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Biochemical Pharmacology 66 (2003) 1571–1579
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Apoptosis in male germ cells in response to cyclin A1-deficiency and cell cycle arrest

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Received 5 March 2003; accepted 22 April 2003

Abstract

Male mice homozygous for a mutated allele of the cyclin A1 gene (*Ccna1*) are sterile due to a block in cell cycle progression before the first meiotic division. Meiosis arrest in *Ccna1*$^{-/-}$ spermatocytes is associated with desynapsis abnormalities, lowered MPF activity, and apoptosis as evidenced by TUNEL-positive staining. With time, adult testicular tubules exhibit severe degeneration: some tubules in the older animals are almost devoid of germ cells at various stages of spermatogenesis. The mechanisms by which the cells sense the cell cycle arrest and the regulation of the decision to undergo cell death are under investigation.

Keywords: Cell death; Spermatocytes; Cell cycle; Meiosis; Apoptosis; Germ cells; Cyclin A

Mammalian gametogenesis provides a unique system in which to study the interplay between cell cycle regulation and apoptosis. The question of cell cycle regulation during mammalian gametogenesis is not understood but has been the subject of several reviews [1,2]. Male and female germ cells have stages of cell cycle regulation in common, including a mitotic proliferative stage, entry into meiosis, completion of a reductive division, and entry into a quiescent state awaiting signals at fertilization. Although the end point of meiosis—the production of haploid gametes—is clearly the same, the timing of these events and the developmental stage at which they occur is strikingly different between spermatogenesis and oogenesis. As will be discussed in greater detail below, it is increasingly clear that a balance between cellular proliferation and cell death, particularly via apoptotic pathways, plays an important role in the mechanism by which the proper number of healthy gametes are formed [3–7].

1. The cell cycle machinery

The key components of the machinery that drives cells through the cell cycle are composed of a complex including the regulatory subunit, a cyclin, and a catalytic cyclin-dependent serine/threonine kinase. MPF was originally described as an activity found in unfertilized frog eggs that were capable of inducing germinal vesicle breakdown and resumption of meiosis when injected into immature oocytes [8]. MPF is composed of cyclin B and p34^{cdc2} (Cdk1). The association of cyclin B with Cdk1 is required for regulating its phosphorylation state, which in turn controls its kinase activity.

Cyclins have been identified in a variety of organisms ranging from yeast to man based on amino acid homology

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Abbreviations: Ccna, cyclin A gene; MPF, maturation (or mitosis) promoting factor; Cdk, cyclin-dependent kinase; TUNEL, terminal dUTP nick end-labeling; Atm, ataxia telangiectasia mutant; FasL, Fas ligand; Akt, protein kinase B; FKHRL1, forkhead-like transcription factor; FADD, Fas-associated death domain; DISC, death-inducing signaling complex; JNK, Jun N-terminal kinase; DAP, death-associated protein; MKK, mitogen activated kinase; PTEN, phosphotase and tensin homolog; PIP-3, phosphoinositol-3,4,5-triphosphate.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S0006-2952(03)00513-6

and by functional complementation of yeast cell cycle mutants. The cyclins have been divided into at least ten classes in higher vertebrates, based on their amino acid similarity and the timing of their appearance during the cell cycle (reviewed in [9–11]). The complexity of the mammalian system is underscored by the existence not only of multiple classes of cyclins but also of multiple members of the A-, B-, and D-type cyclin families. Studies from our laboratory and others have revealed patterns of cell-type and cell cycle-stage specificity of *in vivo* expression of several of the cyclins that had not been detected previously in cultured cell systems (reviewed in [1,12]).

2. The A-type cyclins

Of the mammalian cyclins identified to date, the function of A-type cyclins has been less well understood, in part due to the fact that there are no homologues in the yeasts. We have shown that there are two distinct cyclin A genes in mammals, one of which, *Ccna1*, is testis-specific and restricted to the germ line [13,14]. Two A-type cyclin genes have now been documented in human and frog as well [15,16]. In the adult mouse testis, *Ccna1* mRNA and protein are present in late pachytene to diplotene spermatocytes [14] but not at significant levels during the second division of meiosis [17]. No *Ccna1* mRNA has been detected in ovaries or oocytes by Northern or *in situ* hybridization analysis. The originally described mammalian cyclin A gene, *Ccna2*, was expressed in both spermatogonia and pre-leptotene spermatocytes, suggesting functions in mitotic and pre-meiotic cell cycles [13,17]. Furthermore, both somatic and germ cells of the adult ovary express *Ccna2* mRNA and protein.[1] The different expression patterns suggest distinct functions for *Ccna1* and *Ccna2* in the somatic and germinal lineages, which also differ between the male and female.

In cultured cells, cyclin A2 functions during both G1-S and G2-M phases [18–20]. Cyclin A2/Cdk2 shares some common properties with other G1/S or G2/M-phase cyclin/kinase complexes as well as several features that are unique. Unlike cyclin B1, cyclin A2 can bind and activate both Cdk1 and Cdk2 proteins [21–23]. While both cyclin A2/Cdk and cyclin B/Cdk phosphorylate histone H1, only cyclin A2/Cdk complexes were shown to associate with and phosphorylate the Rb-related protein p107 *in vitro* [24]. Differences in the consensus phosphorylation sites for cyclin A2/Cdk2 vs. cyclin E/Cdk2 complexes have been determined [25] and cyclin A2/Cdk2 was more efficient than cyclin E/Cdk2 in phosphorylating the transcription factor E2f-1 [26]. Cyclin A or E/Cdk2 and cyclin D/Cdk4 can all phosphorylate pRB; however, the phosphorylation sites are different [27].

Much less is known about cyclin A1, in particular as to how it may differ functionally from cyclin A2. As noted above, yeasts lack obvious A-type cyclin homologues and eukaryotes such as *Drosophila* have a single A-type cyclin [28]. Mouse cyclin A1 and A2 share 44% identity at the amino acid level; however, they share a much higher level of identity (84%) within the two highly conserved cyclin boxes (Fig. 1) [13,14]. A major question of interest is the extent to which the two genes are functionally redundant or distinct.

Ccna2 is expressed ubiquitously in cultured cells and in a broad variety of tissues in the adult mouse and during embryogenesis [13,14]. Perhaps not surprisingly then, targeted mutagenesis of murine *Ccna2* resulted in early embryonic lethality, at about the peri-implantation stage [29]. This embryonic lethality has obviated understanding the role of cyclin A2 in other aspects of mammalian development, including the germ line. In contrast, the strikingly restricted expression of *Ccna1* led us to hypothesize that its primary site of function is in the male germ line, specifically at the first meiotic division.

3. Targeted mutagenesis of cyclin A1 in the mouse

To test this hypothesis and to begin to address possible redundancy of the two A-type cyclin genes, we generated cyclin A1-deficient mice by targeted mutagenesis of the *Ccna1* gene [30]. *Ccna1*$^{-/-}$ males were sterile due to a block of spermatogenesis before the first meiotic division, whereas females were normal. Meiosis arrest in *Ccna1*$^{-/-}$ males was associated with desynapsis abnormalities and a striking reduction in the activation of MPF kinase at the end of meiotic prophase. We subsequently determined that although both cyclin B1, cyclin B2, and Cdk1 were present in the cyclin A1-deficient spermatocytes, MPF kinase activity was reduced [31]. This suggested that cyclin A1 is essential for spermatocyte passage into the first meiotic division in male mice, a function that cannot be complemented by the concurrently expressed B-type cyclins. It also suggested that cyclin A1 and an associated Cdk may play a role in activating MPF.

4. Cyclin A1-deficient spermatocytes undergo apoptosis

The spermatogenic arrest in cyclin A1-deficient animals was characterized by the presence of abnormal appearing diplotene spermatocytes and as assessed by TUNEL staining, the presence of apoptotic cells (Fig. 2) [30]. To determine whether the apoptosis in the cyclin A1-deficient mice was triggered by the cell cycle arrest rather than by defects in synapsis and recombination, we turned to an

[1] Liao C, Ravnik SE, Zhang Q, Wang XY, Mulrahd S, Wolgemuth DJ. Differential expression of cyclin A2 during oogenesis in the mouse ovary. In preparation.

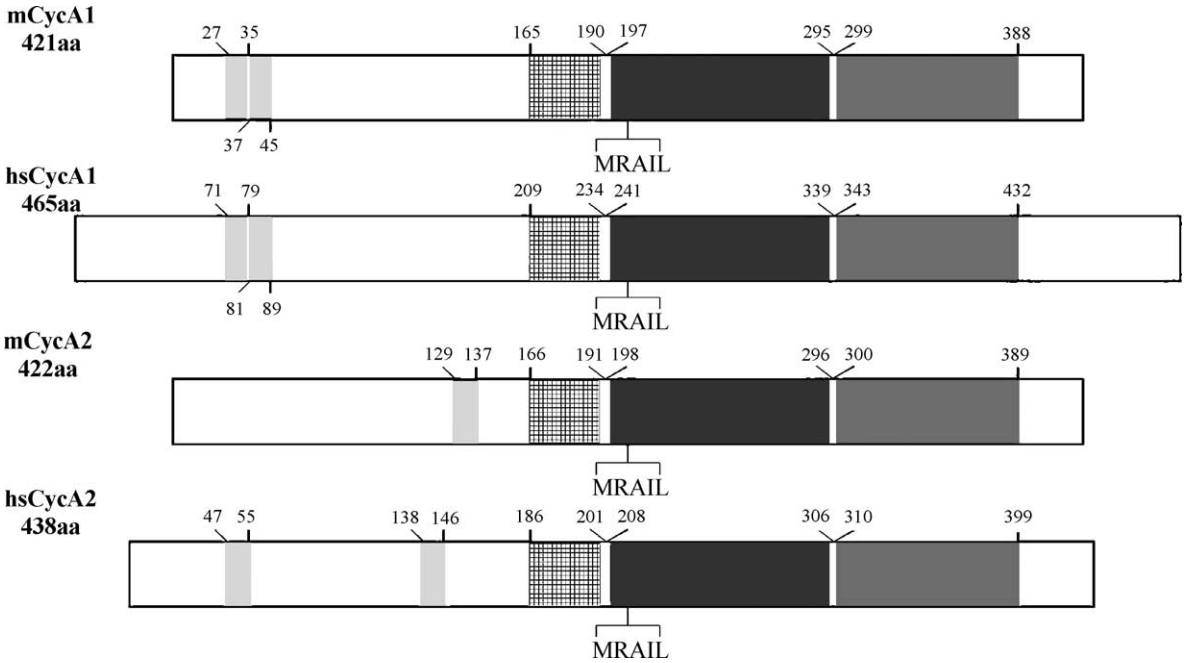

Fig. 1. Cartoon of the organization and structural similarities between cyclin A1 and cyclin A2 and between mouse and human. Light gray: destruction box; hatched: N-terminal helix; black: N-terminal cyclin box; dark gray: C-terminal cyclin box; MRAIL: motif involved in Cdk interaction.

in vitro system developed by Handel and colleagues in which MPF can be activated pharmacologically with okadaic acid [32]. Although the exact mechanisms are not well defined, treatment of pachytene spermatocytes with okadaic acid can induce chromosome condensation and activation of MPF. Treatment of cyclin A1-deficient spermatocytes resulted in the formation of seemingly normal metaphase I bivalents [31] and abrogated apoptosis. An interesting aspect of the cyclin A1-deficient feature is that with time, the testicular tubules undergo further degeneration, such that virtually germ cell-depleted tubules, which resemble Sertoli cell-only syndrome in human infertility, are seen [30].

5. The decision by meiotic prophase germ cells to undergo apoptosis is not unique to cyclin A1-deficiency

The use of targeted mutagenesis in the mouse model has revealed a number of genes that clearly function in the events of meiotic prophase. Models of prophase I arrest in male mice (Table 1) result from the disruption of genes with a variety of functions, from transcription factors to DNA repair enzymes to protein chaperones or from failure of sex chromosome synapsis. The phenotypes share a common outcome: the germ cells somehow sense defects in meiosis and initiate apoptosis. The list of genes also underscores another feature of meiotic progression—that

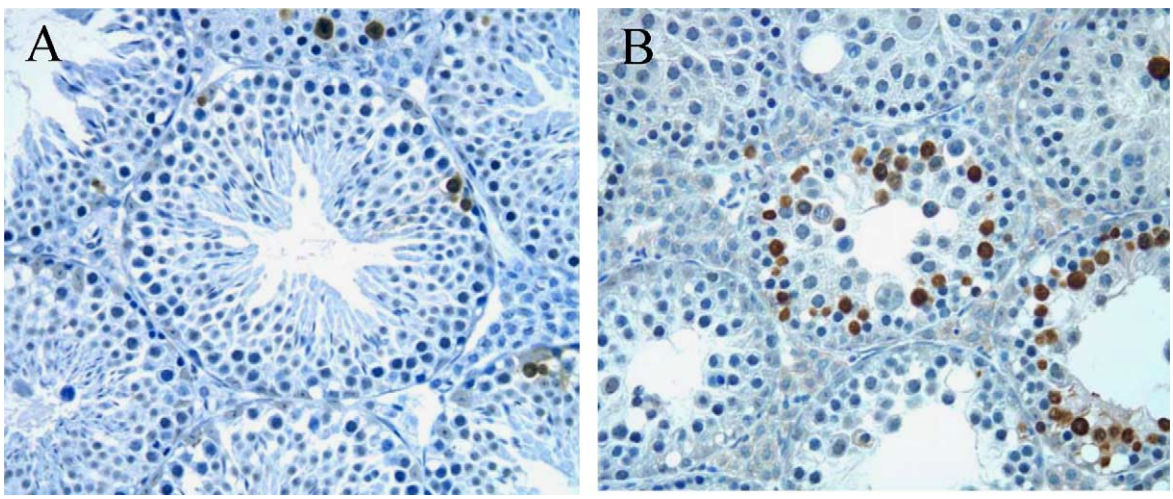

Fig. 2. TUNEL staining of wild-type testes (A) and cyclin A1-deficient mice. TUNEL-positive cells are shown by brown staining. More TUNEL-positive spermatocytes are found in a *Ccna1*$^{-/-}$ testis (B) compared with a wild-type testis.

Table 1
Apoptosis occurs in meiotic prophase cells in various mutant strains

Genes	Arrest point	Synapsis phenotype	References
A-myb	EP	Not reported	[93]
Atm	Z/EP	Frequent asynapsis	[94]
Dmc1	Z/EP	Asynapsis	[95]
Msh4	Z	Frequent asynapsis, nonhomologous pairing	[96]
Msh5	Z/EP	Frequent asynapsis, nonhomologous pairing	[97]
Scp3	Z	Asynapsis	[98]
Spo11	Z	Asynapsis	[37,38]
XSxr(a)0	LP/MI	Asynapsis of the sex chromosome	[34]
Hsp70.2	LP	Failure to desynapse	[35]
Mlh1	LP	Failure to desynapse	[99]
Ccna1	LP/D	Incomplete desynapsis	[30]

Abbreviations: Z, zygotene; EP, early pachytene; LP, late pachytene; D, diplotene; MI, metaphase I.

there are multiple stages at which the cells can be arrested—suggesting that multiple checkpoints sense and respond to meiotic failure. This is illustrated in cartoon form in Fig. 3, as compared to mitotic checkpoints. The existence of multiple checkpoints is supported by observations that apoptosis is induced by signaling pathways that are both dependent and independent of p53. For example, $ATM^{-/-}$ spermatocytes are partially rescued by mutant *p53* [33], while apoptosis induced by sex chromosome asynapsis appears to occur independent of p53 [34].

Similarly, mice lacking Hsp70.2, a putative chaperone protein for the assembly of the Cdk1/cyclin B1 complex, showed increased apoptosis of mid-pachytene spermatocytes that is also p53-independent [35], although p53 does appear to be required for the radiation-induced apoptosis of spermatogonia [36]. We are currently examining if apoptosis in cyclin A1-deficient mice is affected by the absence of p53 by breeding our $Ccna1^{-/-}$ mice onto a p53-deficient background. Interestingly, in those genes that function in both male and female germ cells, the timing of the cell death can vary. For example, while mutations in Spo11 produced abnormalities in both male and female meiotic prophase cells, apoptosis occurs during early prophase in the male, while oocytes progress to the diplotene stage [37,38]. This also suggests that checkpoints responding to meiotic failure may be sexually dimorphic [37].

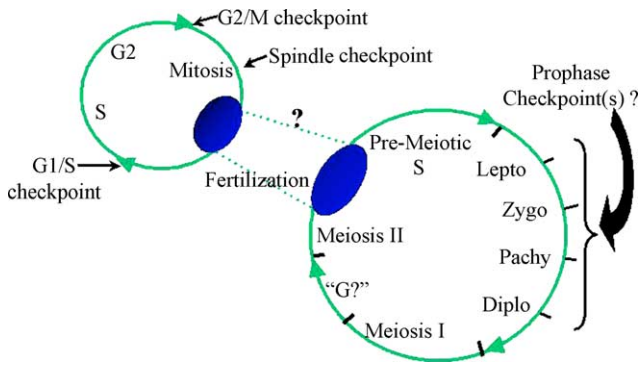

Fig. 3. Known mitotic vs. proposed meiotic checkpoints in mammalian germ cells.

6. Significance of germ cell death during spermatogenesis

The importance of cell death and apoptosis in particular in the reproductive system is seen early in development when a decision is made for regression of the male (Wolffian) or female (Mullerian) duct systems [39]. Spermatogenesis is a highly ordered developmental process of continuous germ cell maturation, which includes mitotic proliferation of spermatogonia, entry into meiosis, recombination, reduction division of spermatocytes, differentiation of haploid spermatids, and elongation and release of spermatozoa into the tubule lumen. These processes occur in intimate association with somatic cells within the tubule, the Sertoli cells, and under a complex milieu of hormones, growth factors, etc. Sperm production is a regulated balance between germ cell division, including between 9 and 11 cell divisions of spermatogonia and two meiotic divisions, and germ cell loss. In the early post-natal rodent testis, apoptotic cell death occurs during the first wave of spermatogenesis and is critical for subsequent normal testicular function [40–42]. In adult animals, germ cell death is important for ongoing rounds of spermatogenesis and helps to regulate sperm output (reviewed in [6,7,43–45]). Homeostasis of the various testicular cell types appears to involve considerable wastage of germ cells, since it has been estimated that only 25% of the possible number of spermatogonia actually enter into the meiotic stages [7,46]. In normal spermatogenesis, degenerating spermatogonia have been shown by several investigators to exhibit classic features of apoptotic cell death while degenerating germ cells at more advanced stages may not (discussed in [5,7,47]).

7. Regulators of cell death in germ cells

Among the myriad of molecules involved in regulation of apoptosis, several members of the Bcl2 family have been shown by molecular genetic approaches to be important for normal spermatogenesis. The Bcl2 family members (named because the anti-apoptotic *Bcl2* gene is up-regulated by transposition in B-cell lymphomas) are important sensors that receive signals and transmit the information to downstream cell death effectors (reviewed in [48–50]). Some Bcl2 family members promote cell survival, while others antagonize it. The relative levels of these factors with apparently opposing functions within any given cell probably determine whether the cell undergoes apoptosis. This competitive interaction of the pro- and anti-survival Bcl2 family members regulates the activation of the cell

Table 2
Expression patterns of Bcl-2 family members in the testis from published studies

GENES	SC	LC	SG	SP	ST	REFERENCES
Bcl-2	(-)	Low stained		C (P Stg VI onward)	√	[91] (Mice)
	C	C		C (P VII-XII; D XIII; 2° SP (XIV)		[56] (Rats)
Bcl-x_l			√	√	(-)	[91] (Mice)
Bcl-w	√		√	√	RS	[62] (Mice)
	√				ES	[61](Mice) *
	√		√	√		[57] (Mice) *
	√ (all stages except VII & VIII				(-)	[52] (Mice) *
Bax			?	Early Stg ?	(-)	[92] (Mice)
	√	√	C (granular or punctuate)	C (granular or punctuate)	RS and ES	[56] (Rats)
	√		√	√		[57] (Mice) *
	√	√	√	√	√	[91] (Mice) *
				√		[55] (Mice) *
Bcl-x_s		√				[91] (Mice)
Bak	√		√	√		[57] (Mice)

*: Conflicting observations; C: cytoplasm; P: pachytene; D: diplotene; SC: Sertoli cells; SP: spermatocytes; LC: Leydig cells; ST: spermatids; SG: spermatogonia; Stg: stage; RS: round spermatids; ES: elongated spermatids; ?: ambiguous; dark gray: antiapoptotic; light gray: proapoptotic.
Data drawn from [52,55–57,61,62,91,92].

death machinery, including the caspase proteases that are key effectors of apoptosis.

The Bcl2 family members exhibit complex patterns of expression in the testis and complicating our understanding of their function are conflicting expression data within the literature (see Table 2). However, several members of the Bcl2 family appear to be essential for male germ cell homeostasis, as determined by transgenic and mutational analysis. There is general agreement that the pro-apoptotic protein Bax is abundantly expressed in germ cells between 1 and 3 weeks after birth and may be involved in the normally occurring wave of apoptosis in spermatogonia and spermatocytes that has been suggested to be essential for establishing the critical balance between the numbers of Sertoli cells and germ cells [41]. Testes of $Bax^{-/-}$ mice contain excessive numbers of spermatogonia and pre-leptotene spermatocytes and never establish normal adult spermatogenesis [51,52]. Because spermatogenesis is never normal in the absence of Bax, its function in cell death in later stages of germ cell development is not known. Further complicating our understanding of Bax function in the normal adult testis, *Bax* expression has been variably reported to be present in spermatogonia, spermatocytes, spermatids, Sertoli cells, or combinations thereof [53–58].

Of the anti-apoptotic family members, Bcl-x_L has been implicated to function in germ cell homeostasis. It is highly expressed in testes during the first wave of spermatogenesis [41] and the number of cultured primordial germ cells was dramatically increased by ectopic expression of *Bcl-x_L* [59]. Expression of a *Bcl-x_L* transgene in germ cells disrupted the first wave of spermatogenesis by reducing the level of apoptosis, resulting in a pathology that resembled that of $Bax^{-/-}$ mice [41]. While there were some conflicting observations regarding the effects on apoptosis of spermatogonia in testes overexpressing Bcl-2, in both studies male sterility was the result [41,60]. It has been suggested that these two anti-apoptotic factors may function similarly in the germ cell lineage [44]. In the adult testis, Bcl-x_L is less abundant than in immature testis and appears to be restricted to spermatocytes and spermatids [53].

The anti-apoptotic factor Bcl-w appears to have a different function. In *Bcl-w* mutant mice, embryonic and early post-natal testicular development appears normal. However, the incidence of apoptotic germ cells increases dramatically between 2 and 4 weeks of age, resulting in substantially reduced numbers of post-meiotic germ cells in young adult males [61,62]. The testes of 6-week-old *Bcl-w* knockout mice contained numerous apoptotic cells, many of which were syncitial in appearance [62]. By 6 months of age, many tubules in the Bcl-w-deficient mice were severely depleted or devoid of germ cells at all stages. Whether this severe depletion of germ cells is a primary or secondary cause of loss of Bcl-w remains to be determined, however, such a loss of germ cells with time has been observed in several model systems involving loss or gain of function of apoptosis-regulating genes (e.g. Bcl2; [60]), cell cycle regulating genes (e.g. cyclin A1; [30]), etc. As *Bcl-w* is also expressed in low levels in Sertoli cells, spermatocytes and round spermatids, it may function in these cells as well. Indeed, a cell autonomous function for Bcl-w in both germ cells and Sertoli cells has recently been suggested by genetic experiments [63]. Other members of the Bcl-2 family are expressed in the testis (reviewed in [44]) but their function has yet to be determined.

One key to triggering apoptosis appears to be the protein receptor Fas (Apo1/CD95), found on the surface of many different types of cells (reviewed in [64]), including germ cells [65]. Fas/FasL interactions are involved in both the proliferative and cell death response induced in a variety of cell types by a variety of stimuli [64,66–69]. The role of FasL is best studied in the immune system, where it is expressed in activated T lymphocytes. The function of FasL is apparently to allow T cells to kill target cells which express Fas, by triggering the caspase cascade and prompting apoptosis, as well as to regulate T cell activity by triggering apoptosis in other T cells [70–73]. In the testis, FasL is expressed in Sertoli cells and Fas in germ cells ([65], reviewed in [74]).

There are at least two potential mechanisms that appear to be involved in events downstream of Fas/FasL action. One involves the serine–threonine kinase Akt (also termed protein kinase B). Akt kinase is believed to be constitutively activated in the presence of trophic factors and to sequester a phosphorylated form of the FKHRL1 in the cytoplasm. This prevents the binding to the FKHRL1 binding site in the FasL gene and thereby promotes survival over apoptosis [68].

There are several separate downstream pathways possible following binding of FasL to Fas. After ligation, Fas multimerizes and recruits the adaptor molecule FADD. Together they form a DISC, which in turn causes autocatalytic activation of caspase-8 and initiation of apoptosis by cleavage and activation of downstream effector caspases such as caspase-3, -6 and -7, leading to irreversible cleavage of proteins necessary for maintaining cell structure, DNA synthesis and repair (type I cells) [75]; in type II cells the processed caspase-8 is insufficient. The caspase cascade has to be amplified by allowing caspase-8 to cut Bid and form truncated Bid (tBid). There is then a subsequent release of mitochondrial pro-apoptotic factors (for example, cytochrome *c* and SMAC/DIABLO) to form the caspase-9-activating apoptosome. Caspase-9 activates further downstream procaspases-3 (reviewed in [76]). In another downstream pathway, Fas recruits the adaptor protein DAXX, which links the receptor to the JNK pathway [77]. In yet another pathway, Fas signals are activated by DAP kinase, which is localized to the cytoskeleton and the function of which is upstream of caspase-8 [78]. Fas may also signal through MKKs or by the PTEN, which dephosphorylates PIP-3 [79,80]. Fas can also be directly activated at the plasma membrane independently of its ligand by UV light, which thereby also induces apoptosis [81]. There have been several recent studies on possible involvement of the Fas/FasL system in the induction of apoptosis in germ cells. The Fas system has been implicated to play an important role in the induction of germ cell apoptosis in testis after drug treatment regimens [65,82–84]; induced cryptorchidism [85,86]; reperfusion in testes after ischemia [87–89]; and in response to meiosis arrest [90]. Which of the Fas/FasL pathways outlined above functions during germ cell apoptosis in the normal testis or in response to the stress or noxious stimuli listed above is not well defined nor is it known if the Fas/FasL pathway is activated in cyclin A1-deficient mice.

8. Conclusions and remaining questions

It is increasingly clear that tight regulation of cellular proliferation and apoptosis is a normal part of development of mammalian gametogenesis, in both spermatogenesis and oogenesis. It is critical for maintaining the proper balance of germ cells to somatic cells in the developing gonad and during cyclical expansion of stem cells. It is also critical for sensing defects in the normal progression of the meiotic cell cycle—germ cells that are arrested in meiosis or that have aberrant synapsis, etc. should not be permitted to form defective gametes.

How do germ cells sense that meiosis has been perturbed—whether a defect in assembly of the recombination machinery, chromosome condensation, etc.? Once sensed, how does the germ cell activate a cell death pathway? Which of the various pathways are activated? Are there different sensing pathways depending on when during meiotic prophase the cells are arrested? What happens if the preferred pathway is not operational—can the cells activate a second pathway as a ‘fail-safe’ mechanism? Is perturbation of meiosis, as compared to mitosis, particularly sensitive to a decision to undergo apoptosis? And why apoptosis as compared to other mechanisms for killing cells—is this the most efficient and ‘clean’ way to get rid of defective cells? These are just a sampling of the myriad of important questions that remain to be answered.

Acknowledgments

This work was supported in part by a grant from the NIH to D.J.W. (HD34915), a grant from the Wenner Gren Foundation to C.L., and a grant from The Croucher Foundation to S.S.W.C.

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