

## Apoptosis in the germ line

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### Abstract

**Apoptosis is a critical process for regulating both the size and the quality of the male and female germ lines. In this review, we examine the importance of this process during embryonic development in establishing the pool of spermatogonial stem cells and primordial follicles that will ultimately define male and female fertility. We also consider the importance of apoptosis in controlling the number and quality of germ cells that eventually determine reproductive success. The biochemical details of the apoptotic process as it affects germ cells in the mature gonad still await resolution, as do the stimuli that persuade these cells to commit to a pathway that leads to cell death. Our ability to understand and ultimately control the reproductive potential of male and female mammals depends upon a deeper understanding of these fundamental processes.**

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### Introduction

The term 'apoptosis' was coined by *Kerr et al.* (1972) to describe the process of programmed cell death in multicellular organisms. It differs from the necrotic cell death that follows acute cellular injury in being a carefully orchestrated process involving well-established biochemical entities that drive apoptosis via the 'intrinsic' or 'extrinsic' pathways (*Adams & Cory 2007*). The extrinsic pathway is activated by tumor necrosis factor family receptors triggered by pro-apoptotic ligands such as Apo2L/TRAIL and CD95L/FASL binding to their cognate receptors (DR4/DR5 and CD95/FAS respectively). This induces the clustering of these receptors followed by recruitment of FAS-associated death domain and caspase 8 or 10, which constitute the death-inducing signaling complex. In contrast, the intrinsic apoptotic process represents a default pathway for cells that are suffering from disruption of cellular homeostasis as a consequence of stress induced by a wide variety of factors including attack by reactive oxygen species (ROS), detachment from extracellular matrix, heat, hypoxia,  $\gamma$ -irradiation, DNA damage, or just lack of pro-survival factors. It is regulated by the interplay of the pro- and anti-apoptotic members of the B-cell lymphoma/leukemia 2 (BCL2, Bcl-2) family of proteins (*Youle & Strasser 2008*). The pro-survival

members, including BCL2, BCL2L1 (Bcl-x<sub>L</sub>), MCL1 (Mcl-1), RFC1 (A1), and BCL2L2 (Bcl-w), are essential for survival in a cell type-specific manner. The multi-BH domain pro-apoptotic subfamily members, BAX, BAK1 (BAK), and possibly also BOK, are critical for activation of the downstream events that result in cell death. Finally, the pro-apoptotic BH3-only proteins, including BCL2L11 (BIM), BBC3 (PUMA), BID, BAD, BMF, BIK, HRK, and PMAIP1 (NOXA), initiate apoptosis signaling by activating BAX and BAK1, either through direct binding or, indirectly, through binding to pro-survival BCL2 family members, thereby unleashing BAX and BAK1 (*Cragg et al. 2009, Michalak et al. 2009*). Activation of BAX/BAK1 proteins is followed by release of cytochrome *c* and other apoptogenic factors from the mitochondria, resulting in the formation of the apoptosome, which promotes activation of caspase 9 with subsequent activation of the effector caspases (e.g. caspases 3 and 7).

Functionally, apoptosis is thought to play a major role in tissue morphogenesis during development, in the physiological regulation of cell number, and in mediating the cellular response to adversity. Within the germ line, apoptosis may serve all of these functions depending on the stage of development, the nature of the extracellular environment, the status of intracellular metabolism, the integrity of the cell, and whether it is the male or female germ line under consideration.

## Apoptosis in the male germ line

### *Spermatogenesis*

The testis produces spermatozoa from spermatogonia in a complex developmental cascade involving proliferation, meiotic maturation, and subsequent differentiation of germ cells in the germinal epithelium lining the seminiferous tubules. This process is called spermatogenesis and its duration varies considerably between species ranging from 35 days in mice to 50 days in rats and 70 days in humans. Although daily sperm production from paired testes is impressive (~1000 new spermatozoa produced every second in man) up to 75% of the theoretical sperm yield may be lost during spermatogenesis, depending on the species. Degeneration and death among male germ cells can occur in any phase of spermatogenesis and is attributable to intrinsic or acquired disturbances of cell viability resulting in apoptosis.

### *Apoptosis in the testes*

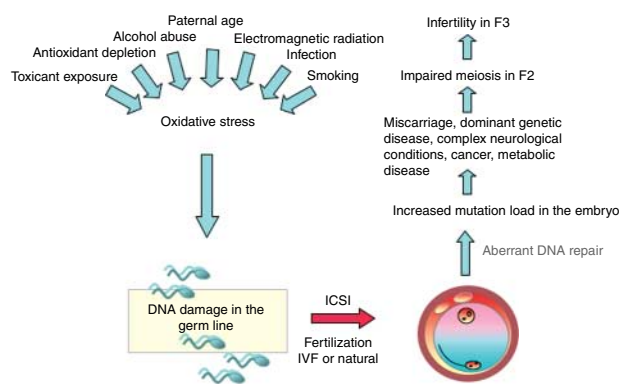
Apoptosis in male germ cells occurs also during fetal life in pro-spermatogonia/gonocytes when the testes are differentiating and adjustments have to be made to achieve the optimal ratio of germ cells to Sertoli cells. During this developmental process, excess premeiotic spermatogonia are removed by an early wave of apoptosis that accompanies the first round of spermatogenesis in the testes. Impairment of apoptosis through the functional deletion of key mediators of this process generates a male infertility phenotype due to an imbalance in germ cell and Sertoli cell numbers (Rodriguez *et al.* 1997). Later in life, apoptosis is involved in the removal of germ cells that are damaged as a result of exposure to environmental toxicants, chemotherapeutic agents, or heat (Wang *et al.* 2007). A role of apoptosis in the etiology of spontaneous male infertility has also been suggested by virtue of the excessively high numbers of apoptotic germ cells observed in the testes of some infertile males (Lin *et al.* 1997). In addition, apoptotic markers including caspase activation and phosphatidylserine exteriorization have been discovered in the mature spermatozoa of male infertility patients (Barroso *et al.* 2000, Paasch *et al.* 2004, Almeida *et al.* 2009, Grunewald *et al.* 2009, Aitken & Curry 2011). Since apoptosis in mature spermatozoa appears to be critically involved in the induction of a subfertile state, it is this aspect of the apoptotic process that we shall focus on in the first part of this review.

### *Apoptosis in spermatozoa*

Interest in the ability of mature spermatozoa to undergo apoptosis has surfaced recently with the discovery that human spermatozoa frequently exhibit high levels of

DNA fragmentation, an archetypal signature of this process (Aitken & De Luliis 2007, 2010). This DNA damage is a significant factor in defining the functionality of spermatozoa and has been linked with a wide variety of adverse clinical outcomes including impaired fertilization, disrupted preimplantation embryonic development, poor implantation rates, and an increased incidence of miscarriage (Aitken & De Luliis 2007, Zini *et al.* 2008, Aitken *et al.* 2009). DNA damage in the male germ line is also thought to have an impact on the health and well-being of children and presumably underpins the relationships that have been repeatedly observed between paternal age, which is positively correlated with sperm DNA damage (Singh *et al.* 2003) and morbidity in the offspring including: i) dominant genetic diseases such as achondroplasia; ii) complex neurological conditions including spontaneous schizophrenia, epilepsy, bipolar disease, and autism; and iii) an increased rate of death in the F1 generation associated with congenital malformations, injury, and poisoning (Sipos *et al.* 2004, Reichenberg *et al.* 2006, Frans *et al.* 2008, Zhu *et al.* 2008, Green *et al.* 2010).

Some of this morbidity is certainly due to an age-dependent increase in the mutational frequency carried by the germ line as a result of replication errors in the spermatogonial stem cell population. These mutations appear to persist in the germ line because they confer upon the mutant germ cells a selective advantage that leads to their preferential clonal expansion within the testes; for example, this appears to be the case for Apert's syndrome and some germ cell tumors (Goriely *et al.* 2009). However, another potential mechanism for male-mediated mutagenic change involves the aberrant repair of sperm DNA damage in the oocyte following fertilization (Fig. 1). Thus, once fertilization has occurred, the oocyte will suppress DNA synthesis in both the male and female pronuclei as it engages in a round of DNA repair (Shimura *et al.* 2002). If the oocyte makes a mistake at this point, a mutation may be created, which, because it precedes the S phase of the first mitotic division, will be in every cell in the body. As a consequence of such aberrant repair, apoptosis-mediated DNA damage in spermatozoa could translate into an increased mutational load carried by the offspring. These mutations might directly impair the viability or health of the F1 generation as in the case of achondroplasia (Hurst & Ellegren 2002, Aitken *et al.* 2004, Aitken & De Luliis 2007) or, if the organization of the DNA is sufficiently disrupted, impair meiosis in F2, further exacerbating the damage to the germ line (Joffe 2010). Through such mechanisms, an environmentally induced, apoptosis-mediated insult on DNA integrity in the male germ line could have trans-generational impacts on the health and fertility of future generations of descendants (Fig. 1). Furthermore, modern-assisted conception techniques such as ICSI only enhance the risk of genetic damage being either created in or



**Figure 1** Hypothesis suggesting one of the mechanisms by which DNA damage in human spermatozoa can increase the mutational load subsequently carried by the embryo. This scheme proposes that a variety of environmental factors can conspire to induce a state of oxidative stress in the male germ line. The DNA-damaged spermatozoa then fertilize the egg and the latter rapidly engages in a round of DNA repair. If this DNA repair is incomplete or inaccurate then mutations may be generated that will influence the survival and well-being of the F1 generation. It has also been suggested that if, as a result of this process, the DNA possesses profound structural defects, this may ultimately influence the normality of meiosis, and hence, fertility in future generations.

transferred to the progeny by facilitating fertilization with DNA-damaged spermatozoa *in vitro* that would have been rejected *in vivo*.

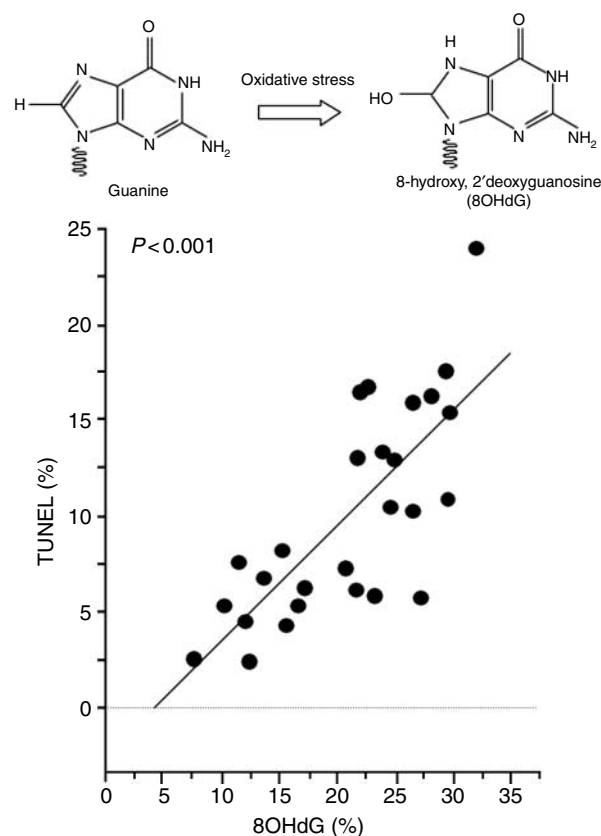
### Biological significance of apoptosis in spermatozoa

Given the biological importance of DNA damage in the male germ line, it is essential that we now determine the cause of this pathology. In this context, three major hypotheses have been advanced: i) DNA fragmentation in spermatozoa may be the result of unresolved strand breaks created during the normal process of spermiogenesis in order to relieve the torsional stresses involved in packaging DNA into the sperm head. Normally, these 'physiological' strand breaks are corrected by a complex process involving H2Ax phosphorylation and the subsequent activation of nuclear poly(ADP-ribose) polymerase and topoisomerase (Meyer-Ficca *et al.* 2009). However, if spermiogenesis should be disrupted for some reason then the restoration of these cleavage sites might be impaired and the spermatozoa, lacking any capacity for DNA repair in their own right, would be released from the germinal epithelium still carrying their unresolved strand breaks. ii) These strand breaks could also represent the induction of an incomplete, abortive apoptotic response during spermatogenesis that achieved endonuclease-mediated DNA cleavage but failed to compromise cell viability. iii) Alternatively, the strand breaks may be the end result of oxidative stress. A recent analysis of DNA damage in the male germ line has demonstrated an extremely close relationship between DNA fragmentation (TUNEL) and oxidative

DNA base damage in spermatozoa, as reflected by the presence of 8-hydroxy, 2' deoxyguanosine (8OHdG), a major metabolic product of DNA oxidation (De Iuliis *et al.* 2009b; Fig. 2). Furthermore, cause and effect have been confirmed by demonstrating that the direct exposure of normal spermatozoa to ROS ( $H_2O_2$ ), or levels of electromagnetic radiation that will trigger endogenous ROS generation, results in a subsequent increase in oxidative DNA damage and DNA fragmentation (Aitken *et al.* 1998, De Iuliis *et al.* 2009a). Since the oxidative stress seen in defective human spermatozoa appears to involve mitochondrial superoxide generation, and mitochondrial ROS is a typical feature of apoptosis (Koppers *et al.* 2008, De Iuliis *et al.* 2009a), a significant involvement of apoptosis in the etiology of DNA damage in the male germ line seems likely.

### DNA damage is correlated with impaired spermiogenesis

Another important factor to be considered in relation to the induction of DNA damage in the male germ line is the role played by impaired spermiogenesis (the differentiation of haploid round spermatids into spermatozoa).

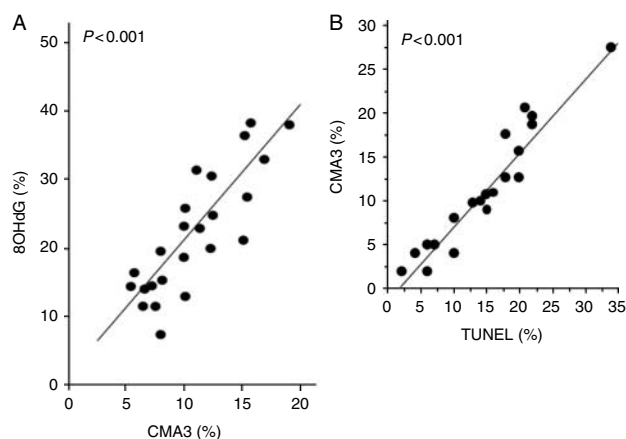


**Figure 2** Powerful correlation observed between DNA fragmentation in human spermatozoa and oxidative DNA base damage as indicated by the presence of 8-hydroxy, 2' deoxyguanosine (8OHdG). Modified from De Iuliis *et al.* (2009a, 2009b).

Thus, several authors have found that DNA damage in spermatozoa is highly correlated with the efficacy of sperm chromatin protamination, measured using the probe chromomycin (CMA3; Bianchi *et al.* 1993, De luliis *et al.* 2009b). This link between defective spermiogenesis and DNA damage is further supported by the fact that several independent studies have also recorded correlations between DNA damage in human spermatozoa and elements of the conventional semen profile (specifically sperm count and morphology), which, in turn, reflect the efficiency of the spermatogenic process (Lolis *et al.* 1996, Iranpour *et al.* 2000, Irvine *et al.* 2000). Spermiogenesis is itself highly susceptible to oxidative stress because isolated spermatids have a limited capacity for both DNA repair and glutathione replenishment (Den Boer *et al.* 1990). Throughout this phase of development these haploid germ cells are highly dependent on the nurturing activity of Sertoli cells, which possess high levels of antioxidant enzymes including superoxide dismutase and the transferase, peroxidase, and reductase activities of the glutathione cycle (Bauché *et al.* 1994). It may also be significant that spermiogenesis is entirely dependent on the regulated translation of preexisting mRNA species. Experimental studies in *Escherichia coli* indicated that severe oxidative stress can induce protein mistranslation through impairment of an aminoacyl-tRNA synthetase editing site (Ling & Söll 2010). If protein translation should be disrupted in this way when differentiating spermatids are placed under oxidative stress, it would explain the close relationship between such stress and disrupted spermiogenesis. Indirect evidence for this relationship is seen in the experimental induction of oxidative stress in the testes with the organophosphorous pesticide, methyl-parathion (Piña-Guzmán *et al.* 2006). Administration of this compound was found to generate oxidative stress that then impaired spermiogenesis, resulting in high levels of DNA damage in the spermatozoa.

### Defective spermiogenesis and apoptosis

It is not just the process of spermiogenesis that appears to be vulnerable to oxidative stress; the defective spermatozoa produced as a consequence of this process also appear to be sensitive to this form of attack for two reasons. First, if chromatin remodeling has been disrupted, then the spermatozoa's DNA will be relatively nucleohistone rich. The persistence of histones impairs normal chromatin compaction (by excluding protamines), leaving the DNA relatively open and vulnerable to oxidative damage (Bennetts & Aitken 2005). This conclusion is supported by the extremely close correlations that have been recorded between CMA3-based assessments of chromatin remodeling during spermiogenesis, DNA fragmentation, and oxidative DNA damage (Fig. 3; De luliis *et al.* 2009b, Aitken & De luliis 2010). Secondly, defective spermatozoa generated in an



**Figure 3** Close relationship between the efficiency of chromatin remodeling as monitored by CMA3 fluorescence and DNA damage in spermatozoa. (A) Correlation between CMA3 and 8OHdG. (B) Correlation between CMA3 and DNA fragmentation as measured by the TUNEL assay. Modified from De luliis *et al.* (2009a, 2009b).

environment of oxidative stress and impaired spermiogenesis have a tendency to default to an apoptotic pathway associated with cell senescence. Mature spermatozoa have the potential to exhibit many of the features of apoptosis including activation of caspases 1, 3, 8, and 9, annexin-V binding, mitochondrial generation of ROS, and DNA fragmentation (Barroso *et al.* 2000, Paasch *et al.* 2004, Koppers *et al.* 2008, Aitken & De luliis 2010). Although many of the reagents that have been shown to induce apoptosis in somatic cells (staurosporine, lipopolysaccharide, 3-deoxy-D-manno-octulosonic acid (Kdo), and genistein) are ineffective with human spermatozoa, these cells will default to the intrinsic apoptotic pathway in response to oxidative stress. Thus, exposure of human spermatozoa to H<sub>2</sub>O<sub>2</sub> can readily trigger an apoptotic cascade characterized by the activation of caspase 3 and the appearance of annexin-V binding positivity (Lozano *et al.* 2009). Furthermore, preexposure of human spermatozoa to antioxidants, such as melatonin or catalase, will prevent this apoptotic response to oxidative stress (Espino *et al.* 2010, Libman *et al.* 2010). Such an apoptotic cascade can also be precipitated by a variety of factors that induce oxidative stress in spermatozoa by triggering free radical generation by the mitochondria, including exposure to radio-frequency electromagnetic radiation (De luliis *et al.* 2009a), unsaturated fatty acids (Aitken *et al.* 2006, Koppers *et al.* 2010), and exposure to the phosphoinositide 3 kinase (PI3 kinase) inhibitor, wortmannin (A Koppers & RJ Aitken, unpublished observations).

### Function of apoptosis in spermatozoa

We propose two major roles of apoptosis in mature spermatozoa. First, this is a mechanism for functionally deleting damaged germ cells. In somatic tissues, the end

point of apoptosis is the activation of caspases that then stimulate a cytoplasmic endonuclease, caspase-activated DNase (CAD) that, in combination with other nucleases released from the mitochondria (endonuclease G and apoptosis-inducing factor), move to the nucleus to induce DNA cleavage and complete the execution process. However, in spermatozoa this process is prevented by the physical architecture of the cell (the mitochondria and nucleus are in different cellular compartments) and the condensed nature of the chromatin. Apoptosis induces motility loss, which deletes the spermatozoa in an immediate functional sense, however, this process is inefficient and may not completely eliminate the cells' competence for fertilization (Aitken *et al.* 1998, De Iuliis *et al.* 2009a, Koppers *et al.* 2010). As a result, it is perfectly possible for apoptotic spermatozoa, possessing high levels of DNA fragmentation, to fertilize the oocyte (Aitken *et al.* 1998), particularly when ICSI is involved (Twigg *et al.* 1998). Further evidence that DNA damage in spermatozoa is not the efficient executioner and that it is in somatic cells is seen in the correlations that have been repeatedly observed between DNA damage in the germ line and miscarriage or morbidity in the offspring. In such cases, conception must have occurred irrespective of the DNA damage in the sperm nucleus (Aitken *et al.* 2009). This is the price the spermatozoon pays for its highly compactible chromatin, for while the latter might protect the DNA to some extent (Bennetts & Aitken 2005), it also impedes the ability of apoptosis to rapidly eliminate these cells. The only element of the apoptotic cascade which can induce DNA damage is the ROS released from the mitochondria and it is for this reason that most of the DNA damage seen in human spermatozoa is oxidative in nature (De Iuliis *et al.* 2009b). More extensive DNA fragmentation may occur *postmortem* as a result of the activation of endonucleases that are integrated into the sperm chromatin, thus finalizing the destruction of the cell (Sotolongo *et al.* 2005).

The second function of apoptosis in spermatozoa is that it represents a form of regulated senescence that permits the fast, efficient, silent removal of these cells from the male and female reproductive tracts once viability has been lost. The reason for this is that the presence of specific apoptotic markers, such as phosphatidylserine, on the surface of apoptotic cells informs incoming phagocytes that removal of the target cells should be silent, in the sense that there should be no oxidative burst or production of pro-inflammatory cytokines (Kurosaka *et al.* 2003). This occurs, for example, when senescent neutrophils are being removed by macrophages from the sites of tissue repair (Leitch *et al.* 2008). Since it is the fate of a majority of spermatozoa to die in the female reproductive tract following insemination, it is essential that the effective removal of these dead and moribund cells is achieved in the absence of inflammation (Rossi & Aitken 1997).

The presence of apoptotic markers on the sperm surface permits such a silent phagocytic removal to occur.

While spermatozoa are supposed to undergo apoptosis in the female reproductive tract following insemination, it is possible that the high levels of apoptosis and DNA damage seen in the spermatozoa of subfertile males represent the premature activation of this regulated senescence pathway (Almeida *et al.* 2009, Aitken & De Iuliis 2010). Such reasoning may explain why the ejaculates of male infertility patients are frequently associated with enhanced levels of leukocytic infiltration (Aitken *et al.* 1994, Tremellen & Tunc 2010).

### Apoptosis and pro-survival factors

If apoptosis represents a default pathway of regulated senescence for mammalian spermatozoa, what normally prevents these cells from entering this pathway? The key to this process is the phosphorylation status of two key defenders of cellular integrity – PI3 kinase and its target AKT (protein kinase B). When these molecules are not adequately phosphorylated, a key substrate molecule, BCL2-associated death promoter (BAD), also becomes dephosphorylated. This leads to BAD breaking away from its binding protein, 14-3-3 and in this unfettered state, moving rapidly to the mitochondria. When BAD reaches the mitochondria, it participates in a process of pore formation that leads to the leakage of mitochondrial factors into the cytoplasm such as Endo G, AIF, and, most important of all, cytochrome *c*. Spermatozoa possess their own form of cytochrome *c* and it is three to five times more powerful an inducer of apoptosis than the somatic version of this protein (Liu *et al.* 2006). It is also a particularly powerful scavenger of ROS and may help limit the release of these powerful mediators of inflammation to the outside of the cell (Liu *et al.* 2006).

If the PI3 kinase pathway is essential for the maintenance of cell viability then we might ask what pro-survival factors normally stimulate this pathway in order to keep spermatozoa from defaulting to this program of regulated senescence and apoptosis. Such factors must exist. Traditionally, when we are performing IVF, spermatozoa are suspended in simple balanced salt solutions, the critical components of which are thought to be calcium, bicarbonate, and a protein source such as albumin to help remove cholesterol from the plasma membrane. In such media, capacitation will occur in a matter of 2–5 h, but the cells have lost their motility in 12–24 h (Alvarez *et al.* 1987). However, *in vivo* sperm survival is an order of magnitude longer since these cells must be able to survive for up to a week in the epididymis as they complete their maturation and up to another week in the female tract as they wait for an egg to arrive. Clearly, *in vivo* pro-survival factors are operating that are missing from our *in vitro* culture media.

In order to understand the nature of these pro-survival factors we need to understand what receptors are on the

surface of these cells that might be able to maintain high levels of PI3 kinase activity and thereby prevent apoptosis. To achieve this aim, we have undertaken a detailed proteomic analysis of human spermatozoa, which has revealed a number of potential receptors that might fulfill this role (Baker *et al.* 2007). One of the receptors identified in this screen was for prolactin. We have subsequently demonstrated that the human spermatozoon has four different isoforms of the prolactin receptor on its surface, the long and three variants of the short form, one of which is unique to the male gamete. Critically, prolactin stimulates PI3 kinase activity, maintains AKT phosphorylation, and has a powerful pro-survival effect on human spermatozoa (Pujianto *et al.* 2010). There is no reason to believe that prolactin is the only or even the most powerful pro-survival factor of human spermatozoa; however, it is the first such factor to be identified. Its discovery heralds a new era in our understanding of sperm cell biology by emphasizing the dependence of these cells on survival factors that, through activation of PI3 kinase–AKT phosphorylation, prevent spermatozoa from entering an apoptotic pathway that culminates in cell death.

## Apoptosis in the female germ line

### Cell dynamics in the ovary

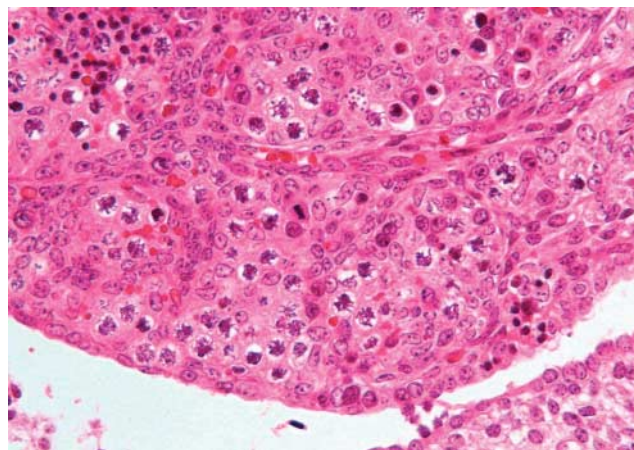
From its earliest differentiation until the time when it is no longer capable of ovulation, the ovary exhibits constant remodeling, whereby new cells and tissues are formed and others degenerate. The population of female germ cells is itself a reflection of the balance between their survival and death in prenatal and postnatal life. Controlled self-destruction of germ cells (apoptosis) is thought to predominate in fetal life, in contrast with the postnatal ovary where elimination of oocytes in growing follicles is a characteristic feature of atresia. However, whether this process is driven primarily by the oocyte or is a secondary response to the degeneration of granulosa cells is uncertain at the present time and requires further research. The development and growth of germ cells and follicles in mammals has been thoroughly reviewed by Byskov & Nielsen (2003).

It is estimated that more than 99% of the germ cells generated during ovarian development are lost through mechanisms involving apoptosis, with most never reaching ovulation. Germ cells are lost in the greatest numbers immediately prior to and during the process of primordial follicle formation, which occurs during embryonic and early postnatal development. Accumulating evidence from knockout mouse studies, expression analyses, and *in vitro* models of oogenesis supports a role of the BCL2-regulated apoptotic pathway in the developmentally regulated death of primordial germ cells, oogonia, and oocytes during the embryonic period. Further research is needed to identify which

members of the BCL2 family are involved and when during the embryonic period. Alternative mechanisms, such as autophagy, are also emerging as key regulators of primordial oocyte death during the early postnatal period. While atresia is thought to originate in the oocyte in primordial follicles, once these follicles enter the growth pathway to become primary to antral follicles, apoptosis begins in the somatic granulosa cells, eventually leading to death of the oocyte and elimination of the follicle. Follicular atresia will not be addressed in this review.

### Establishment of the ovarian pool of primordial follicles

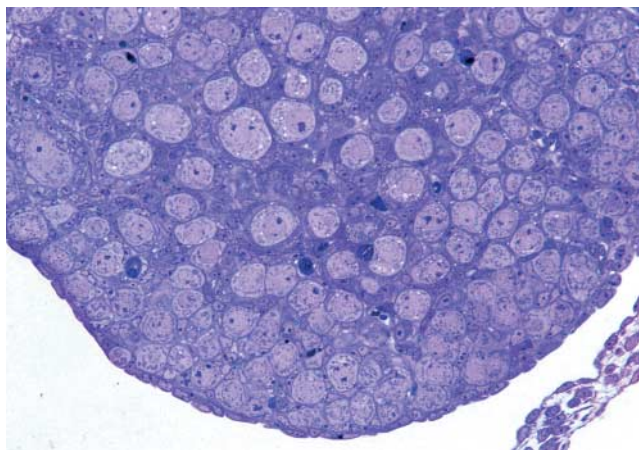
Much of our knowledge on the formation of primordial follicles comes from studies in mice. Oogenesis begins at E7.5 with the allocation of the embryonic precursors of adult gametes (oocytes), known as primordial germ cells. Approximately 45 cells make up the founder population and these migrate from their origin at the base of the allantois through the hindgut to colonize the genital ridge by E11.5 (McLaren 2000). Once in the gonadal anlage, the primordial germ cells migrate centrally forming primitive sex cords after which they cease motility and are called oogonia. However, these cells proliferate extensively during their migration and continue to do so as oogonia after colonization of the gonads (De Felici *et al.* 2005). The oogonia then begin forming nests, beginning at E10.5 in mice, and are connected by intercellular bridges. Proliferation ceases for some oogonia by E13.5, with the number of germ cells peaking at ~26 000 (Borum 1961, Tam & Snow 1981). The oogonia subsequently enter the first stages of meiosis and differentiate into primary oocytes (Fig. 4) at which point proliferation is no longer possible (McLaren 1988). At this time their intercellular bridges are



**Figure 4** Mouse ovary embryonic day 17. The germ cells (oocytes) are in prophase I of meiosis and show thickened chromosomes, characteristic of pachytene. Oocytes are clustered in nests in which somatic or pregranulosa cells do not enclose individual germ cells. Most of the small, scattered pyknotic elements are apoptotic oocytes.

dissolved and individual oocytes become enclosed in a layer of pregranulosa cells. Around the time of birth, the enclosed oocytes arrest in meiotic prophase I at diplotene and become primordial follicles (Fig. 5) with the entire process of follicle formation being complete by postnatal day PN7 (Kerr *et al.* 2006, Pepling 2006). The process of human primordial follicle endowment is analogous, with the peak in germ cell number at mid-gestation, and the completion of primordial follicle assembly before birth (Motta *et al.* 1997).

The number of primordial follicles established within the ovary during development is the combined result of both extensive proliferation and death of primordial germ cells, oogonia, and oocytes. It is important to note that once formed in primordial follicles, oocytes cannot undergo proliferation by cell division. Thus, the continuous death of germ cells throughout embryonic development and into early neonatal life results in the loss of two-thirds or more of germ cells generated, such that in C57/BL6 mice fewer than 10 000 germ cells remain to make up the ovarian reserve at birth and only 2000 primordial follicles remain at PN7 (Kerr *et al.* 2006). Death occurs at all stages of oogenesis, but a wave of attrition involving a large proportion of the germ cells present has been documented in the mouse at E13.5–E15.5, when oogonia cease mitosis and enter into meiosis. A second wave of loss occurs during the breakdown of oogonial nests between E17.5 and PN1 in mice, which is coincident with the assembly of oocytes into primordial follicles (Coucovanis *et al.* 1993, Ratts *et al.* 1995). The strains of mice used by these authors (C57BL and ICR wild-type (WT) female mice respectively) may be significant since variation in oogonial loss is thought to be heavily dependent on genotype. The germ cells lost during these waves before the formation of oocytes in primordial follicles exhibit



**Figure 5** Mouse ovary postnatal day 1. All of the oocytes are arrested at diplotene of meiosis I and most are enclosed by granulosa cells, forming primordial follicles. With the exception of erythrocytes in capillaries, all other densely staining elements are oocytes at various stages of degeneration.

nuclear condensation, cell shrinkage and fragmentation, and DNA laddering with positive TUNEL staining, and PARP1 immunoreactivity, all of which are characteristics typically associated with apoptosis (Coucovanis *et al.* 1993, Pesce & De Felici 1994, Pepling & Spradling 2001, Ghafari *et al.* 2007, Lobascio *et al.* 2007a, 2007b).

#### **Developmentally programmed cell death during ovarian development: BCL2-mediated apoptosis**

There is mounting evidence to suggest that the death of primordial germ cells, oogonia, and oocytes, which occurs during the ovarian development in both mice and humans, is mediated through the actions of the BCL2 family of proteins (Ratts *et al.* 1995, Rucker *et al.* 2000, Flaws *et al.* 2001, Jefferson *et al.* 2006, Greenfield *et al.* 2007, Lobascio *et al.* 2007b, Albamonte *et al.* 2008, De Felici *et al.* 2008).

Gene overexpression and knockout studies in mice have demonstrated that deregulation of the BCL2-regulated apoptosis pathway has a dramatic effect on the initial number of primordial follicles making up the ovarian reserve. Deletion of the pro-apoptotic protein BAX yields increased oocyte numbers as primordial follicles in neonatal ovaries when compared with WT (Greenfield *et al.* 2007). The role of BAX in primordial germ cell, oogonial, and oocyte death is also supported by the observation that BAX expression is upregulated in ovaries during follicular endowment and in cultured fetal oocytes undergoing apoptosis (De Felici *et al.* 1999). BAX expression in oocytes of the human fetal ovary has been detected from the 14th week until term (Vaskivuo *et al.* 2001). If the anti-apoptotic protein BCL2L1 is deleted, the neonatal ovaries have a deficiency of oocytes apparently due to a failure to survive during embryonic life (Rucker *et al.* 2000). Deletion (Ratts *et al.* 1995) or overexpression (Flaws *et al.* 2001, 2006) of BCL2 leads to either reduced oocyte endowment or enhanced oocyte endowment respectively. Collectively, these studies provide clear evidence of the role of the BCL2-regulated apoptotic pathway in establishing the pool of primordial follicles. However, there remain a large number of other members of the BCL2 (and BH3-only proteins) family, which have not been examined with respect to regulating the primordial pool.

#### **Apoptosis in the early postnatal ovary**

Contrary to germ cell death during prenatal ovarian development, the contribution of classical apoptotic pathways to the developmentally driven death of oocytes in the postnatal ovary is less clear. Apoptosis appears to be one of many mechanisms for eliminating oocytes from the germ line once the initial pool of primordial follicles has been established. Tinggen *et al.* (2009)

evaluated the expression of four morphological or biochemical markers typically associated with apoptosis: nuclear condensation/cell shrinkage, active caspase 3, cleavage of poly(ADP-ribose) polymerase 1 (PARP1), and DNA fragmentation within the primordial follicle population of prepubertal and young adult mice (PN6-26). These hallmarks were not commonly observed in primordial follicles in any of the ovaries examined, despite the fact that empirically derived follicle numbers and mathematical models of follicular dynamics indicate the loss of 155 primordial follicles per day per ovary during this stage of development. These observations were supported by [Rodrigues \*et al.\* \(2009\)](#), who also failed to detect significant numbers of PARP1, caspase 3, and TUNEL-positive oocytes in the postnatal ovary ([Rodrigues \*et al.\* 2009](#)). However, these authors did provide evidence to suggest autophagy as an alternative mechanism for oocytes loss in the postnatal ovary, at least during the perinatal period.

### **Biological significance of apoptosis during primordial follicle endowment**

The reason behind the massive depletion of germ cells during prenatal and neonatal ovarian development remains unknown, though several hypotheses have been suggested. The first is death by defect, where germ cell loss may relate to poor quality due to reduced DNA integrity. This loss of DNA integrity in the fetal oogonia may be related to high levels of oxidative DNA damage in these cells, which may, in turn, depend on such factors as maternal nutritional status during pregnancy ([Murdoch \*et al.\* 2003](#)). Mice bearing mutations in genes essential for chromosome pairing, homologous recombination, DNA repair, and synapse formation during the early stages of the first meiotic prophase (which occurs immediately prior to meiotic arrest and primordial follicle assembly) invariably have ovaries devoid of oocytes and are infertile ([Barlow \*et al.\* 1996, 1998](#), [Pittman \*et al.\* 1998](#), [Yoshida \*et al.\* 1998](#)). These studies indirectly suggest that low-quality oocytes, arising as a result of errors made during meiotic recombination or following exposure to high levels of oxidative stress, are eliminated from the ovary. Alternatively, failure of germ cells to accumulate adequate energy supplies and cytoplasmic organelles may mark them for elimination, while a surfeit of organelles and energy stores may promote survival ([Pepling & Spradling 2001](#)). A second hypothesis involves death by self-sacrifice. [Pepling & Spradling \(2001\)](#) believe that this is necessary to break the intercellular bridges between oogonia, allowing the somatic cells to permeate the nests and form primordial follicles. A third possibility is that some cells undergoing death may serve as nurse cells, supplying neighboring oogonia with additional nutrients and organelles via their connecting intercellular bridges ([Pepling & Spradling 2001](#)). The depleted

nurse cells are subsequently eliminated during nest breakdown. A final hypothesis is death by neglect, whereby apoptosis may be triggered in germ cells that do not receive adequate cell-extrinsic growth factor or somatic cell support ([Tilly 2001](#)). It is possible that several of these processes could be responsible for the depletion of germ cells, oogonia and oocytes.

The collective evidence from the aforementioned studies suggests that primordial germ cell-, oogonial-, and oocyte-death may be required for the endowment of primordial follicles of sufficient quality to support female fertility and to ensure development of healthy offspring ([Guigon & Magre 2006](#)). However, this wave of death must be tightly controlled to ensure that there are sufficient oocytes to support a fertile life span of adequate length. Numerous studies demonstrate that a reduction in the supply of primordial follicles results in premature ovarian failure, which has significant negative consequences for female fertility and health, including early risk of osteoporosis and heart disease ([Shuster \*et al.\* 2010](#)). Conversely, it is not yet clear how interventions that increase follicle numbers might impact fertility, both in terms of fertile life span and oocyte quality, as well as the health of the offspring. BCL2 overexpression mice have significantly more primordial follicles at PN4 than WT mice, but by PN60 the number of follicles was no different to WT ([Flaws \*et al.\* 2001](#)), suggesting that germ cells that fail to die during development are slated for death during postnatal development. By contrast, inactivation of the pro-apoptotic BAX gene elevated follicle numbers and significantly prolonged fertility into advanced age. In a unique study, [Perez \*et al.\* \(2007\)](#) showed that loss of BAX prolonged the life span of the ovary into advanced age but did not lead to a higher incidence of ovarian tumors. Equally important, many age-related problems such as bone and muscle loss were minimized. However, it is not clear from these studies if the rescue of follicles fated for death by depletion of BAX compromised oocyte quality and threatened offspring health. Thus, it is likely that the retention of adequate follicle numbers must be balanced with the critical need to eliminate damaged or defective oocytes to ensure optimal oocyte quality and healthy offspring development. How this balance is maintained is yet to be clearly elucidated.

### **Apoptosis in the male and female germ lines**

It is clear from this review that apoptosis is a critical process in regulating the availability of healthy germ cells that will differentiate into gametes and ultimately participate in fertilization. In both the male and the female gonad, apoptosis is clearly important during embryonic development in regulating the size of the spermatogonial stem cell and primordial follicle – pools that will ultimately define an individual's reproductive potential. Thus, it is through apoptosis in the germ line



that the optimal ratio of germ cells to nurse cells (Sertoli cells and prefollicular cells) is established to ensure the establishment of a functional gonad. It is also through this apoptotic process that the quality of the germ cells entering the reproductive pool is ensured. Quality control of the germ line is also a major function of apoptosis following puberty. In males, spermatozoa are produced throughout adult life although the quality of these cells may be extremely variable, especially in man (Aitken *et al.* 2009). As soon as spermatozoa become oxidatively stressed they appear to default to an apoptotic pathway designed to ensure that inferior quality gametes will not participate in the process of fertilization. Similarly, in the ovary there is a massive loss of primordial follicles with the apparent purpose of ensuring the quality of oocytes advancing to ovulation and conception.

In males, the mechanisms responsible for depletion of defective spermatozoa bear many of the hallmarks of the intrinsic apoptotic cascade (mitochondrial ROS generation, caspase activation, and phosphatidylserine exposure) but the final step in this process, endonuclease-mediated DNA cleavage, cannot occur because the nucleus and mitochondria are in different compartments of the cell and the chromatin is too densely packed to permit endonuclease entry. The extensive fragmentation of sperm DNA seen in infertile patients may be a *postmortem* change induced by the entry of extracellular nucleases into the spermatozoa following cell death and a loss of membrane integrity. Such a proposal would be in keeping with the presence of powerful nucleases in the fluids of the lower male reproductive tract and the fact that most TUNEL-positive human spermatozoa are no longer viable (Boaz *et al.* 2008, Aitken *et al.* 2010). By contrast, in the female germ line there is no compartmentalization of the nucleus and mitochondria as we see in spermatozoa. As a result, there would appear to be no physical reason why the oocyte would not be competent to undergo apoptosis at any stage of its existence. Indeed, while spermatozoa might be capable of surviving for several days post ejaculation, the same cannot be said for the oocyte following ovulation. Once this cell is released from the ovary it has a relatively short period of free existence before it dies an apoptotic death (Perez *et al.* 2005) or is rescued by the act of fertilization. Prior to their release from the gonads, male germ cells are competent to undergo apoptosis following the activation of meiosis, however, the mechanisms regulating this apoptosis are unclear. There is ample evidence of apoptosis in granulosa cells, leading to the demise of growing oocytes, although whether the oocyte itself can undergo apoptosis while a component of the primordial follicle reserve, once recruited into the growing follicle pool or even following ovulation, is less certain. In the mature testes, differentiating precursor germ cells also undergo apoptosis and again this may be driven directly by the

germ line, responding to high levels of DNA damage with the activation of an apoptotic cascade which may be p53 mediated (Odorisio *et al.* 1998, Kaushai & Bansal 2009) or indirectly via the disruption of Sertoli cell function. Unlike granulosa cells, the latter do not withdraw support from the germ line by undergoing apoptosis themselves, rather they trigger apoptosis in the germ line by stimulating FAS and FAS ligand expression (Lee *et al.* 1999).

Clearly, many questions regarding the control of apoptosis in the germ line remain. What is certain is that our ability to manipulate the reproductive life span of mammals is dependent on resolving the precise nature of the apoptotic processes affecting these cells, as well as the physiological triggers that bring about their activation.

### 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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