REVIEW

Hormonal regulation of male germ cell development

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Abstract

Over the past five decades, intense research using various animal models, innovative technologies notably genetically modified mice and wider use of stereological methods, unique agents to modulate hormones, genomic and proteomic techniques, have identified the cellular sites of spermatogenesis, that are regulated by FSH and testosterone. It has

been established that testosterone is essential for spermatogenesis, and also FSH plays a valuable role. Therefore understanding the basic mechanisms by which hormones govern germ cell progression are important steps towards improved understating of fertility regulation in health diseases.

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Introduction

The past decade has been a critical period in our discoveries of how the complex process of spermatogenic cell development is regulated. These discoveries have been aided through the use of innovative technologies, most notably genetically modified mice, the wider use of best practice stereological methods that allow the rigorous mapping of cell populations, unique agents to modulate hormones, and the genomic and proteomic revolution. Most data come from rodent models, although data are emerging from humans, and similarities in basic processes between species are evidenced. It has been firmly established in rodent models since the 1970s that testosterone is essential for spermatogenesis. The data over the last decade prove that FSH also plays a valuable role, even though it was previously debated. The future challenges for germ cell biologists are to determine the causes and to develop strategies to address the common problem of male infertility. Herein, we aim to provide an overview of male germ cell regulation with relevance to clinical disorders of fertility.

Germ cell development involves mitotic and meiotic divisions and the commitment to 'differentiate or die (survive)' in a process requiring master regulators, FSH and testosterone. FSH and testosterone act at multiple sites in spermatogenesis, either alone or in concert. In rodents, FSH predominantly supports spermatogonial development, while it appears that testosterone provides little support (reviewed in McLachlan *et al.* (2002a) and Ruwanpura *et al.* (2008a,b)). Testosterone partly supports spermatocyte maturation; however, it is critical (at least in rats) in facilitating round to elongated spermatid progression. The release of spermatids from Sertoli cells

(termed spermiation) requires both testosterone and FSH (reviewed in McLachlan *et al.* (2002a)). In humans, spermatogonial development, meiosis, and spermiation are the three main processes that are regulated by gonadotrophins (McLachlan *et al.* 2002b, Matthiesson *et al.* 2005, 2006).

A stable germ cell population is determined by the balance of death (apoptosis) and division, which are influenced by many biochemical factors. The highly coordinated nature of spermatogenesis requires intimate functional and junctional communications between Sertoli and germ cells (Kierszenbaum & Tres 2004, reviewed in Mruk & Cheng (2004) and Sluka *et al.* (2006)). It is generally accepted that germ cells do not contain receptors for hormones; thus, these endocrine factors exert their biological effects on spermatogenesis via the receptors located in or on the plasma membrane of Sertoli cells (Parvinen 1982). Sperm output relies on the establishment of a normal adult Sertoli cell population, which is set around puberty in mammals (for rodents around 2 weeks after birth; Orth 1984, Orth *et al.* 1988, for humans 11–13 years; Zivkovic & Hadziselimovic 2009). Prior to puberty, there is an early wave of spermatogonial apoptosis (effecting 70% of spermatogonial death), which is required to maintain a critical ratio between germ and Sertoli cells (Rodriguez *et al.* 1997). However, it is considered that the Sertoli cell population may not tightly regulate germ cell populations in the testis as originally thought, as transplantation of rat germ cells into the testis of an infertile mouse has shown that germ cells, not Sertoli cells, control the timing and organization of spermatogenesis (Franca *et al.* 1998).

New findings show that hormones, predominantly FSH and testosterone, act as survival factors to regulate

pathway-specific apoptotic genes and proteins, rather than proliferation (Nandi *et al.* 1999, Woolveridge *et al.* 1999, Chausiaux *et al.* 2008, Ruwanpura *et al.* 2008a,b). The intrinsic (mitochondrial) and extrinsic (death receptor) apoptotic pathways play roles in executing germ cell death, although as yet other pathways are unexplored: in the case of spermatogonia, this appears to be almost exclusively via the intrinsic apoptotic pathway in FSH-suppressed rodents (Ruwanpura *et al.* 2008a,b) and in gonadotrophin-deficient men (Ruwanpura *et al.* 2008c). While testosterone regulates rodent spermatocyte and spermatid survival via both the intrinsic and extrinsic pathways (Ross *et al.* 1998, Nandi *et al.* 1999, Woolveridge *et al.* 1999, Chausiaux *et al.* 2008). Understanding these mechanisms may provide new approaches to the development of therapeutic interventions for infertility.

Mechanisms of germ cell survival

There are 11 different types of cell survival mechanisms that are known; 10 of which proceed according to genetically programmed mechanisms, while necrosis is the only non-genetically programmed cell death mechanism which occurs in pathological conditions (Melino *et al.* 2005). However, only three of the genetically programmed cell death mechanisms, such as caspase-dependent apoptosis (Sinha-Hikim & Swerdloff 1999, Shaha 2007), anoikis (Show *et al.* 2004), and caspase-independent apoptosis (Lockshin & Zakeri 2004), are described in the testis. Caspase-dependent apoptosis (hereafter referred to as 'apoptosis') is the most common and is distinct from other forms of cell death due to its involvement in caspase activation via pathways employing death receptors and/or BCL2 family regulators. Apoptosis has been defined morphologically, but it is now clear that other forms of death also feature one or more hallmarks of the apoptotic morphological process. Apoptosis has gained intense attention due to its disturbances in a range of human testicular pathologies, such as cancer (reviewed in Mayer *et al.* (2003)). During normal spermatogenesis, apoptosis regulates the homeostasis of Sertoli cells to germ cell number by inducing programmed cell death to eliminate germ cells that fail to replicate their DNA accurately during cell division. Apoptosis can be also induced by specific stimuli, such as deprivation of hormones, exposure to ionizing radiation, various chemotherapeutic drugs, cell injury, and cell stress.

Biochemical and molecular mechanisms of apoptosis

In the testis, apoptosis represents molecules-directed mechanism involving cysteine proteases (termed caspases) apoptosis promoters, and adaptors to govern cell death at least via two major pathways (Fig. 1). There are two classes of

apoptotic caspases, initiator and executioner caspases, depending on their position of entry into the apoptosis cascade. The initiator caspases, caspases 8, 9, 10, and 12, are the first to get activated in the apoptotic pathway, and then they are involved in the activation of executioner caspases, caspases 3, 6, and 7, in the latter part of the apoptotic pathway (Fig. 1). Caspases are synthesized as inactive pro-enzymes (procaspases) of 32–56 kDa length (reviewed in Shi (2002)), and they reside in the mitochondrial membrane space or in the endoplasmic reticulum (ER; caspase 12) and are then released into the cytosol for activation. Thereafter, activated caspases translocate to the nucleus to perform the cleavage of other proteins and DNA degradation (Krajewski *et al.* 1999).

It is important to recognize that not all of the known caspases are cleaved in every cell in response to the same apoptotic stimuli or within a single cell type in response to different apoptotic stimuli. This heterogeneity likely reflects differential activation of caspases within differing cell types for different apoptotic stimuli. The caspase activation of target cells in the testis is triggered via two major pathways, either by intracellular signals resulting in DNA damage (intrinsic pathway) or by a specific signal delivered through cell surface 'death' receptors (extrinsic pathway; Fig. 1).

The intrinsic (mitochondrial) pathway

The intrinsic pathway involves members of BCL2 family proteins that control the release of cytochrome *C* from the mitochondria into the cytosol (Fig. 1). In normal cells, BCL2 family proteins are found in the outer membrane of the mitochondria, and are bound to adaptor protein APAF1 (Zou *et al.* 1997). BCL2L2 (formerly BCLW) is an important pro-survival member of the BCL2 family (Print *et al.* 1998, Ross *et al.* 1998) that participates in the regulation of apoptosis by dimerizing with the pro-apoptotic factor BAX (Yan *et al.* 2000). It is generally held that the ratio of pro-apoptotic to pro-survival BCL2 family proteins is the critical determinant of cell fate. For example, an excess of BCL2L2 results in cell survival, while an excess of BAX results in cell death via this pathway (Sinha-Hikim & Swerdloff 1999). Apoptotic signals have been suggested to be involved in the modulation of antioxidant defenses, resulting in increased sensitivity to the reactive oxygen species and the induction of the mitochondrial permeability transition (Erkkila *et al.* 1999). In turn, this causes the release of cytochrome *C* into the cytosol and excess BAX to enter into the mitochondria through the open pores of the membrane (reviewed in Adams & Cory (1998), Ashkenazi & Dixit (1998) and Green (2000)). The procaspase 9 and APAF1, together with cytochrome *C*, trigger the activation of caspase 9, and this in turn activates 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, resulting in apoptosis (reviewed in Adams & Cory (1998), Ashkenazi & Dixit (1998) and Green (2000)).

Figure 1 A simplified schematic diagram of the caspase-dependent apoptotic pathway involved in mammalian testicular cells describing three main molecular mechanisms: intrinsic (mitochondrial), extrinsic (death receptor), and endoplasmic reticulum (ER) pathways. The *intrinsic pathway* involves the translocation of apoptotic inducer BAX (BCL2 family member) into mitochondria, while BCL2L2 acts as a suppressor of cell death. Upon BAX translocation, cytochrome C is released into the cytosol where it binds to the apoptosis protease activating factor-1 (APAF1). APAF1 and cytochrome C then bind to procaspase 9 to form an apoptosome by activating caspase 9, leading to subsequent proteolytic activation of the executioner caspases 3, 6, and 7, resulting in apoptosis. Caspase 2 has also been shown to activate this pathway by acting on BAX. The *extrinsic pathway* involves ligation of death receptor protein, FAS, to its ligand (FASL). Binding of FAS to FASL induces trimerization of FAS receptor (FASR), which recruits FAS-associated death domain (FADD). The FAS–FADD complex binds to the initiator caspase 8 for activation, which then activates the executioner caspases 3, 6, and 7 causing apoptosis. The *ER pathway* involves the activation of caspase 12 and subsequent activation of executioner caspases. The crosstalk between these pathways occurs at multiple levels. Crosstalk between apoptotic pathways is evidenced by the fact that in some cells, activated caspase 8 leads to a cleavage of the BCL2 protein family member BID. BID can then induce BAX-mediated release of cytochrome C from mitochondria, further committing the cell apoptosis via the intrinsic pathway.

Most knockout mouse models targeting intrinsic pathway components die in utero by mid-gestation, while the caspase 9 knockout mice die 3 days after birth (Hakem *et al.* 1998, Kuida *et al.* 1998) although their testicular phenotypes have not been studied. Expression of most of the intrinsic pathway components such as *Bcl2L2*, *Bax* mRNAs, and proteins (Yan *et al.* 2000, Meehan *et al.* 2001); APAF1 (Honarpour *et al.* 2000); and cytochrome C (Narisawa *et al.* 2002) has been reported in early germ cell types (spermatogonia and spermatocytes) and in Sertoli cells throughout development. On the other hand, no intrinsic apoptotic pathway components (*BCL2L2* and *BAX*) were expressed in spermatids or mature sperm (Beumer *et al.* 2000, Yan *et al.* 2000).

The extrinsic (death receptor) pathway

The extrinsic pathway represents another major mode of caspase-activated cell death, in which the cell surface death receptors called FAS receptors (FASRs) have emerged as the key regulators (Fig. 1). FAS is a transmembrane receptor protein, and contains a ‘death domain’ and initiates apoptosis

following binding to its ligand (FASL) and then to FASR. This trimerization of ligand–receptor complex recruits the intracellular adaptor molecule, FAS-associated death domain, which binds several molecules of caspase 8 pro-enzyme, resulting in proteolytic activation. Then, activated initiator caspases cleave executioner caspases leading to apoptosis (Nagata & Golstein 1995, Nagata 1997; Fig. 1).

The activation of this pathway has been shown in physiological and experimentally induce germ cell apoptosis (Shaha 2007). In rodents and man, FAS has been localized to germ cells, spermatocytes, and spermatids, while FASL to Sertoli cells (Lee *et al.* 1997, Sugihara *et al.* 1997, Pentikainen *et al.* 1999), while neither FAS nor FASL is expressed in spermatogonia (Francavilla *et al.* 2000).

Other apoptotic pathways and crosstalk between pathways

The ER pathway is associated with cell death, where caspase 12 has been considered to be the main initiator caspase. In gonadotrophin-deficient hypogonadal (*hpg*) mice, an up-regulation of the expression of caspase 12 mRNA level

has been observed (Chausiaux *et al.* 2008), suggesting an involvement of this pathway in the testis. Crosstalk between apoptotic pathways occurs at some levels. In some occasions, caspase 8-mediated activation is less efficient, and/or activation of the effectors is inhibited by the members of the 'inhibitor of apoptosis protein' family (Scaffidi *et al.* 1999, Riedl & Shi 2004). In such a case, a caspase 8-generated cleavage product of BID, a pro-apoptotic BCL2 family member, causes the release of cytochrome C from the mitochondria and leads to cell death via the intrinsic apoptotic pathway (Hengartner 2000; Fig. 1). This type of crosstalk has been observed for spermatocyte apoptosis during the first wave of spermatogenesis in rats (Lizama *et al.* 2007). However, whether this crosstalk exists in other germ cell death models such as hormone deprivation remains unknown.

Following biochemical changes, morphological changes ensue; dying cells shrink and detach from the neighboring cells, and the nucleus becomes markedly condensed. The nucleolus and nuclear membrane of apoptotic cell then break and form discrete membrane-bound 'apoptotic bodies'. The most notable morphological feature of apoptosis is the cleavage of genomic DNA into internucleosomal fragments of about 180–200 bps (Douglas *et al.* 1995, Li *et al.* 1995), which is a hallmark of apoptosis. In addition, early modification of the apoptotic cell involves the exposure of phosphatidylserine (PS) on the outer side of the plasma membrane. The PS-exposing apoptotic cells then bind to receptors on the phagocytotic cells such as macrophages, which then engulf the cell fragments. Both *in vitro* and *in vivo* Sertoli cells recognize, engulf, and remove PS-exposing apoptotic spermatogenic cells (reviewed in Nakanishi & Shiratsuchi (2004)).

Mechanisms of germ cell proliferation

Germ cells proliferate through a series of mitotic and meiotic divisions during which genetic stability is maintained through the precisely regulated DNA duplication, repair, and segregation mechanisms of the cell cycle, which can be divided into four discrete phases. During the cell cycle, spermatogonia grow in the G₁ phase, synthesize DNA in the S phase, prepare for mitosis in the G₂ phase, and undergo mitosis and meiosis in the M phase (consists of four subphases: prophase, metaphase, anaphase, and telophase; Fig. 2). There are two meiotic divisions in spermatocyte development. In prophase I, metaphase I, anaphase I, and telophase I of meiosis I, spermatocytes transform to give rise to leptotene, zygotene, pachytene spermatocytes and diplotene cells respectively. In meiosis II, both diplotene cells undergo further reduction without DNA synthesis and give rise to four haploid germ cells, marking the completion of the M phase (Fig. 2; reviewed in Wolgemuth *et al.* (2002)). At the end of the cell cycle, a cell has two potential fates; the cell can enter either a new cycle or into a quiescence phase (G₀).

Figure 2 A schematic diagram representing the cell cycle of germ cells. The cell cycle has been divided into four discrete phases: S phase (DNA synthesis), M phase (mitosis and meiosis), and two gap regulatory phases G₁ and G₂ prior to M phase and S phase respectively. M phase can be further divided into four subphases: prophase, metaphase, anaphase, and telophase. At the completion of cell cycle after M phase, a cell has two fates: the cell can either start a new cycle or enter a quiescence phase, G₀ (e.g. Sertoli cells after puberty). During M phase, spermatogonia undergo mitosis, while spermatocytes undergo two meiotic divisions. During meiosis I, at prophase I: leptotene, at metaphase I: zygotene, at anaphase I: pachytene and at telophase I: diplotene, spermatocytes are observed. During meiosis II, diplotene spermatocytes undergo further meiotic divisions to eventually give rise to haploid spermatids.

For example, Sertoli cells around puberty enter G₀ phase and remain quiescent. A cell can stay in G₀ phase for a varying amount of time before resuming proliferation. For example, undifferentiated spermatogonia become arrested in G₁–G₀ phase at stages II–III of spermatogenesis, and resume proliferation at stage IX and then undergo 9–11 mitotic divisions during spermatogonial development (de Rooij 2001). The cell cycle can be stopped at specific checkpoints (G₁ and G₂), or the cells can be withdrawn from the cell cycle at any phase. Generally, cells only proliferate if they are stimulated by external factors such as mitotic factors or growth factors, in the absence of which they are directed to G₀ phase, a process that can be reversed upon

appropriate stimulation. Failure to receive survival factors restricts the cell's ability to proceed to the next phase and triggers apoptotic pathways resulting in death (Rodriguez *et al.* 2006).

Hormonal regulation of spermatogenesis

Germ cell proliferation and survival depend heavily on gonadotrophin-dependent mechanisms (McLachlan *et al.* 2002a), the subject of this review, although some aspects appear to be gonadotrophin independent (Allan *et al.* 2004). FSH exerts its biological effects via G protein-coupled FSH receptors (FSHRs) found on the plasma membrane of the Sertoli cells (Simoni *et al.* 1997, Heckert & Griswold 2002). FSH signaling on Sertoli cells is known to activate at least about five signaling pathways, such as cAMP and protein kinase, MAP kinase, calcium, phosphatidylinositol 3-kinase, and phospholipase A₂ pathways (reviewed in Walker & Cheng (2005)). These pathways eventually activate cAMP-responsive element (CRE)-binding protein (CREB), and then transcribe downstream target genes of spermatogenesis. Testosterone is synthesized by Leydig cells upon LH stimulation (de Kretser *et al.* 1971, Wahlstrom *et al.* 1983). Testosterone exerts its biological effects on spermatogenesis via androgen receptors (ARs) that are localized in the Sertoli cells (reviewed in Silva *et al.* (2002)). There is also evidence for a non-genomic action of androgens, in which testosterone elicits responses through secondary messengers such as cAMP and signaling pathways different from classical AR-mediated transcription (reviewed in Walker (2003)). Cellular responses following secondary messenger or signaling pathways in Sertoli cells include the phosphorylation of CREB and CRE modulator, and these transcription factors have also been shown to be regulated by FSH (West *et al.* 1994, Scobey *et al.* 2001). Despite common pathways being activated by FSH and testosterone, some differences exist, for example, testosterone does not up-regulate cAMP production in Sertoli cells (reviewed in Walker & Cheng (2005)). In recent years, the characterization of the signaling pathways regulated by FSH and testosterone has been an important step toward the understanding of how these hormones support spermatogenesis. For example, observations of non-genomic effects of androgens are found to be transient and fast (20–30 s), while modulation of gene transcription via the ARs takes at least 30–45 min (Shang *et al.* 2002). Therefore, it is likely that further studies on the non-genomic signaling will identify additional hormone-regulated genes and factors that are required to support spermatogenesis.

Roles of FSH in spermatogenesis

The size of the Sertoli cell population is set in early postnatal life, and it is a major determinant of sperm output in the adult animal. As spermatogenesis is initiated, Sertoli cells undergo

a period of proliferation in rodents, and numerous *in vivo* and *in vitro* studies have shown FSH to be a key regulator (Sharpe *et al.* 2003). The general view of Sertoli cells in adulthood is that the population is fixed and unmodified by hormonal manipulation after puberty (Sharpe *et al.* 2003). Yet, new evidence derived from gonadotrophin-deficient hamsters and men shows that Sertoli cells retain proliferative ability and can re-acquire features reminiscent of immature Sertoli cells (Meachem *et al.* 2005a, 2007, Tarulli *et al.* 2006). The short-term manipulation of FSH levels in rats and data from transgenic mice models have identified various sites of FSH action in the first wave of spermatogenesis and in adulthood (reviewed in McLachlan *et al.* (2002a)), including the recent findings of roles in spermatogonial survival and adhesion complexes in spermiation (Beardsley & O'Donnell 2003, Ruwanpura *et al.* 2008a,b). On the other hand, the individual role of FSH action in human spermatogenesis is largely undefined. These issues are difficult to address in men for many reasons, including ethical, practical, and physiological considerations. Despite these difficulties, the quality data emerging on gonadotrophin regulation of human spermatogenesis suggest that FSH and/or LH have impact on spermatogonia, meiosis, and spermiation (Zhengwei *et al.* 1998a,b, McLachlan *et al.* 2002c, Matthiesson *et al.* 2005, 2006).

Short-term studies

We have divided the effects of FSH into short and long term purposely. Numerous short-term studies on mammals have reported the effects of FSH manipulation on spermatogenesis (reviewed in McLachlan *et al.* (2002a)). Although data provide important information, the data are incomplete due to the complex and dynamic nature of spermatogenesis. However, maintenance and restoration of full spermatogenesis (thus long-term effects), in cases where there are no testosterone actions, have not been possible until now due to the unavailability of homologous FSH preparations and/or efficient models to fully withdraw hormones. Important lessons can be learnt from both short- and long-term studies. The role of FSH is better defined for adult spermatogenesis than in the first wave; however, it is generally considered that, subtle or otherwise, differences are apparent. For gonocytes, we have documented that FSH plays no role in their maturation into type A spermatogonia *in vivo*, as no changes in apoptotic and proliferation rates have been observed after acute FSH suppression (Meachem *et al.* 2005b). In agreement, gonocyte survival is not affected by FSH treatment in organ cultures of fetal and neonatal rat testes (Boulogne *et al.* 1999). However, FSH combined with other factors such as follistatin, activin, or c-kit has promoted gonocyte maturation (Meehan *et al.* 2000) and survival (reviewed in Orth *et al.* (2000)). Additionally, gonocyte migration appears to be dependent in part on the interaction of c-kit and its FSH-regulated ligand, stem cell factor (reviewed in

Orth *et al.* (2000)). Therefore, it is likely that FSH influences a set of interacting factors to regulate gonocyte development.

Spermatogonia FSH plays a major role in the regulation of spermatogonial population in rodents (reviewed in McLachlan *et al.* (2002a)). During the 15 dpp of first wave of spermatogenesis, FSH acts as a survival factor rather than as a proliferative factor (Meachem *et al.* 2005b). This contrasts with *in vitro* data suggesting that FSH is essential for the proliferation of undifferentiated type A spermatogonia in immature testis culture, whereas FSH combined with activin (which inhibits FSH secretion) markedly depressed their proliferation (Boitani *et al.* 1993, 1995). Therefore, there are differences that exist between the subpopulations of spermatogonia as to whether FSH acts as a mitogenic factor, and this needs to be addressed. In adult rats, short-term manipulation of FSH levels has resulted in a time-dependent change in type A/intermediate spermatogonial number (reviewed in McLachlan *et al.* (2002a)), with the most sensitive types being the A₃–A₄ spermatogonia in stages XIV–I (Meachem *et al.* 1999), which are the stages corresponding to maximal FSHR number (Kangasniemi *et al.* 1990), mRNA expression (Heckert & Griswold 1991), and the highest cAMP production (Parvinen *et al.* 1980). FSH acts as a survival factor for spermatogonia (Krishnamurthy *et al.* 2000), and we have shown it to act in a stage-specific manner on stages XIV–III and VI–VIII tubules (Ruwanpura *et al.* 2008a). While others have reported that FSH stimulates DNA synthesis in spermatogonia and preleptotene spermatocytes at stages I and VIII–IX *in vitro* of adult rat seminiferous tubule segments (Henriksen *et al.* 1996). Inconsistencies with regard to FSH acting as a mitogenic factor may arise from the use of different experimental models. The reason for FSH having little to no effect on proliferation *in vivo* may be the potentially low concentration of biologically active FSH is present and thereby providing support sufficient for proliferation.

Our laboratory demonstrated that acute FSH suppression induces spermatogonial apoptosis via the intrinsic apoptotic pathway, and not via the extrinsic apoptotic pathway in immature and adult rats, as evidenced by increased activated caspase 9-positive spermatogonia, but not by activated caspase 8-positive spermatogonia (Ruwanpura *et al.* 2008a,b). This may occur due to developmentally controlled changes in the expression of intrinsic pathway regulators such as BCL2 family proteins during the first wave of spermatogenesis. Anti-apoptotic genes such as *Bcl2l2*, *Bcl2*, and *Bax* are expressed in juvenile spermatogonia, but only *Bcl2l2* is detected in spermatogonia of adult mice. *Bcl2l2* mRNA was shown to be up-regulated by FSH *in vitro*, and the *Bcl2l2* knockout mice had elevated spermatogonial apoptosis indicating the potential influence of BCL2 family members on cellular responses (Meehan *et al.* 2001). Additional evidence for the intrinsic pathway regulation of spermatogonial survival was provided by the *Bax* gene knockout mice, wherein spermatogonia accumulate suggesting failed physiological apoptosis (Knudson *et al.* 1995, Russell *et al.* 2002), and

when *Apaf1* was removed via gene-targeted deletion, high numbers of degenerating spermatogonia were observed. (Honarpour *et al.* 2000). Collectively, these data reveal that FSH regulates spermatogonial apoptosis via the intrinsic pathway in rodents.

In humans, spermatogonia are highly sensitive to gonadotrophins as evidenced by decreases in type B (~10–20% of control) and type A pale (40% of controls) spermatogonia following 12 weeks of gonadotrophin suppression (Zhengwei *et al.* 1998a, McLachlan *et al.* 2002c). These observations held true in later androgen-based contraceptive studies involving the addition of a gonadotrophin-releasing hormone antagonist or a 5- α reductase inhibitor to further withdraw gonadotrophic/androgenic support for spermatogenesis (Matthiesson *et al.* 2005). However, differences were noted between primates and humans in terms of sensitivities of spermatogonial subtypes to FSH and/or androgens. Several studies using monkeys have indicated that type A spermatogonia, followed by type B spermatogonia, are reduced following gonadotrophin suppression (reviewed in Meachem *et al.* (2001)) as a result of altered proliferation of type A pale spermatogonia (Simorangkir *et al.* 2005), and also, it must be noted that the species of monkeys used can yield differences in outcomes and thus conclusions drawn (Marshall *et al.* 2005). The specific effect of FSH and LH on spermatogonial development in humans is poorly understood. However, data from our laboratory show that FSH appears to maintain spermatogonia up to pachytene spermatocytes (Matthiesson *et al.* 2006), suggesting that FSH plays an important role that is similar to that shown in the rats. The data provided the new finding that gonadotrophins in men act as survival factors for spermatogonia by regulating the intrinsic apoptotic pathway, not the extrinsic apoptotic pathway, while playing no role in proliferation (Ruwanpura *et al.* 2008c). In gonadotrophin-suppressed men, it has not been possible to distinguish whether survival of type A and/or B spermatogonial subpopulations is affected, but since type A spermatogonial numbers are unchanged in these men after 6 weeks of gonadotrophin suppression (McLachlan *et al.* 2002c), it is speculated that B spermatogonia undergo apoptosis.

Spermatocytes/spermatids FSH plays some role in meiosis and spermiogenesis in adulthood, although our understanding of the first wave is rudimentary. Some studies have suggested that FSH is important for the progression of meiosis in the first wave of rat spermatogenesis (Vihko *et al.* 1991), but this has not been studied in the last decade. In adults, FSH appears to support meiosis as shown by a maintenance of preleptotene spermatocytes and partial maintenance of pachytene spermatocytes following the administration of GnRH antagonist with FSH (Chandolia *et al.* 1991, Sinha-Hikim & Swerdloff 1995). Whether these are direct effects remains uncertain as FSH may act indirectly by the regulation of AR number (and its associated pathways, Verhoeven & Cailleau 1988) or that of androgen-binding protein (Louis & Fritz 1979).

However, acute FSH suppression has led to a reduction in pachytene spermatocyte numbers at stage VII in the presence of normal levels of androgens (Meachem *et al.* 1999), suggesting a direct effect of FSH. FSH plays a role in spermiogenesis (McLachlan *et al.* 1995, Meachem *et al.* 1998) perhaps by regulating adhesion and junctions between Sertoli cells and spermatids (Muffly *et al.* 1994, Sluka *et al.* 2006). Our laboratory has identified that sperm fail to be released after passive FSH immunization (Saito *et al.* 2000), indicating a role of FSH in spermiation process.

FSH (in the presence of normal levels of androgens) supports spermatocyte survival by regulating both the intrinsic and extrinsic apoptotic pathways during the onset of meiosis (Ruwanpura *et al.* 2008b). In adult rodents, FSH, together with testosterone, supports the survival of meiotic cells and spermatids via both the intrinsic and extrinsic pathways (Pareek *et al.* 2007, Chausiaux *et al.* 2008, Ruwanpura *et al.* 2008a). Increases in FAS and BAX protein and their mRNA levels (Pareek *et al.* 2007), increases in activated caspase 9- and caspase 8-positive spermatocytes (Ruwanpura *et al.* 2008a) after GNRH antagonist treatment, and increases in the expression of *Casp8* and *Casp9* mRNAs in *hpg* mice (Chausiaux *et al.* 2008) suggest a synergistic action of FSH and testosterone in regulating both the intrinsic and extrinsic pathways. However, in adult rats, acute FSH suppression (in the presence of normal levels of androgens) alone had little or no effect on caspase activity of meiotic and spermatogenic cells (Ruwanpura *et al.* 2008a), suggesting that testosterone promotes later germ cell survival in rats. The data regarding whether FSH acts as a mitogenic factor for spermatocytes are conflicting. Adult rodent data of ours and others provide no evidence that FSH modulates their proliferation (McLachlan *et al.* 1995, Krishnamurthy *et al.* 2000, Ruwanpura *et al.* 2008a).

In humans, it is not clear whether FSH plays a direct role in meiosis and spermiation. However, a study investigating the relative role of FSH and LH in the maintenance of human spermatogenesis shows some indications that FSH appears to be more effective than LH (or intratesticular androgens) in supporting pachytene spermatocytes when simultaneously administered with LH (Matthiesson *et al.* 2006). Similar to rodents, FSH, together with testosterone, acts as a survival factor for spermatocytes and spermatids by regulating both the intrinsic and the extrinsic apoptotic pathways in men. Human seminiferous tubules cultured in a medium without FSH or testosterone showed significantly increased DNA fragmentation in primary spermatocytes and elongating/elongated spermatids via the intrinsic and extrinsic apoptotic pathways (Tesarik *et al.* 1998, 2002, Pentikainen *et al.* 1999, Vera *et al.* 2006).

Long-term studies

Earlier studies claimed that testosterone alone is capable of restoring spermatogenesis qualitatively in adults (Buhl *et al.* 1982, Awoniyi *et al.* 1992) without stimulating pituitary FSH

(McLachlan *et al.* 1994). Data from our laboratory demonstrated that FSH does not have the ability to rescue full spermatogenesis following chronic FSH and androgen action depletion in rats; however, mild increases in spermatogonia and flow on the effect of other germ cells up to pachytene spermatocytes have been observed (SM Ruwanpura, PG Stanton, DM Robertson, RI McLachlan, Y Makanji and SJ Meachem, unpublished observations). Short-term studies clearly show that upon FSH stimulation, spermatogonia are restored in the presence of either low or normal levels of androgens (McLachlan *et al.* 1995, Meachem *et al.* 1998), leading to the assumption that even a low level of androgen action is essential for potentiating the FSH response. Similarly, transgenic mice models show that FSH supports early germ cell development, but that it is incapable of supporting full spermatogenesis (Allan *et al.* 2004, Chang *et al.* 2004, De Gendt *et al.* 2004). The testis of the *hpg* mice lacking both FSH and LH showed that testosterone alone can qualitatively complete the first wave of spermatogenesis (Singh *et al.* 1995), although effects on gonocyte numbers are not apparent (Baker & O'Shaughnessy 2001). Accordingly, the AR Sertoli cell knockout mice show arrest at the late spermatocyte (Chang *et al.* 2004, De Gendt *et al.* 2004), and suggest that FSH alone can support mitosis and meiosis during the first wave, but that androgen action is absolutely necessary for the completion of meiosis and spermiogenesis. *tgFSH/hpg* mice and *hpg* mice after FSH administration (Singh & Handelsman 1996, Allan *et al.* 2004) show increases in early germ cell number up to early spermatids but the absence of spermatozoa. Although mice deficient in FSH protein or receptor show that FSH is not needed for fertility, these mice have reduced levels of sperm (Dierich *et al.* 1998, Krishnamurthy *et al.* 2000, reviewed in Sairam & Krishnamurthy (2001) and Wreford *et al.* (2001)). Transgenic models of FSHRKO, FSH β KO, and *tgFSH/hpg* mice have shown changes in Sertoli cell number (reviewed in Sairam & Krishnamurthy (2001) and Allan *et al.* (2004)), and thus effects on early germ cell populations may be a direct effect of perturbed Sertoli cell proliferation during the first wave of spermatogenesis. Additionally, it has been suggested that the transition of round spermatids into elongated spermatids is a target of direct FSH action as identified in the FSHRKO and FSH β KO transgenic models (reviewed in Sairam & Krishnamurthy (2001)). In these transgenic models, it has been demonstrated that FSH supports both survival and proliferation of germ cells. Adult *tgFSH/hpg* mice provide compelling evidence that FSH supports spermatogonial proliferation and the stimulation of meiotic and post-meiotic germ cell development (Haywood *et al.* 2003). In immature *hpg* mice testes, an up-regulation of the intrinsic pathway transcripts caspase 9 and *Apaf1* was documented in spermatogonia, as assessed by microarray analysis, indicating the involvement of intrinsic apoptotic pathway in the spermatogonial death in response to deficiency in gonadotrophins (Chausiaux *et al.* 2008) although it is anticipated to be a FSH-specific effect.

Role of testosterone in spermatogenesis

It is beyond question that testosterone is needed for adult spermatogenesis. The main targets of testosterone action are meiosis and spermiogenesis with ample data being provided in rodents. In immature testes, testosterone is essential for gonadal formation and testicular differentiation. Over the last 5 years, evidence is mounting that testosterone is involved in Sertoli cell differentiation in early postnatal life (Buzzard *et al.* 2003, Haywood *et al.* 2003, reviewed in Sharpe *et al.* (2003) and Walker (2003)). It has been reported that testosterone in conjunction with thyroid hormone and retinoic acid may be involved in suppressing Sertoli cell proliferation *in vitro* (Buzzard *et al.* 2003), leading to the cessation of proliferation of Sertoli cells (reviewed in Walker (2003)), and that testosterone may act as a suppressor of Sertoli cell development. Although data seem to be model dependent in that Sertoli cell numbers remain unchanged in mice lacking Sertoli cell androgen (De Gendt *et al.* 2004) and LH receptors (Allan *et al.* 2004), Sertoli cell numbers remained unchanged. In contrast, the phenotype of hypogonadic rat appears to be a result of Leydig cell dysfunction, displaying low levels of testosterone with high levels of gonadotrophins (Yagi *et al.* 2007), and suggests that the lack of testosterone in this model leads to Sertoli cell apoptosis in early development. Despite the role testosterone plays in Sertoli cells, the importance of testosterone in the first wave of spermatogenesis requires further investigation.

Germ cells

Gonocytes The role of testosterone in germ cell development during the first wave of spermatogenesis is ill-defined. One study has documented that the lack of testosterone can contribute to the occurrence of multinucleated gonocyte proliferation (Scott *et al.* 2007) during the early stage of the first wave of rat spermatogenesis. However, AR knockout mice with testicular feminization did not demonstrate any occurrence of multinucleated proliferating gonocytes, suggesting an androgen-independent effect (Ferrara *et al.* 2006); however, this may be due to differences between species.

Spermatogonia There is no evidence to suggest that testosterone is required for spermatogonial development in the first wave of spermatogenesis and adult rats. In addition, transgenic mice models reported that testosterone plays no role in spermatogonial development (Haywood *et al.* 2003, reviewed in Holdcraft & Braun (2004)). What is clear is that supraphysiological levels of serum (Meachem *et al.* 1998, Shetty *et al.* 2001) and testicular testosterone are detrimental to spermatogonial development. This is shown elegantly in irradiated/chemotherapy-treated rats and in the juvenile spermatogonial mutant mice, where suppression of supraphysiological testicular testosterone (fourfold normal) is required for the induction of spermatogonial development

(Shetty *et al.* 2001). Similar to rodents, it is presumed that the reduction in spermatogonial numbers in men following gonadotrophin suppression may be purely due to the reduced levels of FSH rather than to those of testosterone.

Spermatocytes/spermatids Testosterone action is critical for the completion of meiosis, entry into and progress through, and spermiogenesis in rodents (reviewed in McLachlan *et al.* (2002a) and Haywood *et al.* (2003)). In immature rats, testosterone is an absolute requirement for the completion of the first wave of spermatogenesis during puberty (Cameron *et al.* 1993, Marathe *et al.* 1995). Similarly, AR Sertoli cell knockout mice have also displayed arrest at late spermatocytes and beyond (Chang *et al.* 2004, De Gendt *et al.* 2004), suggesting that the completion of meiosis and spermiogenesis is dependent on androgen action in the first wave. In adults rats, gonadotrophin suppression induced by testosterone implants had a modest impact on the initiation of meiosis by suppressing early spermatocyte number and pachytene spermatocytes at stages I–VII with reduced number of spermatids (Meachem *et al.* 1997, 1998), and this is further reduced upon the elimination of residual testosterone in these models with an anti-androgen (Meachem *et al.* 1997, El Shennawy *et al.* 1998, O'Donnell *et al.* 1999). Similarly, in adult *hpg* mice, testosterone alone facilitated the completion of meiosis, as evidenced by the production of round spermatids from pachytene spermatocytes (Haywood *et al.* 2003). Several studies from our laboratory have pinpointed particular sites in rat spermiogenesis at which testosterone action is required. Testosterone is important for the conversion of step 7 round spermatids into step 8 spermatids by regulating the adhesion between Sertoli round/elongating/elongated spermatids at the apical ectoplasmic specialization (ES) adherens junctions (O'Donnell *et al.* 1996, 2006, Wong *et al.* 2005). However, testosterone suppression had no effect on the structure of the ES complexes (O'Donnell *et al.* 2006). It has been shown that testosterone regulates ES-restricting proteins such as β 1-integrin, Tyr-phosphorylated focal adhesion kinase (p-FAK), and c-SRC. Therefore, it is believed that testosterone regulates the p-FAK–c-SRC protein complex via the ERK signaling pathway (Wong *et al.* 2005, Cheng & Mruk 2009). In addition, testosterone together with FSH has been found to support spermiation (Saito *et al.* 2000).

The precise mechanism by which testosterone regulates spermiogenesis is not defined; however, stimulation of adhesion molecules and/or regulation of cell survival are highly likely. The role that testosterone plays in germ cell survival during the first wave of spermatogenesis has not been explored in the last decade; however, studies have shown that testosterone regulates spermatocyte and spermatid survival *in vivo* and *in vitro* conditions of immature rats (Tapanainen *et al.* 1993, Marathe *et al.* 1995). A recent study has reported the regulation of both the intrinsic and extrinsic pathway components, *Bcl2l2*, *Casp8*, and *Casp9*, in *hpg* mice at 13 dpp;

however, the specific contribution of testosterone to this process is not known (Chausiaux *et al.* 2008). In adults, several studies have shown that testosterone withdrawal from rat testis leads to an increase in meiotic and spermatogenic cell apoptosis in a stage-dependent manner, and suggested that testosterone may act as a survival factor (Billig *et al.* 1995, Henriksen *et al.* 1995, Marathe *et al.* 1995, Bakalska *et al.* 2004). Few studies have investigated the molecular mechanism by which testosterone affects germ cell apoptosis in rodents. It has been suggested that acute testosterone withdrawal by EDS treatment leads to an increased spermatocyte and spermatid apoptosis with a significant increase in FAS content (Nandi *et al.* 1999). Nonetheless, the co-localization of FASL and FASR correlated with spermatocyte and spermatid death after testosterone withdrawal (Woolveridge *et al.* 1999). Collectively, these suggest the involvement of the extrinsic apoptotic pathway. In addition, a significant increase in BAX was observed after EDS-induced androgen withdrawal in rat testis (Woolveridge *et al.* 1999). It has also been shown that BCL2L2 may be important as *Bcl2l2*-deficient mice are sterile due to the loss of spermatocyte and spermatid apoptosis (Ross *et al.* 1998). Since BAX and BCL2L2 act together as a dimer via the intrinsic pathway during apoptosis, it can be postulated that testosterone withdrawal induces spermatocyte and spermatid apoptosis via the intrinsic pathway. However, whether the intrinsic pathway is regulated by testosterone in rodents requires further investigation.

In humans, using available clinical models of chronic gonadotrophin suppression, it has been deduced that testosterone is important for the conversion of round spermatids (Matthiesson *et al.* 2006). Unlike rat models, detachment of round spermatids from Sertoli cells has not been observed in gonadotrophin-suppressed men (Zhengwei *et al.* 1998b, McLachlan *et al.* 2002c), despite appropriate time periods being studied. Despite the presence of normal numbers of elongated spermatids prior to spermiation in testis biopsies taken from men after 6 weeks of combined testosterone and progestin treatment, most men are oligospermic throughout the treatment phase, suggesting that spermiation failure underlies the initial suppression of sperm counts (McLachlan *et al.* 2002c, Matthiesson *et al.* 2005, 2006). Conversely, concomitant administration of human chorionic gonadotropin (hCG) with male hormonal contraception demonstrated normal spermiation (Matthiesson *et al.* 2006), suggesting the importance of testosterone for spermiation. In humans, testosterone alone or together with FSH acts as a survival factor for spermatocytes and spermatids by regulating both the intrinsic and the extrinsic apoptotic pathways (Tesarik *et al.* 1998, 2002, Pentikainen *et al.* 1999, Vera *et al.* 2006). This was also observed in the non-human primate model, where increases in spermatocyte and spermatid apoptosis were observed in rhesus monkeys following gonadotrophin suppression (Zhou *et al.* 2001, Zhang *et al.* 2003). It has also been suggested that administration of exogenous testosterone to

rhesus monkeys induced spermatocyte and spermatid apoptosis by increasing FAS/FASL (Zhou *et al.* 2001) and BCL2/BAX (Zhang *et al.* 2003) expression, indicating the activation of both the extrinsic and intrinsic apoptotic pathways respectively.

FSH and testosterone co-operativity

Even though FSH and testosterone have independent roles in spermatogenesis, they also act in a co-operative manner to promote quantitative spermatogenesis presumably by modulation of post-receptor events within Sertoli cells. Both FSH and testosterone act in a stage-dependent manner and act at different cellular sites during spermatogenesis in order to optimize the spermatogenic process. Synergy between FSH and testosterone is observed at spermatocyte development, wherein only partial maintenance or restoration of meiosis is achieved by individual hormone suppression or replacement, while combined exposure is much more effective (reviewed in McLachlan *et al.* (2002a)). There are few studies to suggest that testosterone together with FSH promotes spermiogenesis by promoting adhesion of round spermatids to Sertoli cells (O'Donnell *et al.* 2006, Sluka *et al.* 2006). FSH and testosterone synergy is mainly demonstrated in spermiation. The suppression of both hormones led to further induction of spermiation failure to about 90% compared with their individual effects, which were about 15% (Saito *et al.* 2000), and this process is likely mediated by β 1-integrin in an ILK-independent mechanism (Beardsley & O'Donnell 2003).

It has been suggested that both FSH and testosterone act as survival factors of germ cells in immature and adult rats by regulating both the intrinsic and extrinsic apoptotic pathways (Russell *et al.* 1993, Tapanainen *et al.* 1993, Sinha-Hikim & Swerdloff 1995, Meachem *et al.* 2005b, Ruwanpura *et al.* 2008a). The molecular mechanisms of FSH and testosterone synergy remain largely undefined, but there is a possibility that FSH may affect AR action by regulating AR number and androgen-binding proteins (Ottenweller *et al.* 2000). It is not known whether FSH and testosterone co-operativity is required for the proliferation of germ cells; however, there is a possibility that only a small amount of either hormone may be required to promote proliferation.

Observed differences in the ability of FSH and testosterone to either maintain or restore spermatogenesis may be explained in part by differences in experimental models/designs, rather than by their intrinsic activities. There are many reports that a lower dose of either hormone is equally effective in promoting spermatogenesis when in the presence of other hormones (Meachem *et al.* 1998, SM Ruwanpura, PG Stanton, DM Robertson, RI McLachlan, Y Makanji and SJ Meachem, unpublished observations).

Summary

Even though hormones act on all phases of spermatogenesis in rodents, there are only three phases of spermatogenesis that are regulated by gonadotrophins in men: i) the maturation of type A spermatogonia to type B spermatogonia, ii) meiosis, and iii) spermiation, both acutely and chronically (Fig. 3). FSH acts on the development of spermatogonia by controlling their survival mechanisms in rodents; however, the individual effect of FSH and LH on human spermatogenesis is ill-defined, and based on rodent data, one could speculate that FSH plays an important role to support spermatogonia. FSH and testosterone action is important for the progression of meiosis, perhaps by the regulation of the survival via the

intrinsic and the extrinsic apoptotic machineries, and also spermiation in both rodents and humans. However, there are differences between rodent data and human data as to whether hormones play any role during spermiogenesis by involving adhesion complex proteins in humans. Male germ cells need hormones to survive by controlling the intrinsic and extrinsic apoptotic pathways. 'Do germ cells proliferate independent of hormones, but rather via genetic control' is one of the main questions needed to be answered to understand the hormonal and genetic control of spermatogenesis. It is important to investigate whether human data are similar to rodent data, and this will allow us to investigate the effect of specific hormones in germ cells and molecular pathways which underpin these regulations (Fig. 4).

Figure 3 A diagram summarizing the sites, cellular mechanisms, and molecular pathways underpinning FSH and testosterone (T) actions on immature and adult rodent spermatogenesis based on the findings from the literature (in black) and unsolved issues (in red).

Figure 4 A diagram summarizing the sites, cellular mechanisms, and molecular pathways underpinning gonadotrophin (FSH and testosterone (T)) actions on human spermatogenesis based on the findings from the literature (in black) and unsolved issues (in red).

Conclusions

Since the turn of the millennium, there has been a considerable improvement in our understanding of the regulation of germ cell progression. Mouse models, better and wider application of morphometric analysis, evolution of technology such as microscopy and more sensitive hormone assay, new and better molecular probes, and of course the genomic revolution have all underpinned this progress. In terms of the hormonal control of specific spermatogenic sites, most controversies have been remedied, with attention being turned to understand mechanistic actions that each hormone procures. Even though there are appreciable species differences, there are also likenesses with

data demonstrating that different sites of spermatogenesis either rely on hormones almost exclusively or co-jointly. For example, data show that FSH predominantly regulates spermatogonial development, while testosterone regulates the latter phase of spermiogenesis, while both FSH and testosterone seem to be equally important in supporting spermatocyte development.

Now more than ever, the molecular pathways that underpin the hormonal response are being identified, and although we are only at the tip of the iceberg, it is unequivocal that the proteomic revolution will play a major hand in unraveling the precise signaling mechanisms essential for proper germ cell development, arguably more than the insurgence of sophisticated genetic technology. Post-receptor events still

need identification, but research on how non-genomic events play a role in germ cell progression is emerging, and this area of research will undoubtedly be important for putting all the pieces of the puzzle together in an already complex mix of intercellular and intracellular interactions. Limitations still exist in understanding the specific effects of some hormones, particularly FSH, with the foremost reason being the lack of available modulating agents, a problem not encountered for androgens/steroids, although our deeper understanding is attributed to models using genetically modified mice and immunoneutralization of endogenous FSH.

In order to impact clinical management of infertility and diseases such as testicular cancer, understanding the origins of disease is vital. Understanding the precise control process of spermiogenesis and spermatogonial development by hormones is required to achieve a better efficacy and wider and more profound spermatogenetic suppression, and therefore to the development of the world's first available male hormonal contraceptive.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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