et al[218]
			PDE4D		
			PDE10A		

death to eradicate germ cells that fail to replicate their DNA properly during cell division. Apoptosis can be induced by specific stimuli, such as deprivation of hormones, exposure to ionizing radiation, various chemotherapeutic drugs, cell injury and cell stress.

Not all degenerating germ cells display the classical morphology of apoptosis and therefore the exact incidence of adult male germ cell apoptosis remains unclear. Spermatogonia and round spermatids almost certainly follow apoptotic death, since they demonstrate many of the classical morphological and biochemical features of apoptosis, as for example labeling of nuclei by terminal deoxynucleotidal transferase and compaction of DNA at the nuclear margin.[83] Apoptotic round spermatids often degenerate *en masse* as multinucleated symplasts. During apoptosis, germ cells are either phagocytized by SCs or sloughed into the tubule lumen. However, the extent of spermatocyte and elongated spermatid apoptosis is less clear; some can be labeled as terminal deoxynucleotidal transferase and annexin V without showing the characteristic nuclear changes usually associated with apoptotic death, possibly due to the unusual DNA configuration and morphology of these cells.[83]

Whether germ cells survive or die is decided by a composite network of signals including paracrine signals such as SCF, leukemia inhibitory factor (LIF) and Desert Hedgehog (Dhh),[84] as well as endocrine direct or indirect signals such as pituitary gonadotrophins, estrogens and testosterone, among others.[53,85] In addition, germ cells respond to external signals and to their internal milieu by triggering intracellular signaling pathways that ultimately determine their destiny.

The mitochondrial or intrinsic pathway of apoptosis in the human

The intrinsic pathway, which involves members of BCL-2 family proteins that regulate the release of cytochrome c from the mitochondria into the cytosol, seems to be crucial 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. Bcl-2-associated X protein (BAX), BAK and BIM, among others)]. In normal cells, BCL-2 family proteins are found in the outer membrane of the mitochondria, bound to the adaptor protein APAF1.[86] BCL-2L2 (formerly BCL-W) is a significant pro-survival member of the BCL-2 family[87] that takes part in apoptotic modulation by dimerizing with the proapoptotic factor BAX[88] whose expression is a feature of germ cell apoptosis *in vitro*. It is generally agreed that the ratio of proapoptotic to pro-survival BCL-2 family proteins is the critical determining factor of cell destiny. Apoptotic signals have been proposed as being involved in the regulation of antioxidant defences, resulting in enhanced sensitivity to reactive oxygen species and the induction of the mitochondrial permeability transition.[89] In turn, this causes the release of cytochrome c into the cytosol and the entrance of surplus BAX into the mitochondria through the open pores of the membrane.[90,91] Pro-caspase 9 and APAF1, together with cytochrome C, activate caspase 9, with subsequent activation of executioner caspases 3, 6 and 7. The activated executioner caspases are involved in the cleavage of intracellular proteins such as poly (ADP-ribose) polymerase, lamin, actin and gelsolin, leading to apoptosis.[90,91]

The pro-survival protein BCL-2L2 has a significant function in the regulation of the testicular germ cell population. The incidence of germ cell apoptosis in Bcl-2l2 knock-out mice increases dramatically between 2 and 4 weeks of age.[87] Other members of the BCL-2 family are expressed in the testis, but their exact role in spermatogenesis is still obscure (i.e. Bad, Bok, Bcm and Boo9, among others). There is evidence 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.[92] It appears that these proteins play a specific role in the processes of cellular differentiation and maturation during the human spermatogenesis process. In humans, BCL-2 and BAK are mainly expressed in the compartments of spermatocytes and differentiating spermatids.[92]

Human male germ cells show a preferential expression of BCL-X at the stage of spermatogonium, whereas BAX is mainly expressed in the nuclei of round spermatids. BAD can be observed by immunochemistry in the acrosome region of several 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-2L2, p53 and

p21 cannot be detected. The apoptotic promoter BAX preferential expression in human round spermatids may suggest that round spermatids may be especially prone to apoptosis when DNA is damaged. Expression of most of the intrinsic pathway constituents such as Bcl-2l2, Bax mRNAs and proteins,[88] APAF1[93] and cytochrome c[94] has been reported in early germ cell types including spermatogonia and spermatocytes as well as in SCs throughout development.

Aged men present a significantly lower apoptotic rate in spermatogonia in comparison with controls.[95] However, in the latter study,[95] the balance of spermatogonial proliferation and apoptosis showed no significant difference between the group of aged men and the control group. This could be one of the reasons explaining why the spermatogonial population in aged men is 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 reduction of the population of human primary spermatocytes per SCs in aged men. Furthermore, it has been demonstrated that the expression of BCL-XL is inversely related to the apoptotic rate in human primary spermatocytes, indicating that BCL-XL may contribute to the regulation of human primary spermatocyte apoptosis.[95]

The death receptor or extrinsic pathway of apoptosis in humans

The death receptor or extrinsic pathway represents another major pathway of caspase-dependent cell apoptosis. This pathway includes the cell surface death receptors called FAS receptors (FASRs) which have emerged as the key regulators. FAS is a transmembrane receptor protein and contains a 'death domain'. Among the apoptotic receptors comprising the tumor necrosis factor receptor superfamily, CD95/APO-1 (FAS) is the best characterized. FAS is abundantly expressed in various tissues, particularly in activated T and B cells, thymocytes, hepatocytes and heart tissue.[96] Of particular interest is the observation that FAS ligand (FASL, a cell surface molecule) is constitutively expressed by cells in immune privileged sites such as the testis[97] and the anterior chamber of the eye.[98,99] The binding of FAS protein to its ligand and then to FASR activates the apoptosis as a result of the recruitment of the intracellular adaptor molecule, FAS-associated death domain, which binds several molecules of caspase 8 proenzyme, leading to proteolytic activation. Subsequently, activated initiator caspases cleave executioner caspases, resulting in apoptosis.[100] Both physiological and experimentally induced germ cell apoptosis has been shown to follow the activation of this pathway.[78] Pentikainen et al[101] have demonstrated that the FAS-FASL system regulates germ cell apoptosis in the human testis. In humans and in rodents, FAS has been identified in germ cells, spermatocytes and spermatids and FASL in SCs.[72,101,102] Antibodies antagonistic to FASL block human germ cell apoptosis *in vitro*.[101] On the other hand, neither FAS nor FASL is expressed in spermatogonia.[103]

The control of SCs-derived factors over the fate of male germ cells

Germ cells are dependent not only upon paracrine signals but also on signals derived from SCs through direct membrane contact. Membrane-bound SCF is expressed on precursors of SCs within the embryonic genital ridge and its receptor, the c-kit tyrosine kinase, is expressed on the surface of adjacent primordial germ cells. The first wave of spermatogenesis also requires the presence of SCF. In adults, c-kit is expressed on the surface of spermatogonia, while membrane-bound SCF is expressed on the corresponding basal regions of SCs. *In vivo* studies have demonstrated that the blockage of SCF/c-kit interaction in adults results in increased incidence of apoptosis in spermatogonia and spermatocytes. The c-kit gene is located at the white spotting (W) locus in the mouse and on chromosome 4 in the human,[104,105] whereas its ligand, SCF, has been identified as an analogue of the murine Steel (Sl) gene and in humans is located on chromosome 12 encoded by 9 exons.[106] Both in the adult and in the postnatal testis, c-kit is detected in the proliferating spermatogonia A1-A4 and is also present in interstitial somatic Leydig cells.[107,108] On the other hand, it appears that only SCs produce SCF in the testis.[109] The SCF/c-kit system is involved in different functions in the testis, including germ cell adhesion, cellular proliferation, cell migration and anti-apoptotic activities. In experimental studies, homozygous mutations of W and Sl loci resulted in the absence of functional production of c-kit or SCF, respectively, and were associated with the absence of germ cells in the post-

natal testis. These alterations of spermatogenesis are related to defects in primordial germ cells migration and/or induction of apoptosis.[110] Therefore, one of the key regulators of spermatogenesis seems to be the SCF/c-kit complex.

In addition, SCs may utilize the FAS system to control the destiny of germ cells. With regard to the human testis, the expression and the activity 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.[72,101,111,112] As discussed earlier, caspase inhibitors inhibit programmed human germ cell death, suggesting that FAS-associated human germ cell apoptosis is mediated via the caspase pathway.[78] Experimental studies have recorded an up-regulation of FAS in germ cells undergoing apoptosis after *in vivo* administration of SCs toxicants.[72] However, in human testis, the withdrawal of survival factors during an enhanced apoptotic process was not accompanied by up-regulation of the expression of FAS. This may point to the existence of additional pathways leading to enhanced apoptosis in the human testis. Alternatively, it has been hypothesized that FAS activation may be more efficient in unfavorable conditions, thus increasing the ability of the FAS-FASL complex to induce apoptosis in human germ cells.[101]

SC apoptotic signaling is a hormonally regulated process and a deregulation in SC apoptotic signaling will affect germ cells, compromising spermatogenesis. Dias et al hypothesized that the lack of insulin could lead to alterations in apoptotic signaling. Using cultures of rat SCs they examined the effect of insulin deprivation on several markers of apoptotic signaling. SCs cultured in insulin deprivation demonstrated a significant decrease in mRNA levels of p53, Bax, caspase-9 and caspase-3, followed by a significant increase of Bax and decrease of caspase-9 protein levels compared with control cultures.[113] Caspase-3 activity was also decreased in SCs cultured in insulin deprivation conditions. These results indicate that insulin deprivation decreases caspase-dependent apoptotic signaling in cultured rat SCs evidencing a possible mechanism by which lack of insulin can affect spermatogenesis and fertility.[113] Sex steroid hormones could also play a role in the regulation of mitochondria-related pro-apoptotic factors. Simoes et al investigated the influence of E_2 and DHT on the apoptotic signaling pathways in immature rat SCs, evaluating the mRNA expression of p53, the pro-apoptotic Bcl2 family member Bax, caspase-3 and caspase-9 as well as the protein expression of caspase-9, Bax, the anti-apoptotic Bcl2 and AIF. They also evaluated caspase-3 activity and DNA fragmentation (TUNEL assay) as endpoint markers of apoptosis.[114] The researchers demonstrated that E_2 and DHT act as apoptotic signaling modulators in *in vitro* immature rat SCs. Both DHT and E_2 down-regulated p53, Bax, caspase-9 and caspase-3 mRNA levels. Importantly, DHT alone decreased the protein levels of apoptosis-inducing factor (a caspase-independent death effector released from mitochondria),[115] whereas E_2 alone down-regulated cleaved caspase-9 protein levels (regulated by the release of cytochrome c from the mitochondria and its binding to Apaf-1 to form the apoptosome complex).[116] This evidence may suggest that androgens and estrogens are capable of modulating independent pathways of apoptosis, regulated by different pro-apoptotic factors. Both DHT and E_2 were able to reduce caspase-3 activity and DNA fragmentation, clearly confirming the anti-apoptotic action of DHT and E_2.[114]

Hormonal regulation of germ cell apoptotic death

Reproductive hormones including FSH, luteinizing hormone (LH) and testosterone are known to influence the destiny of male germ cells. In their absence germ cells undergo apoptosis. In addition, within the testes, 1-2% of testosterone is converted to E_2 by the aromatase enzyme.[117] There is evidence for both beneficial and detrimental effects of E_2 on spermatogenesis in humans and in experimental studies.[53,118,119] Estrogen treatment, which is thought to mimic a gradual withdrawal of gonadotrophins, leads to apoptosis of all germ cells including elongated spermatids.[120] On the other hand, is has been demonstrated that E_2 acts as a germ cell survival factor in the human testis *in vitro*.[121] Therefore, it appears that E_2 may have mixed effects on spermatogenesis in experimental models wherein overmuch is detrimental but estrogen receptor signaling is required for the maintenance of normal spermatogenesis.[117]

Within the seminiferous tubules in men, apoptosis

is induced under serum-free conditions *in vitro*.[70] This apoptosis can be suppressed by administration of testosterone, implying that testosterone in the human male is a vital germ cell survival factor. However, the exact apoptotic pathway by which androgen withdrawal induces germ cell death remains elusive. BCL-XL and BCL-2 in the testis are altered following long-term anti-androgen therapy for prostate cancer.[122] Therefore, it is tempting to hypothesize that androgen deprivation changes the expression of the BCL family proteins in germ cells.

Somatostatin (SRIF) is a regulatory peptide hormone produced by neuroendocrine, inflammatory and immune cells in response to ions, nutrients, neuropeptides, neurotransmitters, thyroid and steroid hormones, growth factors and cytokines.[123] It plays a role in the control of the proliferation of male germ cells. Its biological functions are mediated by five receptors (sst1-sst5).[124] In clinical trials it has been demonstrated that the injection of SMS201995, an SRIF analogue, in healthy adult males is followed by a rapid (2h after the injection) rise in serum testosterone level. This enhanced testosterone secretion takes place without a simultaneous increase in LH production, which suggests that SRIF is able to modulate testosterone production at the testicular level.[125] The presence of SRIF and its receptors in human testes[126] clearly points to the existence of auto/paracrine loops controlling local testosterone secretion. Indeed, SRIF receptors sst3, sst4 and sst5 are expressed in human normal testicular tissue, while sst1 and sst2 are usually absent. In the rat testis the receptors sst1, 2 and 3 have been identified mainly in spermatocytes and SCs.[123,127,128] In addition, the accumulation of sst1-3 mRNAs at a high level in round spermatids at stages I-VII and their dramatic decrease at stage IX when round spermatids have already begun their elongating changes possibly point to some effects of SRIF and its receptors on spermiogenesis.[129] Further experimental studies have provided evidence for an inhibitory role of SRIF in the regulation of spermatogonial proliferation.[130] 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 SCs.[130]

The stimulating and challenging issue is now to discover the intracellular apoptotic regulators of germ cells and to unravel their regulation by a variety of death signals. The mechanisms by which several pro-apoptotic and anti-apoptotic genes modulate the process of spermatogenesis will offer new insights into the molecular components of the cellular survival/death machinery within the testis that determine the germ cell fate including their growth, division or death. This knowledge could provide potential applications in the assessment and management of male infertility.

THE EFFECT OF GROWTH FACTORS ON THE APOPTOTIC PATHWAYS IN THE TESTIS AND ON GONOCYTE PROLIFERATION AND DIFFERENTIATION

The presence of the tumor necrosis factor alpha-related apoptosis-inducing ligand (TRAIL) and its receptors has been demonstrated in different human testicular germ cell types.[131] Moreover, both TRAIL and two of its receptors, DR5/TRAIL-R2 and DcR2/TRAIL-R4, have been localized in Leydig cells. In addition, another receptor, namely DR4/TRAIL-R1, is seen in human peritubular cells and SCs. Most probably the TRAIL pathway is involved in the induction of apoptosis in the human testis.[131] The fate of germ cells is regulated by several paracrine signals. LIF promotes the survival of primordial germ cells in culture. Moreover, other factors including interleukin-4 (IL- 4), basic fibroblast growth factor (bFGF), a soluble form of SCF, and the bone morphogenetic protein-4 (BMP-4) also promote primordial germ cells survival *in vitro*. In contrast, other *in vitro* studies showed that transforming growth factor-β (TGF-β) promotes gonocyte apoptosis *in vitro*. LIF, bFGF and ciliary neurotrophic factor promote the survival of gonocytes co-cultured with SCs.[132-134] 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. SCs secrete Dhh, another paracrine signal known to promote germ cell survival indirectly. GDNF, neurturin, persephin and artemin belong to the TGF-β superfamily.[135-138] GDNF mRNA has been detected in many tissues in addition to the brain and kidney, including the intestine, stomach, muscle, cartilage, lung and testis.[139] Expression of GDNF mRNA in the testis is associated with the

proliferation of SCs. In addition, GDNF contributes to the paracrine regulation of spermatogonial self-renewal and differentiation.[140]

The platelet-derived growth factor-A (PDGF-A) and PDGF-B genes encode A and B chains of PDGF and are located on human chromosome 7p and 22q, respectively.[141] Many cells produce PDGF, which exerts its effects on cells through receptor phosphorylation, resulting in cellular responses such as migration, proliferation, contraction and alteration of cellular metabolic activities, as for example matrix synthesis, cytokine production and lipoprotein uptake.[142] The 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 is enhanced in the adult testis, indicating a connection between the PDGF system and the start of spermatogenesis.[143] Since human Leydig cells express both the ligands and receptors of the PDGF system, it can be speculated that the ontogeny of this cell type is strongly determined by PDGF.[143]

The *in vitro* clonogenic method has been used to investigate the growth factors regulating mouse gonocyte proliferation and differentiation.[144] In the latter study, it was observed that epidermal growth factor (EGF) and TGF-β had no inhibitory effect on gonocyte-derived colony formation. Similarly, neither Mullerian inhibitory factor nor Leukemia Inhibitory Factor (LIF) demonstrated any effects on the proliferation of gonocytes. The authors showed that the growth of gonocytes *in vitro* was optimal in the presence of fetal calf serum.[144] Thus, it has been concluded that no specific growth factors (with the probable exception of PDGF[145]) are necessary for the growth of gonocytes *in vitro*.[144] However, other investigators[146] demonstrated that the addition of GDNF to a culture of mouse SSCs had a positive effect on 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. This suggests that GDNF inhibits spermatogonial differentiation.[140] Hasthorpe[145] evaluated the effect of SCF in the differentiation of spermatogonia and demonstrated that SCF did not exert any effect on mouse type A spermatogonia colony-forming cells. This indicates that more highly differentiated spermatogonia represent the target-cellular population of SCF. The majority of gonocytes expresses c-kit mRNA but fails to respond to SCF, pointing to a non-functional receptor. PDGF seems to stimulate the proliferation of gonocytes, but not the proliferation of type A spermatogonia recovered from 15-day-old animals.[145]

Growth factors are key regulators of the fate and lineage determination of undifferentiated spermatogonia. A substantial subpopulation of them has been identified and their role has been slowly unraveled during the last few years. However, important scientific work is still pending in order to elucidate their effects and utilize the acquired knowledge in the development of treatments for male factor infertility.

HORMONAL REGULATION OF GERM CELLS

Hormonal regulation of gonocyte proliferation and differentiation

The addition of activin to a gonocyte culture system rules against any antagonistic effect of SCs (which produce inhibin-bA subunit) on gonocyte proliferation. However, although Hasthorpe[145] observed that activin increased gonocyte colony formation, very little effect on spermatogonia cells was demonstrated in this study.[145] On the other hand, other investigators noticed that the addition of activin to culture systems significantly decreased the population of SSCs,[146] suggesting that the stimulation of spermatogonial proliferation by activin may be exerted on more advanced spermatogonia rather than on SSCs. The inconsistency of the findings may be assigned to the dissimilar methods employed for the selection of a spermatogonial population for culture. In the first study, Hasthorpe[145] used the *in vitro* clonogenic method, whereas in the second study Nagano et al[146] applied a two-step enzymatic digestion method on cryptorchid testes to extract spermatogonia as had been described earlier by Ogawa et al.[147] This particular method results in the recovery of a heterogeneous population of germ cells which also includes testicular somatic cells within the population of the recovered cells. These somatic cells might have had a negative effect on the proliferation of SSCs in the study by Nagano et al.[146] Moreover, the use of feeder layers in the study by Nagano et al[146] might also

have had a negative effect on the proliferation of the SSC population. Generally, the *in vitro* attempts to improve the viability and differentiation of cultured gonocytes by adding several growth factors to the basic medium have not yielded any clear results to date. Activin-A, follistatin and FSH appear to play a role in germ cell maturation during the period in which gonocytes resume mitosis to form the SSCs and differentiating germ cell populations.[148] It has been proposed that germ cells have the potential to regulate their own development initially through the production of endogenous activin-A.[148] SCs were observed to produce the activin/inhibin-bA subunit, the inhibin-A subunit and follistatin, demonstrating that these cells play a key role in the regulation of germ cell maturation and development.[148] 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 SCs, whereas treatment with follistatin and FSH enhanced the population of spermatogonia.[148] Hence, it appears that locally produced activin can stimulate gonocyte proliferation immediately after birth in the rat testis.[148] Therefore, it is possible that activin and follistatin may play a critical role in the development of gonocytes into spermatogonia.

Other investigators demonstrated that FSH has an indirect effect on the gonocytes by inducing SCs expression of follistatin and inhibin.[149] It appears that the maturation of gonocytes to form spermatogonia could result from the combined action of follistatin and inhibin as activin antagonists, with FSH as the stimulus for inhibin production, thus producing effects on both germ cells and SCs that control germ cell maturation. *In vitro* experiments furnished evidence that FSH and activin stimulate SC proliferation during early postnatal testis development.[150,151] Furthermore, the EGF receptor is functional in spermatogonia and it has been proposed that EGF suppresses testicular germ cell differentiation. Haneji et al[152] have established that EGF inhibits the proliferation of adult mouse type A spermatogonia stimulated by FSH, whereas Wahab-Wahlgren et al[153] observed a stimulatory effect of EGF on spermatogonial proliferation in adult rat seminiferous tubules *in vitro*, with a possible important role in the paracrine regulation of spermatogenesis.

Hormonal regulation of advanced spermatogonial development

In vitro culture systems have provided evidence that spermatogonia in an advanced stage of differentiation have different regulatory mechanisms that control their fate in comparison with the mechanisms regulating gonocyte proliferation.[122] Thus, both SCF and its receptor c-kit have a key role in relatively advanced spermatogonial development. It has been shown that SCF acts as a mitogen and survival factor for spermatogonia type A cultured in a Potassium Simplex Optimized Medium, i.e. a potassium-rich medium.[154] In the same study, granulocyte macrophage colony stimulating factor also enhanced the survival of porcine type A spermatogonial cells.

Insulin-like growth factor-I and -II (IGF-I and IGF-II, respectively) as well as insulin have been shown to promote spermatogonial differentiation into primary spermatocytes.[155] These findings corroborate the data furnished by Tajima et al[156] who have demonstrated, *in vitro,* that IGF-I and TGF-α enhances the differentiation of mouse type A spermatogonia in organ culture. On the other hand, neither PDGF nor fibroblast growth factor (FGF) has the above stimulatory effect. Other investigators, employing a Vero cell conditioned medium rich in growth factors and ILs, showed that FSH inhibits human spermatogonia degeneration and stimulates meiosis entry, being further potentiated by testosterone.[157]

In another series of studies, Tesarik et al[158,159] demonstrated *in vitro* that high concentrations of FSH are a requirement for the completion of meiosis and spermiogenesis. In the latter studies, cultured round spermatids, after the addition of high concentrations of FSH into the culture medium, underwent nuclear changes similar to those taking place during the normal spermiogenesis process with morphological characteristics including nuclear condensation, peripheral migration and protrusion. It should be stressed that in an *in vitro* culture system the high concentration of FSH (25 IU/l) is mandatory to obtain alterations in spermatid morphology.

Hormonal regulation of spermiogenesis

Spermiogenesis implies the maturation and differentiation of the early haploid male gamete to a mature spermatozoon. During the spermiogenetic

process, modifications occur in the entire male gamete including nuclear proteins, cellular size, cellular shape, the position and size of proacrosomal granules and the localization of the centrioles. This fascinating transforming process that converts a round immotile haploid gamete to an elongated cell with potential to move is regulated by a complex of factors/mechanisms. Since immunoreactive ABP has been detected in SCs processes that surround the elongated spermatids, it may be suggested that ABP plays a role in spermiogenesis.[160] ABP has a high affinity for androgens, probably contributing to the generation of high androgen concentrations in the vicinity of certain meiotic germ cells.

The exact role that androgens play in spermiogenesis and spermiation is not clear, but the fact that it is crucial can be deduced from most genetically-modified models of AR ablation in which the germ cells do not reach the spermatid stage.[161] However, the process of spermiogenesis can be studied in androgen-withdrawal models and results indicate that the adhesion between SCs and spermatids is androgen-dependent, since androgen action is required to prevent the retention and phagocytosis of mature, elongated spermatids and the premature release of round spermatids.[48,162] Experimental studies[163,164] have suggested that following withdrawal of intratesticular testosterone, 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 could be the result of the loss of the adhesions of the spermatids to the sustentacular SCs.[165] Complementary studies in the rat experimental model have demonstrated released step 8 round spermatids within the epididymal lumen of animals with low intratesticular testosterone profiles.[164] It has been shown that decreased intratesticular testosterone concentration leads to apoptosis of germ cells.[166] In addition, a consequence of reduction in intratesticular testosterone is the loss of adhesion of round spermatids to the SCs, with subsequent slough into the lumen of the seminiferous tubules and occasionally phagocytization by SCs. The Bcl-2-modifying factor (Bmf) is likely to play a significant role in germ cell death in response to diminished intratesticular testosterone profiles.[80] Bmf has been detected in the subacrosomal space of spermatids of steps 4-16 of the spermiogenetic process. The detection of Bmf in this region is not surprising since Bmf is normally sequestered to the actin cytoskeleton via its conserved dynein light chain-binding domain.[167] The actin polymers in the subacrosomal space have been shown to disappear in the late stages of spermiogenesis (step 19) just before the release of mature spermatozoa from the SCs.[80,168] Show et al[80] have provided strong evidence supporting the absence of the Bmf protein from the subacrosomal space near the end of spermiogenesis (step 16), which implies that Bmf is degraded just before the spermatid is released from the seminiferous epithelium.

There are studies indicating that testosterone together with FSH promotes spermiogenesis by promoting adhesion of round spermatids to sustentacular SCs.[169]

Several investigations demonstrate that some patients with spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome may have rare foci of arrested round spermatids somewhere in the testicle.[170-179] The inability of the round spermatid to undergo elongation in the human is accompanied by poor results in assisted reproductive technology programs using ooplasmic injections of round spermatids for the alleviation of male infertility.[180] Amer et al[175] used the term 'complete spermiogenesis failure' for men in whom the most advanced germ cell present in the therapeutic testicular biopsy material is the round spermatid and the term 'incomplete spermiogenesis failure' for non-obstructed azoospermic men with a very limited number of elongated spermatids in their therapeutic testicular biopsy material. It appears that in some men with spermatogenic arrest at the primary spermatocyte stage, a number of germ cells can break the barrier of the premeiotic block and differentiate up to the stage of round or elongating spermatids.[175,177,180] Steps 7 and 8 in the classification of the 16 steps of the rodent spermiogenesis[180] represent the beginning of the elongation of the round spermatid. The elongation of the round early haploid male gamete is accompanied by an increase in the reproductive capacity of this early haploid male gamete.[180] Thus, the beginning of the elongation in the round early haploid male gamete is of paramount importance because it allows its further differentiation *in vivo* and the subsequent development of elongating spermatid, elongated spermatid or spermatozoon within the

seminiferous tubule (otherwise there is arrest at the round spermatid stage). In addition, elongation of the round spermatid is accompanied by higher fertilization, pregnancy rates and live birth rates in assisted reproductive technology programs using ooplasmic injections of immature haploid male gametes.[180] Several biochemical mechanisms may be responsible for the inability of the round spermatid to undergo the elongation process in rodents. O'Donnell and coworkers have shown that suppression of intratesticular testosterone content may be one of the mechanisms.[163] Additional studies are necessary to clarify whether an intratesticular testosterone concentration below a specific threshold causes failure of elongation of round spermatids. If this hypothesis is correct, testicular pathophysiologies affecting optimal intratesticular testosterone content may lead to complete spermiogenetic failure. It should be emphasized that varicocele, the most frequent cause of male infertility, known to occasionally cause azoospermia, is accompanied by a reduced intratesticular testosterone concentration.[181]

There are differences in specific early events of the spermatogenesis process mainly prior to the first meiotic division in the human, the monkey and rodents (see for review Sofikitis et al[182]). However in most of the species, the transformation of the round spermatid to a mature spermatozoon is a common multistep process involving a gradual elongation of the early haploid male gamete, development of the male gamete tail and a protrusion of the male gamete nucleus in proximity with the plasma membrane.[182]

The synergic action of FSH and testosterone is mainly demonstrated in spermiation. The suppression of both hormones resulted in further induction of spermiation failure to a percentage equal to 90% compared with their individual separate effects, which were about 15%.[183] The latter effects are likely mediated by β_1-integrin in an integrin-associated kinase-independent mechanism.[184] Indeed, Beardsley and O'Donnell[184] aimed to determine the effects of acute testosterone and FSH suppression on the morphological and ultrastructural events associated with spermiation. In addition, they studied the localization of cell adhesion molecules (integrins and cadherins) and associated molecules (β-catenin and integrin-linked kinase) during hormone suppression-mediated spermiation failure in an experimental animal model. β_1-integrin and one of its associated kinases, integrin-linked kinase, are candidates for mediating/regulating adhesion between the spermatid and Sertoli cell during spermiation.[185] It has been concluded that spermiation failure after testosterone and FSH suppression is caused by a dysfunction in the final disengagement of spermatids from the Sertoli cell rather than by a dysfunction in earlier events, such as ectoplasmic specialization removal, during spermiation. Immunohistochemical data suggested that β_1-integrin, not in association with integrin-linked kinase, plays a role in such normal disengagement by a "loss of adhesion" mechanism. During spermiation failure, this β_1-integrin-mediated loss of adhesion is perturbed, resulting in retained spermatids with associated β_1-integrin immunostaining.[184]

The role of FSH alone in the control of spermiogenesis has been a controversial issue. In the international literature there are studies supporting the notion that FSH may have a stimulatory effect in the early events in spermatogenesis including spermatogonial proliferation and meiosis. However, testosterone alone is capable of sustaining complete spermatid differentiation.[186,187] Studies by Baccetti et al[188] showed that exogenous FSH administration with or without simultaneous administration of human chorionic gonadotrophin in infertile men improved sperm counts. Moreover, the administration of FSH had a beneficial effect on sperm cytostructural parameters.[188] Other experimental studies[189] have demonstrated that there is an increase in propidium iodide stainability of elongated spermatids and an increased sperm head size in FSH receptor knock-out mice. In light of these findings, it appears likely that a disturbance takes place in the normal replacement of histones by protamines throughout spermiogenesis, leading to inadequate condensation of spermatid nuclei in FSH receptor knock-out mice.[189] FSH provides SCs with the ability to effectively 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.[122,157]

Studies in diabetic patients tend to point to a role of insulin in the spermiogenetic process.[190] In addition, insulin gene family members have been identified in round spermatids of human and rat testes, a fact that

provides additional evidence for a role of insulin and IGF in spermiogenesis.[191] Prolactin is another hormone that may play a potential role in spermiogenesis. Prolactin receptor expression has been detected in round and elongating spermatids of intact sheep testes.[192] However, other experimental studies in rat early round spermatids did not detect prolactin receptor mRNA expression,[193] indicating species-dependent differences in the hormones regulating spermiogenesis. The identification of type I and type II activin receptors in round spermatids suggest that activins may play a role in early haploid male germ cells.[194] Inhibin B in the testicular tissue of adult men is possibly a joint product of SCs and germ cells (including the stages from pachytene spermatocytes to early spermatids[195]). Other studies employing immunohistochemistry and *in situ* hybridization techniques[196] demonstrated that the inhibin-bB subunit is detectable in germ cells from the pachytene spermatocytes up to round spermatids, but not in SCs. The actions of activin may be regulated by actions of follistatin, which has been shown to be produced by SCs, spermatogonia, primary spermatocytes and round spermatids.[197]

Successful spermatogenesis is the cornerstone of male fertility. As SCs support and nourish the developing germ cells, they certainly merit particular attention in the process of spermatogenesis. SCs have multiple roles in germ cell development including physical support, immunoprotection and supply of nutrients and other factors. On the other hand, germ cells have specific metabolic needs, which convert during their transformation into spermatozoa, rendering them dependent on the nourishment provided by SCs. SCs employ different substrates, such as glucose and fatty acids, and pathways to accomplish their metabolic demands, as well as those of developing germ cells. The characterization of SCs' energy metabolism, the preferred substrates, the metabolic sub-products produced and the mechanism of adaptation to different conditions[44] will offer new therapeutic approaches against several infertility-related disorders.

THE ROLE OF THE SC AS A POLICEMAN IN A HIGHWAY REGULATING THE TRAFFIC OF SPERMATOGONIA CELLS WITHIN THE SEMINIFEROUS TUBULUS

Table 2 demonstrates that donor mouse, rat, hamster or human spermatogenic cells transplanted into the testes of recipient immunodeficient xenogeneic animals colonized the recipient seminiferous tubules and initiated donor spermatogenesis within the recipients. It appears that the recipient species SC is able to support donor species spermatogenesis (both meiosis and complete spermiogenesis) within the recipient seminiferous tubules in the studies cited in Table 2. In these experiments, the initiation and maintenance of donor spermatogenesis and spermiogenesis within the recipient testes post-donor germ cell transplantation seems to be inconsistent with the fact that the donor spermatogonia have been injected and remained within the lumen of the seminiferous tubules. It appears that donor spermatogonia have moved from the recipient adluminal to the basal compartment of the tubules. Such cellular movement does not occur naturally in adult mammalian species. The mechanisms regulating the translocation of the injected donor spermatogonia from the lumen towards the basal surface of the recipient tubule cannot be easily explained. In contrast, the success of these experiments suggests very emphatically that the recipient Sertoli cell can regulate translocation of recipient and donor germ cells, respectively, either from the basement mem-

Table 2. The Sertoli cell regulates both donor and xenogeneic recipient spermatogenic cell meiosis and spermiogenesis within the same recipient seminiferous tubule.

Donor and recipient species	Authors
Rat spermatogenesis within mouse testis	Clouthier et al, Franca et al[219, 220]
Hamster spermatogenesis within rat testis	Sofikitis et al[99]
Hamster spermatogenesis within mouse testis	Sofikitis et al, Ogawa et al[99, 221]
Human spermatogenesis within mouse or rat testis	Sofikitis et al[222]

Donor rat spermatogonia had been collected from healthy animals;[219,220] Donor hamster spermatogonia had been recovered either from healthy animals or cryptorchid animals;[99,221] Donor human spermatogonia had been collected from non-obstructed azoospermic men.[222]

brane to the lumen or from the lumen to the basement membrane. Thus, the SC acts as a policeman in a highway regulating the traffic in both directions (i.e. from the lumen of the seminiferous tubule to the basement membrane and from the basement membrane to the lumen). In addition, given that hamster spermatogenesis lasts 35 days and rat spermatogenesis lasts 58 days, it appears that the production of both testicular rat spermatozoa and hamster spermatozoa within the same testis, after transplantation of hamster spermatogonia into immunodeficient recipient rats, is consistent with the hypothesis that the SC regulates the speed of spermatogenesis according to messages that it receives from the respective germ cells (either donor or recipients).[99]

GENOMIC IMPRINTING IN THE CELLULAR PRODUCTS OF MALE MEIOSIS AND SPERMIOGENESIS

The great majority of genes are expressed equally by the two parental alleles. However, a small subgroup of mammalian genes is differentially expressed depending on whether they have been inherited from the mother or the father. The process which differentially marks the DNA in the parental gametes is termed genomic imprinting. Genes whose expression is inhibited after passage through the mother's germline are called maternally imprinted. On the other hand, genes whose expression is inhibited when transmitted by fathers are called paternally imprinted.

Several imprinted genes have been characterized.[198] A few studies have shown that imprinted genes regulate the development of the embryo/fetus. Additionally, it has been proposed that DNA methylation maintains the imprinting of some genes. Abnormalities in genomic imprinting are associated with genetic diseases. Prader-Willi syndrome and Angelman syndrome are two examples of abnormal functional imprinting. It should be emphasized that abnormal functional imprinting is implicated in tumorigenesis.

Previous studies[199-201] have indicated that genomic imprinting is complete at the rabbit round spermatid stage and the mouse primary spermatocyte stage. However, additional studies are necessary in the human to confirm that genomic imprinting has been completed at the round spermatid stage. If genomic imprinting is incomplete in subpopulations of men with late maturation arrest or hypospermatogenesis, abnormalities may not become manifest in early embryonic development but they may be detectable in the fetus or during postnatal life. A question of major clinical importance is whether genomic imprinting has been completed at the human round spermatid stage. To attempt to answer this question, the imprinting of a gene should conceptually be divided into three stages: (i) erasure of the previous imprint, (ii) re-imprinting and (iii) consolidation of the new imprint. Several studies have suggested that erasure of the previous imprint occurs prior to meiosis and that re-establishment of the new imprint begins prior to the pachytene stage of meiosis (see for review Tycko[198]). On the other hand, the fact that DNA methyltransferase enzyme is present in spermatids may be an argument against the thesis that genomic imprinting is complete at the round spermatid stage. However, it should be emphasized that waves of DNA methylation have been demonstrated during early embryonic development, the blastocyst stage and the time of implantation.[202] These observations tend to indicate that even if genomic imprinting is not complete at the round spermatid stage, genomic imprinting may be completed after the transfer of the round spermatid within the ooplasm. The work by Kimura and Yanagimachi[199,203] supports the latter thesis. Fishel et al[202] propose that the genomic imprinting of mouse spermatogenic cells is complete in the testis prior to the male second meiotic division.

The methylation patterns of the alleles of an imprinted gene during male germ development are different. For example, prospermatogonia in the embryo demonstrate an absence of methylation of both the maternal and paternal allele of a maternally expressed gene.[204] The paternal allele of a maternally expressed gene of a prospermatogonium acquires hypermethylation by the 15th day postcoitus. After birth, both parental alleles are fully methylated.

Several investigators have attempted to achieve generation of elongated spermatids or spermatozoa in non-obstructed azoospermic men with early or late maturation arrest.[182] Considering that the imprint establishment in humans has been completed by the time the stage of the round spermatid has been reached,[204] it appears that generation of haploid cells

(from surgically recovered testicular spermatogenic dipoid germ cells) and subsequent ooplasmic injections of the above *in vitro* generated haploid cells represents an attractive mode of treatment for men with early maturation arrest. However, it is unknown whether the rapidly progressing meiosis and early spermiogenesis occurring under conditions present in *in vitro* culture systems allow the completion of the genomic imprinting process within these relative short periods. This is a consideration of clinical importance because abnormalities in the completion of genomic imprinting during *in vitro* spermatogenesis may be manifested after fertilization as tumor susceptibility and/or tumorgenesis.

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