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Review

Male germ cell apoptosis: regulation and biology

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Cellular apoptosis appears to be a constant feature in the adult testis and during early development. This is essential because mammalian spermatogenesis is a complex process that requires precise homeostasis of different cell types. This review discusses the latest information available on male germ cell apoptosis induced by hormones, toxins and temperature in the context of the type of apoptotic pathway either the intrinsic or the extrinsic that may be used under a variety of stimuli. The review also discusses the importance of mechanisms pertaining to cellular apoptosis during testicular development, which is independent of exogenous stimuli. Since instances of germ cell carcinoma have increased over the past few decades, the current status of research on apoptotic pathways in teratocarcinoma cells is included. One other important aspect that is covered in this review is microRNA-mediated control of germ cell apoptosis, a field of research that is going to see intense activity in near future. Since knockout models of various kinds have been used to study many aspects of germ cell development, a comprehensive summary of literature on knockout mice used in reproduction studies is also provided.

Keywords: apoptosis; spermatogenesis; orchitis; hormone; toxicant

1. INTRODUCTION

The body of a multicellular organism consists of trillions of cells. A large number of these cells die every day to maintain tissue homeostasis. For this, a process of death is required, which will not mount an immune response thereby protecting the neighbouring cells. Cell death can occur via several processes, few primary ones being necrosis, apoptosis, autophagy and entosis. Necrosis is caused by external factors such as infection, toxins or trauma and usually elicits an immune response, while death by apoptosis, effected by a cellular programme called programmed cell death, is a type of death that does not cause cell lysis and therefore does not initiate an inflammatory reaction. The term apoptosis was first used by Kerr, Wyllie and Currie in 1972 to describe a morphologically distinct form of cell death showing features like DNA laddering, chromatin condensation and membrane blebbing (Kerr *et al.* 1972). Autophagy involves degradation of a cell's own components through the machinery involving lysosomes and is a tightly regulated process that plays a normal part in cell growth, development and homeostasis (Stromhaug & Klionsky 2001; Overholtzer *et al.* 2007). Entosis is a death process that occurs due to a neighbouring cell eating up a particular cell (Overholtzer *et al.* 2007). Apoptosis is the most well-studied process of cell death, and the tissue in which a high incidence of apoptosis occurs in vertebrate body is the testis where 75 per cent of all male germ cells produced are discarded through the process of apoptosis. Apoptosis has conventionally been considered an irreversible process with caspase activation committing a cell to death and the engulfment genes serving the purpose of dead cell removal. However, the uptake and clearance of apoptotic cells by macrophages may involve more than just the removal of cell debris because blocking of engulfment genes in *Caenorhabditis elegans* embryos enhances cell survival when cells are subjected to weak pro-apoptotic signals (Hoeppner *et al.* 2001). It is extremely important to illuminate the precise mechanisms of male germ cell apoptosis because of the very complex interactions of the diverse kinds of cells present within the testis and their differing ability to respond to different stimuli (Hikim *et al.* 2003). Building a knowledge base on the molecular components of the apoptotic programme in spermatogenic cells under different conditions is an essential step towards the development of novel therapeutic regimens to targeted approaches of male contraception and treatment of germ cell tumours and infertility.

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One contribution of 17 to a Theme Issue 'The biology and regulation of spermatogenesis'.

2. SCOPE OF THE REVIEW

Both from the perspective of improving gamete production in infertile individuals and treatment of

testicular disorders, study of cell death in the testis is of great relevance for the betterment of reproductive health. This review attempts to provide an overview of apoptotic processes in the testis under several heads. First, it is important to know the molecules and pathways involved during development because this determines the future state of fertility. Second, how the toxins affect the testis is an essential issue because prevention of such effects especially in occupational healthcare requires implementation and for that targets for interruption needs to be identified. Other information reviewed here includes the influence of hormones on male germ cell apoptosis especially in view of concern about environmental oestrogens, and latest developments in understanding the role of microRNAs (miRNAs) in the testis. Since there are increased instances of testicular cancers due to environmental or other factors and testicular germ cell tumours (TGCT) are the most frequent solid malignant tumour in men of the age group 15–44 years in developed countries, the available literature on apoptosis in teratocarcinoma cells is reviewed as they are the core components of TGCT.

3. THE PROCESS OF APOPTOSIS

Apoptosis is broadly divided into an initiation phase, a signalling phase and an execution phase in which cells rapidly execute a death programme (Steller 1995). There are two major apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway and molecules of one pathway can influence the other (figure 1; Igney & Krammer 2002). Additional pathways include the perforin/granzyme pathway that induce apoptosis via either granzyme B or granzyme A (Martinvalet *et al.* 2005) and the p53 pathway (figure 1) that is essential for cell growth regulation and apoptosis induced by genotoxic and non-genotoxic stresses (Vogelstein *et al.* 2000). Apoptosis is characterized by membrane blebbing, cell volume shrinkage, chromatin condensation, cytoplasmic vacuolization and disassembly of the cell into membrane-bound remnants termed apoptotic bodies (Majno & Joris 1995) that are eventually picked up by phagocytic cells. The biochemical features of apoptosis include phosphatidylserine exposure to the external leaflet of the plasma membrane, activation of caspase cascades, DNA cleavage and DNA laddering (Fadok *et al.* 1992). The process of apoptosis is important in the context of germ cells because the cells are undergoing both mitosis and meiosis and errors during the process may create a need for induction of cell death to eliminate cells with genetic defects (figure 2).

4. SPERMATOGENESIS

Spermatogenesis is a dynamic and synchronized process of maturation of stem spermatogonia into mature spermatozoa, which takes place in the seminiferous tubules of the testis. This process involves mitotic development of spermatogonia and their differentiation into spermatocytes followed by the formation of spermatids and mature spermatozoa. As in many tissues throughout the body, the number of cells in the seminiferous tubules of the testis is determined by a dynamic balance between cell proliferation and apoptotic cell death (Russell *et al.* 2002). Both spontaneous and increased cell death due to triggering stimuli (deprivation of intratesticular testosterone and gonadotrophins, Sertoli cell toxicants, chemotherapeutic drug, etc.) occur via apoptosis.

5. APOPTOSIS IN MALE GERM CELLS

(a) *Apoptosis via the intrinsic pathway during male germ cell development*

Primordial germ cells originate at the embryo epiblast and eventually migrate to the developing gonad. During this event, there is apoptosis of cells that show aberrant migration. Excess cells generated during this period die by apoptosis that is largely dependent on Bcl-xL and Bax (Rucker *et al.* 2000). *Bcl-x* knockout heterozygous (*Bcl-x*(+/−)) mice exhibit severe defects in male germ cells during development (table 1). When the gonocytes differentiate into spermatogonia, phases of increased apoptosis known as the first wave of spermatogenesis occur. In rats and mice this event takes place between days 10 and 30 after birth. The first wave of spermatogenesis in mice and rats involves caspase 2 (Zheng *et al.* 2006), caspase 3, 8 and 9 indicating involvement of both extrinsic and intrinsic pathway of apoptosis (Moreno *et al.* 2006). In transgenic mice with *Bax* 'knocked out' or overexpressing *Bcl-2* or *Bclx* (Furuchi *et al.* 1996), the early wave of apoptosis is eliminated resulting in the accumulation of spermatogonia and spermatocytes as a result of which the animals are infertile (Knudson *et al.* 1995). While *Bcl-2*-deficient mice display normal spermatogenesis, those expressing lower levels of Bcl-xL demonstrate increased germ cell death similar to *Bax* overexpression (Russell *et al.* 2002). Thus, genetically induced alterations of the balance between apoptosis-protecting and apoptosis-inducing proteins in the testis are sufficient to disturb the normal development of functional spermatogenesis (Russell *et al.* 2002). The dynamic changes in the expression profiles of Bcl-2 family proteins observed are consistent with a model in which germ cells are primed for apoptosis during the first cycle of spermatogenesis by de novo expression of the death effectors Bax and Bad in a p53-dependent manner and these proteins are prevented from triggering further apoptosis after the first spermatogenic cycle has been set up by anti-apoptotic Bcl-2 family proteins Bcl-xL and Bcl-w (Yan *et al.* 2000). *Bcl-x* hypomorphic mice show severe reproductive defects attributed to compromised germ cell development (table 1). The loss of *Bcl-x* function in the hypomorph corrected by the deletion of both copies of the *Bax* gene results in a restoration of germ cell survival (Rucker *et al.* 2000). *Bclw* mutant mice display progressive and nearly complete testicular degeneration (table 1). During the early wave, a high level of *Bax* expression is normally seen in germ cells of the testes (Rodriguez *et al.* 1997).

The role of Bcl2 in germ cell development is evident from the variable impairment of spermatogenesis observed when transgenic mice carrying 6 kb of the inhibin-alpha promoter are generated that express human *Bcl-2* gene product in the gonads. These results support the critical role of *Bcl-2* in male germ cell

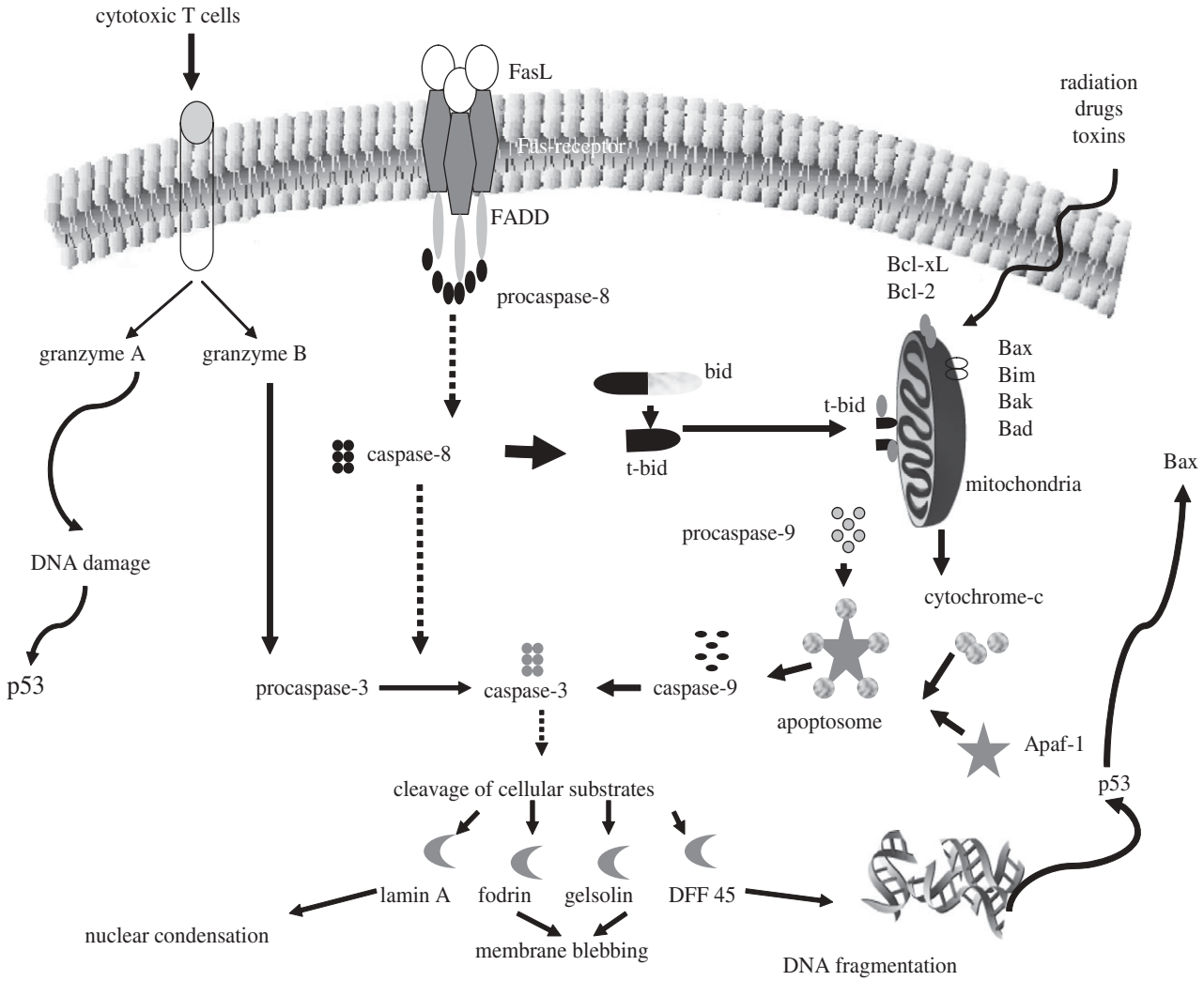

Figure 1. The apoptotic pathways. Cartoon showing primary apoptotic pathways.

development and are consistent with the gender-specific role of the *Bcl-2* family members in reproduction (Yamamoto *et al.* 2001). Bcl6 protein has been detected in testicular germ cells, mainly spermatocytes, of normal mice, but its physiological role is largely unknown although mutants provide some information (see table 1 and review by Shaha 2008). Although testes develop normally in mice lacking either *Bik* or *Bim*, adult *bik*(−/−)*bim*(−/−) males are infertile (see table 1 and review by Shaha 2008).

(b) *Apoptosis via the extrinsic pathway during male germ cell development*

FasL activates Fas which is a 281 amino acid long type II transmembrane molecule. FasL and corresponding receptor Fas both interact as oligomers and the activated Fas receptor complex initiates a pro-apoptotic death signal in the receptor-bearing cell (Janssen *et al.* 2003). Fas receptor and Fas ligand are expressed in the testis (Guazzone *et al.* 2009) and considerable interest has been generated in understanding the role of these proteins in the control of apoptosis in the testis. It has been shown that upregulation of Fas receptor is associated with spermatocyte apoptosis during the first round of spermatogenesis in the rat (Lizama *et al.* 2007). Mice defective in Fas containing a spontaneous loss of function mutation in the Fas gene (homozygous for the lymphoproliferation spontaneous mutation, Fas^{lpr}) or FasL (*gld* mice, homozygous for the $Fasl^{gld}$ mutation) develop obvious lymphoproliferative and autoimmune diseases. Despite the apparent requirement of Fas and FasL for damage-induced germ cell apoptosis, *lpr* (lymphoproliferation) mutant mice are fertile with normal spermatogenesis (Lee *et al.* 1997). These studies suggest that this is due to a 'salvage pathway' that operates to control apoptosis. It appears that in the testes, either the *lpr* mutation has no effect owing to tissue-specific regulation, or a unique system is present to restore proper levels of Fas. The FasL mutant *gld* (generalized lymphoproliferative disease) mice that lack a functional Fas-signalling pathway have a small but significant increase in testis weight and numbers of spermatid heads per testis compared with wild-type mice. In addition, *gld* mice show spontaneous incidence of germ cell apoptosis (Richburg & Nanez 2003). The Fas/FasL system is also involved in germ cell apoptosis in humans. Hypospermatogenesis, such as maturation arrest (MA) and Sertoli cell-only syndrome (SCO), is a result of Fas/FasL-mediated process. Expression of FasL is upregulated in the

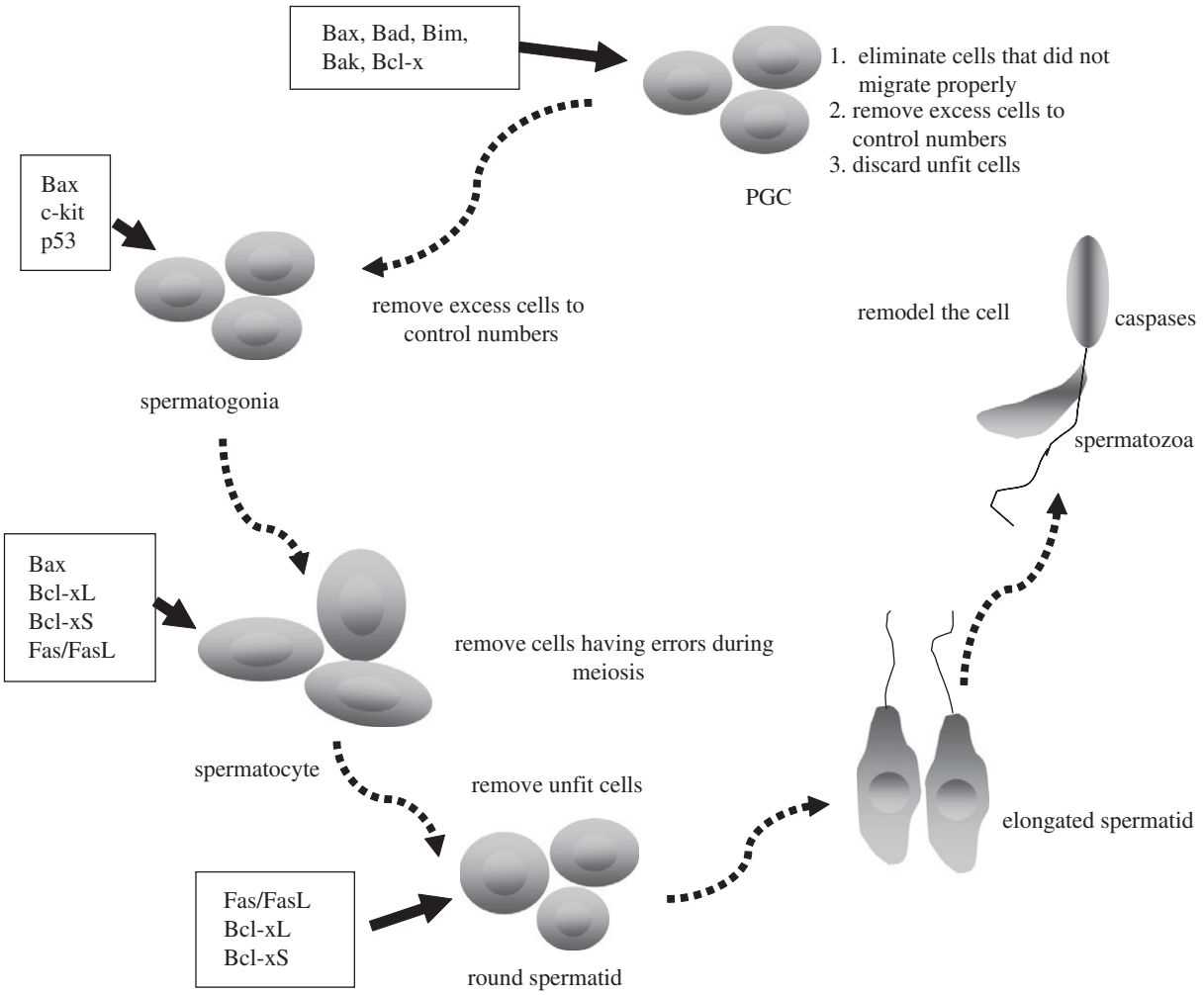

Figure 2. The pro- and anti-apoptotic molecules. Schematic diagram indicates points at which apoptosis occurs during spermatogenesis, the pro- and anti-apoptotic molecules involved and function of the apoptotic process related to the stages of sperm differentiation and maturation.

testes of patients with SCO and MA, which suggests that it may be associated with apoptotic elimination or altered maturation of Fas-expressing germ cells through the activation of caspase-3 (Kim *et al.* 2004).

Studies from our laboratories show that under stimulation with oestradiol, both FasL and Fas are localized to different generations of germ cells (Mishra & Shaha 2005). It has been suggested that in humans, altered meiotic and postmeiotic germ cell maturation might be associated with an upregulation of Fas gene expression capable of triggering apoptotic elimination of defective germ cells (Francavilla *et al.* 2002). It is believed that Fas/FasL expression in the human testis is developmentally regulated and under gonadotropin control. Patients with postmeiotic germ cell arrest show increased Fas expression in germ cells suggesting that the Fas/FasL system may be involved in the quality control mechanism of the produced gametes (Francavilla *et al.* 2002).

(c) *Hormones and male germ cell apoptosis*

In the mammalian testes, follicle stimulating hormone (FSH), luteinizing hormone, human chorionic gonadotropin and testosterone have all been shown to regulate germ cell survival. Exposure to excess hormones or deprivation of hormones can lead to cellular apoptosis in the testis (see review by Shaha 2008). Sertoli cells have receptors for FSH and testosterone that are the main hormonal regulators of spermatogenesis. Hormone removal induces germ cell apoptosis (reviewed in Sofikitis *et al.* 2008). During seminiferous tubule maturation, testosterone and the synergistic action of FSH with oestradiol support germ cell survival while oestradiol alone has an inhibitory, pro-apoptotic effect. It has been shown that both extrinsic and intrinsic apoptotic death pathways are operative in the germ cells following decrease in FSH and testosterone levels; therefore, FSH and testosterone maintain spermatogenic homeostasis by inhibiting death signals for the germ cells (Pareek *et al.* 2007). Excess testosterone can cause death, for example, after injection of testosterone undecanoate, the expression of Fas/FasL in testis increase correlatively in a time-dependent manner, reaching a maximum level on day 30 (Zhou *et al.* 2001). In contrast, testosterone withdrawal stimulates caspase activity and produces DNA fragmentation in Sertoli cells, with only a weak effect on DNA fragmentation and caspase activity in germ cells (Tesarik *et al.* 2002). Evidence has accumulated over several decades now that oestrogen is essential for spermatogenesis and that intratesticular concentrations of oestrogen

Table 1. Summary of data on male germ cell apoptosis using knockout mouse models.

gene symbol	gene name	reproductive phenotype	reference
Bax	Bc12-associated X protein	premeiotic arrest of spermatogenesis, first wave of spermatogenesis affected	Knudson *et al.* (1995)
Hsp70-2	heat shock protein 70-2	meiosis defect and germ cell apoptosis	Dix *et al.* (1996)
Mlh1	MutL homologue 1	apoptosis of pachytene spermatocytes and infertility	Edelmann *et al.* (1996)
Crem	cAMP-responsive element modulator	germ cell apoptosis, late spermatids completely absent	Nantel *et al.* (1996)
HR6B	E2B ubiquitin conjugating enzyme	meiotic arrest, abnormal sperm morphology	Roest *et al.* (1996)
Fshb	FSH hormone β-subunit	decreased testis size	Kumar *et al.* (1997)
Daz1	deleted in azoospermia-like	reduced germ cells by apoptosis; differentiation failure and degeneration of germ cells	Ruggiu *et al.* (1997)
A-myb	protooncogene A-myb	arrest at pachytene spermatocyte stage. Complete absence of postmeiotic cells such as spermatids or spermatozoa. Infertile	Toscani *et al.* (1997)
p53	p53	testicular weight reduction and germ cell apoptosis	Beumer *et al.* (1998), Yin *et al.* (2002)
Bclw	BCL2-like protein apoptosis regulator, BCL-W B1-4 galactosyl-transferase	late meiotic arrest with loss of germ cells; progressive depletion of germ cells through accelerated apoptosis to a Sertoli cell-only phenotype	Ross *et al.* (1998), Russell *et al.* (2001)
Bsg	basigin	block in spermatogenesis at metaphase I	Igakura *et al.* (1998)
Cyp 19	functional aromatase	increased male germ cell apoptosis during spermiogenesis	Robertson *et al.* (1999)
ApafI	apoptotic protease-activating factor I (Apaf-1)	spermatogonial degeneration	Honarpour *et al.* (2000)
Fshr	FSH receptor	decreased testis size	Krishnamurthy *et al.* (2000)
Sycp3	synaptonemal complex protein 3	germ cell apoptosis during meiotic prophase	Yuan *et al.* (2000)
Bcl-6	Bcl-6 protein	spermatocyte apoptosis in metaphase I	Kojima *et al.* (2001)
LHr/hCGr	LH/hCG receptors	germ cell arrest as primary spermatocytes	Lei *et al.* (2001)
TLF	TBP-like factor	complete arrest of spermiogenesis at round spermatid stage	Martianov *et al.* (2001)
TRF2	TATA-binding protein-like protein	postmeiotic spermiogenesis block	Zhang *et al.* (2001)
P18(ink4c) P19(ink4d)	D-type cyclin-dependent kinases (Cdks)	testicular atrophy and germ cell apoptosis	Zindy *et al.* (2001)
MIWI	murine PIWI gene	round spermatid apoptosis	Deng & Lin (2002)
TPAP	testis-specific, cytoplasmic polyadenylate (poly(A)) polymerase	incomplete spermatid elongation and arrest of spermatogenesis	Kashiwabara *et al.* (2002)
SH2-B	SH-2B homologue	small testes owing to increased germ cell apoptosis and reduced sperm counts	Ohtsuka *et al.* (2002)
P53 and FasDouble knock out	cytochrome P45011a, cholesterol side chain cleavage	sequential germ cell apoptosis during development	Yin *et al.* (2002)
Fkbp6	FK506-binding protein	pachytene spermatocyte apoptosis, infertility	Crackower *et al.* (2003)
BclX	Bcl-2 like 1	in male primordial germ cells undergo apoptosis	Kasai *et al.* (2003)
Bc121		around E15.5	
Cks2	CDC28 protein kinase regulatory subunit 2	male germ cell arrest at anaphase	Spruck *et al.* (2003)
BRCA-1	breast tumour suppressor gene 1	apoptosis of pachytene spermatocytes	Xu *et al.* (2003)
Ar	androgen receptor	block in spermatogenesis in premeiotic diplotene, infertility	Chang *et al.* (2004)
ER	oestrogen receptor beta	germ cell apoptosis	Delbes *et al.* (2004)
Mili	Piwi-like homologue 2	spermatogenesis arrested in early prophase I	Kuramochi-Miyagawa *et al.* (2004)
LHbeta	luteinizing hormone beta	apoptosis in round spermatids	Ma *et al.* (2004)
Rxrb	retenoid X receptor beta	accumulation of lipids in Sertoli cells, testicular degeneration and infertility	Mascrez *et al.* (2004)

(*Continued.*)

Table 1. (*Continued.*)

gene symbol	gene name	reproductive phenotype	reference
GRTH/Ddx25	gonadotrophin-regulated testicular RNA helicase	arrest of spermiogenesis by germ cell apoptosis	Tsai-Morris *et al.* (2004)
H1t2	testis-specific and histone H1 variant	abnormal cell restructuring and DNA condensation during the elongation phase of spermiogenesis, reduced fertility	Martianov *et al.* (2005)
Ccna1	cyclinA1	block in spermatogenesis before the first meiotic division	Salazar *et al.* (2005)
Parp-2	poly(ADP-ribose) polymerase-2	delayed nuclear elongation, apoptosis at pachytene and metaphase I	Dantzer *et al.* (2006)
MFP-2	multifunctional protein-2	Sertoli cell apoptosis and infertility	Huyghe *et al.* (2006)
Spo11	meiosis-specific protein Spo11	spermatocytes fail to synapse and progress beyond zygotene stage, infertility	Smirnova *et al.* (2006)
Atm	ataxia telangiectasia	premeiotic germ cells exhibited cell cycle arrest and apoptotic elimination of premeiotic germ cells	Takubo *et al.* (2006)
TSLC1	tumour suppressor of lung cancer 1	spermatid apoptosis leading to infertility	van der Weyden *et al.* (2006)
Rara	retinoic acid receptor A protein	apoptosis of early meiotic prophase spermatocytes, degeneration or germ cells and infertility	Doyle *et al.* (2007)
Cyp26b1	retinoic acid (RA) metabolizing cytochrome P450 enzyme	embryonic germ cell apoptosis	MacLean *et al.* (2007)
Alpha 1-b-AR	alpha 1-b-adrenergic receptor	germ cells undergo apoptosis during meiosis	Mhaouty-Kodja *et al.* (2007)
P63	P63	regulation of gonocyte numbers	Petre-Lazar *et al.* (2006)
LXR alpha	liver X receptor alpha	germ cell apoptosis	Volle *et al.* (2007)
SIRT-1	sirtuin-1	apoptosis in pachytene spermatocytes	Kolthur-Seetharam *et al.* (2009)

are very high (Hess 2003). Since oestrogen receptors are present in the pituitary and spermatogenic cells, oestrogen-like chemicals can act as agonists or antagonists for the hormone and interfere with spermatogenesis and impaired fertility occurs in mice lacking oestrogen receptor-α (Korach 1994). Oestrogen receptor-β (Eddy *et al.* 1996) has been discovered, which is expressed in most of the spermatogenic cells (van Pelt *et al.* 1999). Reports of lowered fertility rates as a consequence of exposure to agents with oestrogenic activity termed as endocrine disruptors are well documented in wildlife populations (Nikula *et al.* 1999). In addition to such examples, it is well established that oestrogen administration to experimental animals during the neonatal period or adulthood can impair sperm production and maturation (McGlynn *et al.* 2008). Our studies demonstrate using both *in vitro* and *in vivo* models that oestrogen induces apoptosis in germ cells through the upregulation of Fas/FasL and that FasL-mediated signal can emanate from different generations of germ cells (Nair & Shaha 2003; Mishra & Shaha 2005). Taken together, the effects of oestrogen on testicular function provide a conceptual basis to examine the speculative link between increased exposure to environmental oestrogens and reduced fertility.

(d) *Toxicant-induced male germ cell apoptosis*

A wide variety of toxins have been tested for their effects on germ cell survival. Most of them have been used to create models for the study of germ cell apoptosis and provide interesting insights into the process. The effects of toxins are summarized in table 2 and for other details check review by Shaha (2008).

Phthalates are ubiquitous environmental toxicants capable of producing testicular atrophy owing to severe germ cell apoptosis in laboratory animals, which is related to increased levels of Sertoli cell FasL (Yao *et al.* 2007; Boekelheide *et al.* 2009).

Nitrobenzene (NB): a component of rubber, pesticides and pharmaceuticals, NB has been identified as a testicular toxicant *in vivo*. Adult, *gld* (generalized lymphoproliferation disease) mice treated with a single oral dose of NB (800 mg kg^{-1}) show a higher apoptotic index as compared with the wild-type C57BL/6 (C57). Similarly, 8-week-old *lpr.lpr* Fas mutant mice treated with NB display a higher apoptotic index as compared with wild-type mice (table 2).

Ionizing radiation: many studies have addressed the effect of either X- or γ-irradiation on cell death in foetal, pre-pubertal and adult rodents (table 2).

Ethanol: among other compounds that affect testis, it is well known that ethanol exposure disrupts the hypothalamic–pituitary–gonadal axis and adversely affects the secretory function of Sertoli cells (table 2). Transgenic mice ubiquitously overexpressing human FasL when treated with 20 per cent ethanol exhibits significantly more apoptotic germ cells than wild-type mice suggesting FasL expression determines the sensitivity of testes to ethanol in mice (Eid *et al.* 2002).

Bisphenol A (BPA): BPA is an organic compound, which is a difunctional building block of several

Table 2. Summary of available literature on toxin-induced male germ cell apoptosis.

toxin	phenotype	apoptotic gene involved	reference
ethane-1,2-dimethanesulfonate	rapid decrease in testosterone levels followed by a characteristic pattern of germ cell loss	Fas	Bakalska *et al.* (2004), Woolveridge *et al.* (2001)
di-(2-ethylhexyl) phthalate	testicular atrophy due to severe germ cell apoptosis	Fas/FasL Apaf-1/caspase-9	Boekelheide *et al.* (2009), Ryu *et al.* (2007), Kuramori *et al.* (2009), Kijima *et al.* (2004)
nitrobenzene	higher apoptotic index	Fas not required	Richburg & Nanez (2003), Allenby *et al.* (1990)
ionizing radiation	foetal gonocyte death apoptosis in adult testicular germ cells	p53	Hughes (1962), Erickson & Blend (1976), Vergouwen *et al.* (1995), Zhu *et al.* (2000), Moreno *et al.* (2001), Chater *et al.* (2007)
ethanol	Sertoli cell vacuolization and germ cell degeneration	Fas/FasL	Koh (2007), Hu *et al.* (2003), Vera *et al.* (2004)
bisphenol A	of Leydig and germ cells	Fas/FasL	Li *et al.* (2009*a*,*b*)
lindane	impair the number of germ cells in the foetal gonads	NF-kB and FasL	La Sala *et al.* (2009), Saradha *et al.* (2009)
2-bromopropane	initiation of primary apoptosis of spermatogonia; reproductive and haematopoietic disorders	downregulation of anti-apoptotic protein Bcl-2 and upregulation of pro-apoptotic protein Bax	Kim *et al.* (1999), Yu *et al.* (2001)
2,5-hexanedione	step 19 spermatids fail to spermeate in rats; apoptosis of male germ cells	Bcl-xL Bcl-xS	Bryant *et al.* (2008), Mishra *et al.* (2006)
4-tert-octylphenol	disrupt testicular development	Bcl-xL reduction	Kim *et al.* (2004)

important plastics and polycarbonate and has been classified as an endocrine disruptor (table 2).

Lindane: lindane, an organochlorine pesticide is known to impair rat testicular functions and fertility through modulation of NF-kB and FasL (table 2).

2-Bromopropane (2-BP): 2-BP, an intermediate in the synthesis of pharmaceuticals, dyes and other organic chemicals, is known to be a male reproductive toxin and epidemiological surveys and animal experimental studies suggest that exposure to 2-BP could result in reproductive and haematopoietic disorders (see table 2 and review by Shaha 2008).

2,5-Hexanedione (2,5-HD): 2,5-HD is a metabolite of the common industrial solvents *n*-hexane and methyl *n*-butyl ketone and is well known to cause apoptosis of male germ cells (Moffit *et al.* 2007). Using an *in vitro* spermatogenic cell apoptosis model, we showed that HD caused a significant increase in reactive oxygen species followed by an enhancement of intracellular Ca^{2+} through the T-type Ca^{2+} channels and cell death is induced through the participation of Bcl-xS and Bcl-xL (see table 2 and review by Shaha 2008).

4-Tert-octylphenol (4-OP): Environmental pollutants that have oestrogenic activity and therefore have the potential to behave like oestrogens are known as endocrine disruptors or environmental oestrogens (Sonnenschein & Soto 1998). 4-OP classified as an environmental oestrogen (Blake & Boockfor 1997) is known to disrupt testicular development and reduce male fertility through interference with synthesis of steroidogenesis (table 2).

(e) *Temperature-induced male germ cell apoptosis*

The scrotal location of the testis allows the germ cells to survive at a lower temperature than that of the main body and germ cell apoptosis occur following exposure to elevated temperatures. Studies using the *gld* and *lprcg* (lymphoproliferation complementing *gld*) mice, which harbour loss-of-function mutations in Fas L and Fas, respectively, show that heat-induced germ cell apoptosis is not blocked in these mice. Therefore, evidence that the Fas-signalling system is not required for heat-induced germ cell apoptosis in the testis is provided by these studies (see review by Shaha 2008). Cryptorchidism, a condition in which the testes are exposed to body temperature rather than scrotal temperature, is a frequent male sexual disorder in mammals, which affects testis functioning (Bernal-Manas *et al.* 2005) through the participation of Bcl-2 and Bax proteins (Xu *et al.* 2000; Zhang *et al.* 2003). P53-dependent apoptosis appears responsible for the initial phase of germ cell loss in experimental cryptorchidism based on a 3-day delay of apoptosis in p53−/− mice. p53−/−, *lpr*/*lpr* double-mutant mice with unilateral cryptorchidism show testicular weight reduction and germ cell apoptosis where the Fas production increased in the time frame of p53-independent apoptosis in the experimental cryptorchid testis of wild-type mice. These results suggest that Fas is involved in heat-induced testicular germ cell apoptosis and that Fas-dependent apoptosis is responsible for the p53-independent phase of germ cell loss in

the cryptorchid testis (Yin *et al.* 2002). Blockade of caspase 2 activation prevents heat-induced germ cell apoptosis in rats by suppressing the MAPK14 (Johnson *et al.* 2008; Li *et al.* 2009*a*,*b*). Serine phosphorylation of *Bcl-2* and activation of the MAPK14-mediated mitochondria-dependent pathway are critical for heat-induced male germ cell death in monkeys (Jia *et al.* 2007).

(f) *Orchitis-induced male germ cell apoptosis*

Autoimmune orchitis is an autoimmune inflammation of the testis that results in degeneration and apoptosis of germ cells owing to a T-cell-mediated mechanism that disrupts testicular immunoprivilege inducing infertility (for reviews see Tung 1995). Recently, it has been shown that extrinsic, mitochondrial and possibly ER pathways are inducers of germ cell apoptosis in experimental autoimmune orchitis and that Bax and Bcl-2 proteins modulate this process (Theas *et al.* 2006).

(g) *Apoptosis in the terminally differentiated spermatozoa*

Terminal differentiation of sperm shares many morphological and biochemical features with apoptosis. However, rather than causing the death of the entire cell, apoptotic proteins are used to specifically eliminate cytoplasmic components, thereby producing a highly specialized living cell. Intracellular bridges between spermatids and the spermatid cytoplasm need to be eliminated during mammalian spermatogenesis. In mammals, at the end of sperm cell differentiation, the cytoplasm collects in the residual body (RB) that displays several features of apoptosis. Although a role of caspases for the removal of bulk cytoplasm during mammalian spermatogenesis remains to be established, data showing that active caspase-3 is present in RBs in the testes of mice are available. The abnormal spermatozoa with residual cytoplasm resulting from caspase inhibition in *Drosophila* bear a striking resemblance to one of the most commonly seen abnormalities known as cytoplasmic droplet. Therefore, it is possible that defects in proper caspase activation may be responsible for this abnormality. The murine septin4 gene (Sept4) has been implicated in diverse cellular functions, including cytokinesis, apoptosis and tumour suppression. A targeted deletion of the Sept4 genomic locus results in viable mice but males are sterile due to immobile and structurally defective sperm showing attached RB. During spermatogenesis, Sept4 mutant sperm showed defects in the elimination of residual cytoplasm during sperm maturation and had increased staining for the caspase inhibitor XIAP. This is consistent with the role of ARTS (a pro-apoptotic protein, one of the four splice variants derived from the Sept4 locus) in promoting caspase-mediated removal of cytoplasm via inhibition of XIAP (Kissel *et al.* 2005). Ectopic overexpression of a protease-dependent caspase homologue c-FLIP(L) in round and elongated spermatids cause a dramatic loss of germ cells resulting in defective spermatozoa (Antonangeli *et al.* in press).

6. APOPTOSIS IN TERATOCARCINOMA CELLS

Testicular carcinoma is the most frequent solid malignant tumour in men of the age group 15–44 years in developed countries, representing 13.4 per cent of new cancer cases (Parkin *et al.* 2005). The most significant risk factor for testicular cancer is cryptorchidism, which increases the risk up to 11-fold (Kinkade 1999). Exposure to endocrine-disrupting chemicals may alter hormonal balance leading to defects in normal apoptosis and thereby increase risk of TGCT (McGlynn *et al.* 2008). These tumours are classified into seminomatous and non-seminomatous germ cell tumours. The seminoma retains a germ cell-like phenotype, whereas the non-seminoma retains embryonic stem cell features and comprises embryonal carcinoma and various mixtures of differentiated teratomatous tissue components (Almstrup *et al.* 2004).

The main drugs used to treat testicular cancer are cisplatin, vinblastine, bleomycin, cyclophosphamide, etoposide, paclitaxel and ifosfamide. Cisplatin is a platinum-based compound and causes DNA intra-stand and inter-stand cross-links, which when not repaired leads to apoptosis. DNA damage by cisplatin results in transcription inhibition, activation of multiple signalling pathways including upregulation of p53 levels, phosphorylation of p38 MAP kinase and Erk1/2 and the others signalling pathways both *in vitro* and *in vivo* (Wu *et al.* 2005; Chauhan *et al.* 2009), which lead to apoptosis in the TGCT. The human TGCT cell lines show an extraordinary sensitivity to treatment with cisplatin and cells undergo apoptotic death upon treatment (Johnstone *et al.* 2002). The mechanism by which cisplatin kills cells is through the generation of reactive oxygen species, also known as one of the intermediates following chemotherapy (Wu *et al.* 2005). UV (10 or 20 $J\,m^{-2}$) radiation causes a massive apoptosis of human teratoma Tera-2 or murine testicular carcinoma F9 cells, both of which contain wild-type p53, but not murine p53 null testicular carcinoma EB-16 cell line. The UV irradiation can induce p53 by suppressing MDM2 expression in a p53-independent fashion and subsequently, massive cell death (Zeng *et al.* 2000). Overexpression of Mcl-1, an anti-apoptotic protein of the Bcl-2 family may function to enhance the viability of testicular germ cells, thereby leading to tumorigenesis (Sano *et al.* 2005).

7. MICRORNAS AS REGULATORS OF MALE GERM CELL APOPTOSIS

miRNAs are a group of highly conserved, non-coding, endogenous small RNAs (typically 19–23 nt long) that strongly regulate gene expression through post-transcriptional gene silencing (Valencia-Sanchez *et al.* 2006) involving mRNA degradation, translational repression, chromatin modification and gene silencing (Carmell *et al.* 2002; Bernstein & Allis 2005). Certain classes of miRNAs are exclusively and preferentially expressed in the testis (Kim 2006; Ro *et al.* 2007) and are mechanistically involved in mammalian spermatogenesis (Kotaja *et al.* 2006*a*; Maatouk *et al.* 2008; Nagamori & Sassone-Corsi 2008; Lian *et al.* 2009).

(a) *Biosynthesis and mode of action of miRNAs during spermatogenesis*

Biogenesis of miRNAs during spermatogenesis starts with the transcription of the miRNA genes in the nucleus of germ cells by RNA Pol II to form the Pri-miRNA. The Pri-mRNAs are capped, polyadenylated and subsequently processed by the RNase III endonuclease, Drosha. Following this event, the miRNA precursors are transported into the cytoplasm and processed from a long hairpin mRNA transcript (about 23-nucleotide duplex) essentially requiring an RNase III endonuclease enzyme known as Dicer. The mature miRNAs are then assembled into an RNA-induced silencing complex (RISC), which subsequently functions on targets by translational repression or mRNA cleavage.

One of the prominent features of nuclear changes during spermatogenesis is the drastic compaction of sperm chromatin resulting in the abrogation of transcriptional activity at later stages of germ cell maturation (Sassone-Corsi 2002; Kotaja *et al.* 2006*b*). During this period, translation of many spermatid-specific genes is arrested and observations indicate that translational arrest of transition protein 2 mRNA occurs through miRNA122a and has been reported to be involved in aberrant spermatogenesis (Yu *et al.* 2005). The spermatids of stages IV–VI in mice have a unique perinuclear structure called the 'chromatoid body'. Recent studies have reported that several RNAs (miRNAs and mRNA), RISC components (Dicer, Ago2, Ago3 and GW182) and Piwi homologue MIWI are indispensable for spermatogenesis (Carmell *et al.* 2007) and are concentrated in the chromatoid body. The mature form of let7-a, a kind of miRNA is concentrated in the chromatoid body (Kotaja *et al.* 2006*a*), which is a characteristic structure in the cytoplasm of spermatocyte and spermatids (figure 3).

(b) *Evidence for specific role of miRNAs in spermatogenesis*

Recent experimental evidence suggests that miRNAs regulate germ cell apoptosis for quality control (Maatouk *et al.* 2008; Papaioannou *et al.* 2009). Mouse maelstrom homologue (MAEL) protein is found in unsynapsed chromosomes of the spermatocyte nucleus and in the chromatoid body. Furthermore, MAEL interacts with the miRNA pathway-associated proteins like mouse vasa homologue (MVH, a germ-cell-specific RNA helicase), piwi-like homologue 2 (MILI) and piwi-like homologue 1 (MIWI) (Argonaute family members; Costa *et al.* 2006). MicroRNA-mediated translational repression involving PIWI/Argonaute family proteins has been widely recognized as a novel mechanism of gene regulation and germ cell numbers during spermatogenesis (Grivna *et al.* 2006).

The conditional knockout mouse lacking proper Dicer1 function have abnormal germ cell differentiation, elongation and sperm motility resulting in infertility (Maatouk *et al.* 2008). The selective ablation of Dicer in mouse Sertoli cells lead to severe impairment of the prepubertal spermatogenic wave owing to defective Sertoli cell maturation and inability to properly support meiosis and spermiogenesis leading to complete absence of spermatozoa and progressive testicular degeneration and subsequent infertility (Papaioannou *et al.* 2009). As Dicer is a component of the miRNA pathway, these studies underscore the importance of precise miRNA biogenesis in male germ cell quality control during spermatogenesis. A recent study using *Ago* 2-mutated mice has established that the critical function of Dicer in spermatogenesis is independent of Argonaute2. During development of male germ cells, miR-17-92 cluster, which is thought to promote cell cycling, and the ES cell-specific cluster encoding miR-290 to -295 (miR-290-295 cluster) were highly expressed in primordial germ cells and spermatogonia. Taken together, it could be concluded that miRNAs are important for the proliferation and/or early differentiation of stem cell population in spermatogenesis (Hayashi *et al.* 2008).

(c) *Clinical significance of miRNAs in male germ cell survival*

The meiotic and haploid phases of spermatogenesis are characterized by high transcriptional activity but suppressed translational activity. Post-transcriptional control of gene expression in these phases can be mediated by sequences in the 5′ and 3′-untranslated regions (UTRs) of mRNAs where they could be regulated by miRNAs, suggesting that miRNAs might play important roles in spermatogenesis (Braun 1998; Kotaja *et al.* 2006*b*; Hayashi *et al.* 2008). These studies suggest possibilities that aberrant miRNA expression could be associated with male infertility. In a study using microarray and quantitative real-time PCR assays with testicular tissues of patients with non-obstructive azoospermia (NOA) versus normal controls identified 154 differentially downregulated and 19 upregulated miRNAs. Altered miRNA expression of NOA in these infertile patients was suggestive of the role of miRNAs in regulating spermatogenesis in human males (Lian *et al.* 2009).

8. KNOCKOUT AND TRANSGENIC MOUSE MODELS IN THE STUDY OF MALE GERM CELL APOPTOSIS

In recent times, the availability of suitable mouse models for the study of male germ cell apoptosis has opened up new avenues in the understanding of the process during normal and abnormal states of spermatogenesis (Knudson *et al.* 1995) and has helped to elucidate the molecular mechanisms of action of the cellular death machinery during germ cell apoptosis thereby providing the basis for the identification of novel targets for male contraception and effective management of male infertility. In the case of male germ cell apoptosis, research using knockout mice allows the effect of specific gene disruptions on the resulting phenotype to be viewed and is often a more preferred system compared with the transgenic mouse model that suffers from the problem of variable expression of the transgene over generations. However, in case of a knockout mouse model, the phenotype is a direct result of the ablation of a targeted gene and can

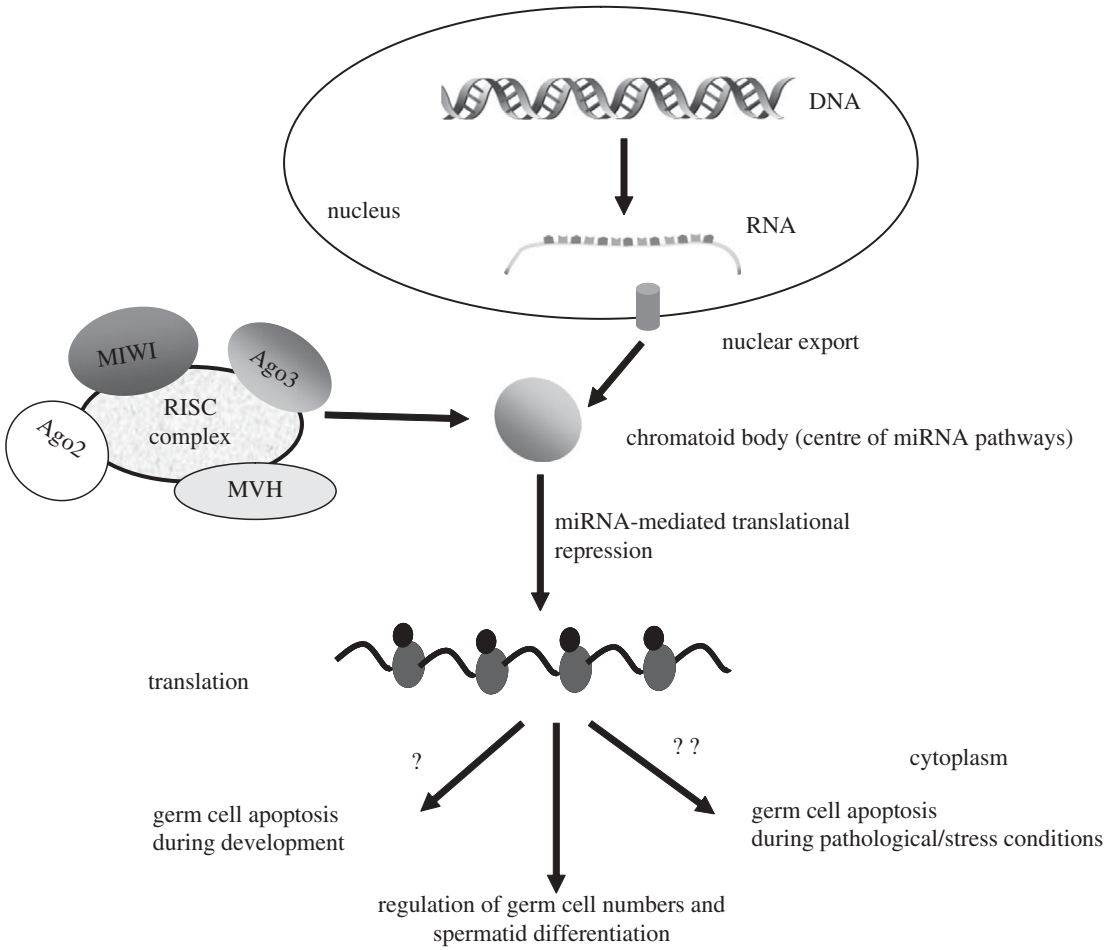

Figure 3. Model of chromatoid body mediated translational repression and apoptosis in postmeiotic male germ cells. In postmeiotic germ cells, the haploid gene transcripts in the nuclei are assembled into ribonucleoprotein particles and exported to the cytoplasm to be stored into the perinuclear chromatoid bodies. The schematic diagram shows events related to apoptosis in postmeiotic germ cells.

provide clues as to the specific biological role of the gene in male germ cell apoptosis. Major findings on the role of pro- and anti-apoptotic proteins derived from knockout mice models include premeiotic arrest of spermatogenesis and first wave of spermatogenesis in *Bax* knockout mice (Knudson *et al.* 1995), significant germ cell apoptosis in *p53* knockouts (Beumer *et al.* 1998), progressive depletion of germ cells through accelerated apoptosis to a Sertoli cell-only phenotype in *Bclw* knockout (Ross *et al.* 1998) and an increase in spermatogonial numbers leading to disruption of spermatogenesis in *Bik*, *Bim* knockouts (Coultas *et al.* 2005). Data from various knockout models are summarized in table 1. Although knockouts provide relevant data, in the reproductive system redundancy plays an important role and data obtained could thus be involving proteins other than the knocked down ones. Therefore, these data should be interpreted with caution and verified through *in vivo* studies with wild-type organisms.

9. APOPTOTIC MARKERS FOR PREDICTION OF SPERM FERTILIZABILITY

Apoptotic markers can potentially be used to assess the fertilizability of spermatozoa. For example, markers for loss of mitochondrial potential are good markers for fertilizability of spermatozoa (Ortega-Ferrusola *et al.* 2009). Increased caspase activity, annexin-V staining and DNA fragmentation can also serve as important markers for sperm survival and ability to fertilize. Sperm with low motility express higher caspase activity and exposure of phosphatidylserine. DNA fragmentation is detected in sperm with lower motility (Weng *et al.* 2002). Therefore, it is evident that markers for apoptosis can also serve as diagnostic parameters for sperm fertilizability. Further research into other apoptotic markers can reinforce this field of study.

10. CONCLUSIONS AND PERSPECTIVES

This review has attempted to highlight the recent developments in the field of male germ cell apoptosis in general including developmental and induced cell death because germ cell apoptosis is involved in every step of testicular development. At present, significant literature is available on the apoptotic pathways in the testis but many aspects are still not understood. Since the testis is a complex organ with different cell types dependent on each other for survival, the challenge is now to identify the functional relevance of inter- and intracellular regulators of germ cell apoptosis. With the realization that miRNAs play a significant role in male germ cell

apoptosis, it is essential to pinpoint the various miRNAs at regulatory points and work out possible induction or inhibition models. Testicular carcinoma of germ cell origin is another model that should be studied to improve modalities of treatment that are toxic at present. The key questions that remain are: (i) are there other apoptotic pathways that are involved in male germ cell apoptosis, like caspase-independent pathways or the granzyme-mediated cell death? (ii) Does autophagy play a role in sperm differentiation or death? (iii) What are the upstream signalling pathways to the apoptotic pathway that actually transmit signals for initiation of apoptosis?

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