PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2010) **365**, 1653–1662
doi:10.1098/rstb.2009.0254

Review

Regulating mitosis and meiosis in the male germ line: critical functions for cyclins

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Key components of the cell cycle machinery are the regulatory subunits, the cyclins, and their catalytic partners the cyclin-dependent kinases. Regulating the cell cycle in the male germ line cells represents unique challenges for this machinery given the constant renewal of gametes throughout the reproductive lifespan and the induction of the unique process of meiosis, a highly specialized kind of cell division. With challenges come opportunities to the critical eye, recognizing that understanding these specialized modes of regulation will provide considerable insight into both normal differentiation as well as disease conditions, including infertility and oncogenesis.

Keywords: cell cycle regulation; mitosis; meiosis; germ cell differentiation; cyclin-dependent kinases

1. THE BASICS OF CELL CYCLE MACHINERY IN MITOTIC CELLS

A basic requirement for cellular proliferation is that the cell replicates its DNA and then segregates the resulting products in an orderly, cyclic manner. This progression through the cell cycle is controlled in part by the sequential activity of the basic components of the cell cycle machinery, the cyclins and the cyclin-dependent serine–threonine kinases, collectively called the cyclin-dependent kinases (Cdks). The cyclins are regulatory subunits that bind, activate and provide substrate specificity for their catalytic partner Cdks (for reviews, see Murray 2004; Sherr & Roberts 2004; Bloom & Cross 2007). The activity of cyclin–Cdk complexes is tightly regulated by a complex network of proteins that function as activators and inhibitors of their kinase activity as well as influencing their transcription, sub-cellular localization and degradation.

Several classes of cyclins have been described in mammalian cells, designated A to I, and also T. Progression through the mitotic cell cycle is governed by the sequential activation and function of these different cyclins, in partnership with Cdks. During the mitotic cell cycle, cyclins from the D-type family regulate progression of cells through the first gap phase, G1 (Sherr & Roberts 1999). D-type cyclins bind and activate Cdk4 and Cdk6 which phosphorylate and functionally inactivate the retinoblastoma protein and related family members. Cyclin E family members are expressed during late G1 and during S-phase progression. E-type cyclins associate with and activate mainly Cdk2, but they can also associate with Cdk1 and Cdk3. Following E-type cyclin activation during S-phase, the A-type cyclin, cyclin A2, becomes functional. Entry into M-phase progression is driven primarily by the B-type cyclins, which associate with and activate Cdk1; however, cyclin A2 also functions prior to cyclin B/Cdk1 activation.

While best known for their association with, and regulation of, Cdks, it is increasingly recognized that several of the cyclins also have functions that are kinase-independent. For example, cyclin D1 has been reported to have a Cdk-independent role as a co-activator of tissue-specific transcription factors (e.g. Zwijsen *et al.* 1997). Studies using mutational analysis have shown that this regulation, specifically the transactivation of p300, PPARγ (Fu *et al.* 2005) and DMP1 (Inoue & Sherr 1998), is independent of the residues required for binding Cdk. Further, recent studies on the function of the E-type cyclins also implicate kinase-independent functions (Geng *et al.* 2007). Specifically, cyclin E1 that is incapable of activating its Cdk2 partner is nonetheless able to support the cell's entry to S-phase, although at a reduced rate. Also, the expression of kinase-deficient cyclin E mutant in cyclin E-null cells was able to rescue MCM loading, to levels similar to those in wild-type cyclin E cells.

2. BASICS OF THE MEIOTIC CELL CYCLE: ANOTHER LAYER OF COMPLEXITY

Meiosis is restricted to germ line cells and has features of cell division that simply do not exist in somatic cells. One striking unique feature, for example, is that there are two metaphase segregation events that occur without an intervening round of DNA synthesis. Another aspect of meiosis that distinguishes it from mitosis is

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

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the behaviour of sister chromatids during the first meiotic division. That is, in mitosis, the sister chromatids separate in the single phase of chromosome segregation, while in meiosis, sister chromatids do not separate from one another until the second round of segregation—the first stage involves segregation of homologous chromosomes. In addition, it is critical that meiosis-unique processes, including the synapsis or pairing of homologous chromosome, recombination and formation of chiasmata, be strictly coordinated with cell cycle progression, as it would be catastrophic for the gamete cell to attempt to move into the cell cycle without these other processes having been completed.

Higher organisms are characterized by having sexually dimorphic gametes. While 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 prior to fertilization, the timing of these events and the stage of development at which they occur differ in the two sexes (reviewed in Wolgemuth *et al.* 1995, 2002; Handel & Eppig 1998). In the mouse model, the germ line is specified early in embryonic development, probably as early as embryonic day 6.0–6.5. The progenitor germ cells migrate from the proximal epiblast to the gonadal ridge, at which point sexual determination based upon genotype occurs. The germ cells then follow either a male pathway, in which the cells enter into a mitotic arrest, or alternatively, a female pathway, in which they enter into pre-meiotic DNA synthesis and meiotic prophase. Thus, germ cells of both sexes undergo mitotic divisions in the embryonic gonad, but the female germ cells enter meiosis during foetal development, whereas this is a postnatal event in the male.

Once the male germ cell has entered meiosis, the process continues without interruption until the haploid sperm is produced. In contrast, the oocyte is arrested in the diplotene stage of meiotic prophase I, where it can remain for months or years depending on the species. Following a growth period, which begins at puberty, the oocyte resumes meiosis, but arrests a second time, at metaphase II. Fertilization then triggers the completion of meiosis and extrusion of the second polar body. Given these striking differences in the sequence of mitotic and meiotic events, it is almost given that the genetic programme underlying this regulation will be distinct between the male and female and will be reflected in a sexual dimorphism in the genes involved in regulating these processes (Handel 1998; Wolgemuth *et al.* 2002).

Mouse spermatogenesis has been a particularly powerful *in vivo* model system to pursue the regulation of mitosis and meiosis in higher eukaryotes, specifically in mammals, for several reasons. First, the biology of spermatogenesis in the rodents is particularly amenable to precise histological analysis. The adult mouse testis contains germ cells in various stages of differentiation, including self-renewing stem cells, mitotically dividing spermatogonia, meiotically dividing spermatocytes as well as spermatids, which are genetically haploid and undergo the remarkable morphological changes and chromatin remodelling that characterize spermiogenesis. Second, the progression of differentiation of these events occurs in a strict temporally and spatially regulated manner, such that classical cellular associations within this cycle of the seminiferous epithelium have been defined. Comparison of changes in such cellular associations upon loss of expression and function of specific genes can provide insight into the stages of differentiation that are affected. Third, the mouse model can be relatively easily manipulated genetically by several experimental strategies. There are also naturally existing mutations in mice that have provided considerable insight into the control of germ cell differentiation, although they have not been particularly useful for understanding the control of cell cycle in the germ line. For example, mutations in the *W* alleles encoding the c-*kit* tyrosine kinase receptor result in aberrant germ cell differentiation in both males and females and can result, for example, in complete loss of the germ cell lineage and, of course, sterility (e.g. Rossi *et al.* 2000, 2003). A series of studies using both a naturally occurring mutation in parallel with targeted mutagenesis approaches revealed the critical role of the gene encoding the transcription factor Plzf in the maintenance of the spermatogonial stem cell population (Plzf knockout (Costoya *et al.* 2004) while concomitantly identifying the spontaneous mutation *luxoid* (Buaas *et al.* 2004) as in fact being the *Plzf* locus).

Conventional gene targeting of both germ-cell-specific genes and genes more widely expressed has been a powerful tool to identify the roles that specific genes play in these processes but has only revealed the 'tip of the iceberg' regarding the complexity of the genetic control of these processes (reviewed in Venables & Cooke 2000; Matzuk & Lamb 2002; de Rooij & de Boer 2003). A compilation of the phenotypes observed from conventional knockouts of the major cyclins (which are the focus of this review) and several of their partner Cdks is found in table 1.

In the paragraphs below, we will summarize our current understanding of the expression and function of the A-, B-, D- and E-type cyclins in the male mammalian germ line. It will be obvious that our understanding of the expression and function of the A-type cyclins is more advanced than for the three other families, which should not be interpreted as reflecting their relative importance but rather our limited knowledge.

3. A-TYPE CYCLINS

There are two distinct A-type cyclins in the mouse (and human) genome, cyclin A1 and cyclin A2 (designated genetically as *Ccna1*/*CCNA1* and *Ccna2*/*CCNA2*, respectively). These two A-type cyclins exhibit strikingly different patterns of expression that have been documented in both mice and humans: cyclin A2 is ubiquitously expressed in mitotically dividing cells, whereas expression of cyclin A1 is highly restricted, being most abundant in the testis (Ravnik & Wolgemuth 1996; Sweeney *et al.* 1996; Yang *et al.* 1997). Mouse cyclin A2 has also been shown to be

Table 1. Effects of the absence of specific cyclins and CDKs on male fertility. n.d., not determined.

cyclin/CDK knockout	expression in WT testis	effect on male fertility	arrest point if infertile	reference
cyclin A1	late pachytene to diplotene	infertile	diplotene	Liu *et al.* (1998*a*,*b*)
cyclin A2	spermatogonia to preleptotene	embryonic lethal		Sweeney *et al.* (1996), Ravnik & Wolgemuth (1999), Murphy *et al.* (1997)
cyclin B1	spermatocytes and post-meiotic spermatids	embryonic lethal		Chapman & Wolgemuth (1992), Brandeis *et al.* (1998)
cyclin B2	spermatocytes and round spermatids	fertile		Chapman & Wolgemuth (1992), Brandeis *et al.* (1998)
cyclin B3	leptotene and zygotene	misexpression leads to infertility		Nguyen *et al.* (2002), Refik-Rogers *et al.* (2006)
cyclin D1	spermatogonia	fertile		Sicinski *et al.* (1995), Beumer *et al.* (2000)
cyclin D2	spermatogonia, pachytene, diplotene, round spermatids to stage V	hypoplastic testis, remain fertile		Sicinski *et al.* (1996), Beumer *et al.* (2000)
cyclin D3	spermatogonia, pachytene, diplotene, round spermatids, elongating spermatids	fertile		Sicinska *et al.* (2003), Beumer *et al.* (2000), Zhang *et al.* (1999)
cyclin E1	n.d.	fertile		Geng *et al.* (2003)
cyclin E2	n.d.	reduced fertility	no consistent arrest point	Geng *et al.* (2003)
Cdk1	differentiating cells	embryonic lethal		Santamaria *et al.* (2007), Ravnik & Wolgemuth (1999)
Cdk2	differentiating cells	infertile	mid-pachytene	Ortega *et al.* (2003), Berthet *et al.* (2003), Ravnik & Wolgemuth (1999)
Cdk4	n.d.	low sperm count, 80% infertile	no consistent arrest point	Rane *et al.* (1999)
Cdk6	n.d.	fertile male		Malumbres *et al.* (2004)

expressed in a broad variety of tissues in the adult mouse and during embryogenesis (Ravnik & Wolgemuth 1996; Sweeney *et al.* 1996). The originally identified A-type cyclin, human cyclin A2, is ubiquitously expressed *in vitro* in tissue culture cells and is upregulated in many cancers (Pines & Hunter 1990; Wang *et al.* 1990).

(a) *A-type cyclins in the mammalian germ line: distinct and sexually dimorphic patterns of expression*

In the mouse testis, cyclin A1 expression has been detected at both the mRNA and protein levels specifically in pachytene and diplotene spermatocytes in stages IX to XII tubules (Sweeney *et al.* 1996; Liu *et al.* 1998*a*). That the mRNA and proteins are detected in close temporal proximity suggests that regulation of cyclin A1 expression is primarily at the level of transcription and that there is little if any regulation at the level of translation. On the other hand, cyclin A2 is expressed in spermatogonia and pre-leptotene spermatocytes and its expression is specifically downregulated early in meiotic prophase, well before cyclin A1 is expressed (Sweeney *et al.* 1996; Ravnik & Wolgemuth 1999).

It is interesting to contrast the expression of the two A-type cyclins between the testis and ovary: in the mouse ovary, cyclin A1 is completely repressed and cyclin A2 is expressed in both oogonia and oocytes in a developmentally regulated manner (Persson *et al.* 2005). The overall levels of expression of cyclin A2 protein decrease from embryonic oocytes to oocytes in postnatal and adult ovaries. Also, cyclin A2 protein expression is nuclear from embryonic days 13.5 to 15.5 and then changes to predominantly cytoplasmic from embryonic day 16.5 to postnatal and to adult ovaries. There are also high expression levels of cyclin A2, Cdk1 and Cdk2 in granulosa cells. Although there had been conflicting reports of cyclin A1 expression in mouse oocytes (Sweeney *et al.* 1996), there was subsequent agreement from gene targeting studies that loss of cyclin A1 function has no effect on oogenesis (Liu *et al.* 1998*a*; van der Meer *et al.* 2004). This question was resolved in a subsequent study that used the cyclin A1-deficient mouse model to unequivocally demonstrate that cyclin A1 is not expressed to any significant levels in oocytes (Persson *et al.* 2005). It is therefore likely that the two A-type cyclins play unique functions in cell cycle progression in the male and female germ lines.

(b) *Regulation of expression of the A-type cyclins in the germ line*

Given the distinct patterns of expression of the A-type cyclins that differ between the two genes and which exhibit such striking differences between the two sexes, there must be specific regulatory elements unique to each A-type cyclin that are critical for their

distinct regulation of expression, and further, there may be important post-transcriptional regulation that is critical as well. There were several studies that have begun to identify critical transcriptional regulatory elements of the human *CCNA1* gene. In one such study, transient transfection of CV-1 cells with a c-Myb expression vector and *CCNA1* reporter constructs suggested that Myb was able to induce *CCNA1* expression and that sequences in a 335 bp fragment upstream of the promoter, which contains several c-Myb binding sites, may be involved in this expression (Muller *et al.* 1999).

However, the most definitive studies are those using a transgenic mouse model and examining the expression of reporter constructs *in vivo* (Lele & Wolgemuth 2004). Transgenic mice carrying constructs consisting of varying lengths of the mouse cyclin A1 regulatory region fused with the reporter gene *lacZ* were generated. Analysis of tissue-specific and testicular cell-type-specific transgene expression indicated that sequences within approximately 1.3 kb of the *Ccna1* putative transcriptional start site were sufficient to direct transgene expression uniquely to late spermatocytes, while maintaining repression in other tissues. However, sequences located between approximately 4.8 and 1.3 kb of the putative transcriptional start site were apparently required to transcribe the reporter at levels needed for consistent X-gal staining. Comparison of the mouse, rat and human proximal promoters revealed regions of high sequence conservation and consensus sequences both for known transcription factors, some of which are co-expressed with *Ccna1*, such as A-myb and Hsf2, and for elements that control expression of genes in somatic cell cycles, such as CDE, CHR and CCAAT elements. Thus, the promoter region within 1.3 kb upstream of the putative *Ccna1* transcriptional start can direct expression of *lacZ* to spermatocytes, while sequences located further upstream may enhance expression.

Although our understanding of the regulation of *Ccna1* is limited, even less is known about the transcriptional regulation of *Ccna2* in either the male or female germ line. There have been several studies, however, examining its regulation in mitotic cell division, mostly in cultured cells (Blanchard 2000). Cyclin A2 expression is repressed in quiescent (G0) cells and early in G1 and is then rapidly induced as cells approach S-phase. *In vivo* foot printing analysis revealed the presence of several putative regulatory regions within a relatively short region (approx. 70 bp) upstream of the initiation site. Two of these elements, designated CRE and CAAT boxes, are bound constitutively throughout the cell cycle by transcription factors of the CREB/ATF and NF-Y families, respectively. The other two sites, known as CCRE/CDE and CHR elements, have been suggested to be negative regulatory elements in the cyclin A2 promoter, and in the promoters of several other cell cycle-related genes, including *Cdc25C*, *Cdk1* and members of the *Myb* family (Lucibello *et al.* 1995; Huet *et al.* 1996). Transcriptional regulatory factors such as E2F, Rb, CDF-1 and perhaps the SWI/SNF chromatin remodelling complex have all been implicated in the function of these elements (Schulze *et al.* 1995; Liu *et al.* 1998*b*; Philips *et al.* 1999; Coisy *et al.* 2004), but they remain poorly characterized. It is of interest that the CDE/CHR elements are also found in the promoter proximal region of both the human and mouse cyclin A1 gene (Lele & Wolgemuth 2004).

As mentioned above, there does not appear to be significant regulation of either *Ccna1* or *Ccna2* at the level of post-transcription in the male germ line. However, this may be different for *Ccna2* in the female germ line. While clearly transcriptional regulation is critical, as evidenced from the complete repression of *Ccna1* expression in oogonia and oocytes, regulation at the level of translation and perhaps sub-cellular distribution may be involved for *Ccna2* in the female germ line. That is, during embryonic days 13.5–15.5, the localization of cyclin A2 protein was predominantly nuclear (Persson *et al.* 2005). At embryonic day 16.5, the localization of cyclin A2 in about 50 per cent of the oocytes began to shift to the cytoplasm. By embryonic day 18.5 only a few clusters of oocytes exhibited nuclear localization of cyclin A2 and the vast majority of expression was in the cytoplasm.

Another interesting example of post-translational regulation of an A-type cyclin has been recently discussed in the context of the *Drosophila* model system (Vardy *et al.* 2009). Although there is only a single A-type cyclin in this organism, its modes of regulation of expression may be relevant to both A-type cyclin's regulation in mammalian germ cells. In *Drosophila* oogenesis, the expression and levels of cyclin A protein must be tightly regulated for proper oocyte differentiation, including the mitotic divisions of the oogonia that give rise to the oocyte and its sister nurse cells, and the induction of meiosis (Lilly *et al.* 2000). Repression of cyclin A translation has been shown to be important early in prophase I in order to maintain the oocyte in meiosis, a repression that is mediated by a combination of deadenylation of the mRNA and function of the inhibitor protein Bruno (Morris *et al.* 2005; Sugimura & Lilly 2006). As meiosis resumed during oocyte maturation, cyclin A protein was concomitantly detected with the polyadenylation of its message. There are also multiple regulatory phosphorylations of the cyclin A protein during the progression of meiosis I, which may in fact involve autophosphoryation and be involved in regulating its stability or rate of degradation (Vardy *et al.* 2009).

(c) *Targeted mutagenesis of the A-type cyclins in the mouse model*

It has been known for over 10 years that mice homozygous null for the *Ccna2* gene die *in utero*, with lethality occurring around the peri-implantation stage (Murphy *et al.* 1997); however, very little is known about the physiological basis of the lethality and even less about the molecular mechanisms involved. These observations demonstrated the essential nature of cyclin A2 function in mammalian development but yield little insight into the cellular and molecular basis of its function and only complicated efforts for understanding its putative function in adult lineages.

In contrast, homozygous null *Ccna1* mice grow and develop normally with the exception of male-specific sterility in the adult animals (Liu *et al.* 1998*a*; van der Meer *et al.* 2004). Examination of the testes of the mutant mice revealed that they exhibited normal meiotic progression until mid-diplotene, with normal formation and resolution of chiasmata (Liu *et al.* 1998*a*; Nickerson *et al.* 2007). However, in late diplotene, rather than undergoing diakinesis and proceeding to a metaphase I arrangement, spermatocytes in the cyclin A1-deficient mice arrested and, as assessed by TUNEL staining, underwent apoptosis (Liu *et al.* 1998*a*; Salazar *et al.* 2005). Interestingly, this arrest and induction of apoptosis occurred later than the meiotic arrest observed in mice deficient in the putative cyclin A1 kinase partner, Cdk2 (Berthet *et al.* 2003; Ortega *et al.* 2003).

(d) *Possible targets and downstream events of cyclin A1 function in male germ cells*

As is the goal of genetic approaches involving loss of function mutations, several features of the phenotype that results from the loss of cyclin A1 function may indeed provide insight into some of the cellular processes and components involved. Changes in timing and progression of various nuclear events that accompany the cell cycle arrest and apoptosis in cyclin A1-deficient spermatocytes have been analysed by Nickerson *et al.* (2007). For example, it has been reported that the Ser139-phosphorylated form of H2AX, or γH2AX, localizes in a characteristic staining pattern at each stage of meiotic prophase in spermatocytes, in particular, in the XY body at the pachytene stage (Mahadevaiah *et al.* 2001). In cyclin A1-deficient spermatocytes, the localization of γH2AX was indistinguishable from that of the control spermatocytes (Nickerson *et al.* 2007). However, at the point of meiotic arrest in diplotene, γH2AX foci were observed first at the centromere and subsequently along the length of the chromosomes. The appearance of γH2AX foci was concurrent with an aggregation of centromeric heterochromatin, which led to the speculation that it may be involved with signalling the induction of apoptosis (Nickerson *et al.* 2007).

The pronounced phosphorylation of histone H3 at serine 10 that has been reported in the late diplotene stage of meiosis (Handel *et al.* 1999) was noticeably reduced in heterozygous *Ccna1*± spermatocytes and undetectable in spermatocytes totally lacking cyclin A1 function (Nickerson *et al.* 2007). Concomitantly, cyclin A1-deficient spermatocytes show reduced staining of aurora B kinase at the pericentromeric heterochromatin. While immunoblot analysis of whole testicular lysates did not indicate a significant difference in levels of aurora B protein between control and A1-deficient testicular lysates, the amount of aurora B protein associated with meiotic chromosomes was clearly different. Interestingly, histone H3 serine 10 is a known target of phosphorylation by the aurora B component of the passenger protein complex (Chen *et al.* 2003) and the point of meiotic arrest in cyclin A1 mice also overlaps with the assembly of the passenger protein complex.

Finally, it is also of interest that Cdk2, a major kinase partner for both A- and E-type cyclins, has been shown to be essential for meiosis in both the male and female germ lines (Ortega *et al.* 2003). Like cyclin A1-deficient spermatocytes, spermatocytes lacking Cdk2 also arrest in meiotic prophase; however, the arrest was observed in mid-pachytene spermatocytes and was accompanied by thin threads of SCP3 staining, perhaps indicating aberrant pairing (Ortega *et al.* 2003). In contrast, obvious differences have not been detected in the appearance of the meiotic chromosomes, notably the appearance of synaptonemal complexes (Liu *et al.* 1998*a*) or in staining of SCP3 (Nickerson *et al.* 2007) in spermatocytes lacking cyclin A1. Further, when cyclin A1-deficicient pachytene spermatocytes were driven into a meiotic configuration by treatment with the phosphatase inhibitor okadaic acid, metaphase I preparations from mutant and normal spermatocytes appeared similar, with no obvious defects in chiasmata (Liu *et al.* 2000).

It has been suggested that a possible reason underlying the two distinct phenotypes may involve differences in the temporal expression and nuclear distribution of cyclin A1 versus Cdk2. Cdk2 protein is localized in the centromeric region, at telomeres, and at foci along chromosomes during pachytene to diplotene (Ashley *et al.* 2001) and is not altered in cyclin A1-deficient mice (Nickerson *et al.* 2007). However, cyclin A1 did not completely co-localize with its putative Cdk2 partner at the centromeres (Nickerson *et al.* 2007). Further, although cyclin A1 is capable of activating Cdk2 (Liu *et al.* 2000), it cannot represent the regulatory subunit for Cdk2 at these early stages of meiotic prophase as it is not expressed until later in meiotic prophase (Sweeney *et al.* 1996). Other Cdk family members have also been studied by targeted mutagenesis, including Cdk1 (Santamaria *et al.* 2007), Cdk4 (Rane *et al.* 1999) and Cdk6 (Malumbres *et al.* 2004; table 1). Not unexpectedly, Cdk1-deficient mice are embryonic lethal while both Cdk4- and Cdk6-deficient mice are viable. There is a quite apparent fertility defect seen in Cdk4-deficient mice, with the females being completely sterile and the males exhibiting an age-dependent infertility. Although the exact causes of the fertility defects remain to be elucidated, it is more likely to be involved with their concomitant diabetic conditions than to primary functions for Cdk4 in the germ cells (Mettus & Rane 2003).

4. E-TYPE CYCLINS

There are also two members of the E-type cyclin family in mammals, cyclin E1 and cyclin E2. Targeted mutagenesis of each of the E-type cyclins revealed an unanticipated function for one of these genes, *Ccne2*, in male fertility (Geng *et al.* 2003; Parisi *et al.* 2003). Surprisingly, reduced fertility in the cyclin E2-deficient male mice was the only obvious phenotype in the single knock-out models of either cyclin E1 or E2. The cyclin E2-deficient males exhibited reduced fertility, with approximately 50 per cent of the males being sterile, and there was an

accompanying reduction (approximately fourfold) in their sperm counts. Although only very preliminary histological analysis was presented in the initial publications, it was clear that there was a reduced cellularity within the tubules and giant cells were observed in the lumens (Geng *et al.* 2003). We have recently undertaken more detailed analysis and observed that spermatogenesis is not arrested at a unique stage, as it is in cyclin A1-deficient testes, and further, that there is a gradation in the level of disruption of spermatogenesis and the loss of cells among the testes of the infertile group (S. S. W. Chung, S. S. Roberts, Y. Geng, P. Sincinski and D. J. Wolgemuth, unpublished data). In the most severely disrupted tubules, later stage spermatocytes and subsequent stages of spermatids were missing. In the less severely affected tubules, more advanced stages, including fully elongated spermatids, could be found.

Mice lacking both cyclin E genes have also been generated and shown to be embryonic lethal, dying during mid-gestation (Geng *et al.* 2003). This lethality could be partially rescued (to birth) by complementation with wild-type tetraploid blastocysts. Mouse embryonic fibroblasts from the doubly cyclin E1- and E2-deficient embryos proliferated normally under conditions of continuous cell cycling, but were unable to re-enter the cell cycle from quiescence. At the molecular level, cyclin E was shown to be loaded into DNA pre-replication complexes, interestingly, in a CDK-independent manner (Geng *et al.* 2007).

5. B-TYPE CYCLINS

There are also two B-type cyclins, which have been well characterized in terms of their mitotic functions, and there may be as many as nine B1-related sequences in the mouse genome (Hanley-Hyde *et al.* 1992; Lock *et al.* 1992). Targeted mutagenesis of *Ccnb1* showed that it is an essential gene, as cyclin B1-deficient mice are embryonic lethal (Brandeis *et al.* 1998). In contrast, *Ccnb2*$^{-/-}$ mice are viable and fertile (Brandeis *et al.* 1998). Although there was an indication that the litter size of homozygous *Ccnb2*$^{-/-}$ matings are slightly smaller, the reason for this observation has not been investigated further; thus its biological significance, if any, is unclear.

Studies of the B-type cyclins in reproduction had demonstrated distinct, developmentally regulated patterns of expression of the mouse *Ccnb1* and *Ccnb2* in both the male and female germ line (Chapman & Wolgemuth 1992, 1993). In the adult testis, *Ccnb2* was present at highest levels in the meiotically dividing spermatocytes, and while *Ccnb1* transcripts are also found in spermatocytes, they are most abundant in post-meiotic round spermatids. Whether this indicates a non-cell cycle progression-related function for cyclin B1 remains to be determined. Lower levels of *Ccnb2* mRNAs were also detected in the early round spermatids. Curiously, neither *Ccnb1* nor *Ccnb2* transcripts were detected in the mitotically dividing spermatogonia. While this could be explained by the sensitivity of the assay, it could also mean that another cyclin could be responsible for activating Cdk1 in these cells.

Although not well studied, a third mouse B-type cyclin, cyclin B3, has also been identified (Nguyen *et al.* 2002). No endogenous cyclin B3 could be detected in a variety of mammalian cell lines that were examined; however, *Ccnb3* mRNA and protein were quite readily detected in leptotene and zygotene spermatocytes and in clusters of oocytes in embryonic ovaries. Studies in which cyclin B3 was ectopically expressed in cultured cells revealed that the protein is predominantly nuclear and that it is degraded upon anaphase entry, following cyclin B1 degradation. Interestingly, it was also shown to be a poor activator of Cdk2 kinase, at least in this cultured cell model (Nguyen *et al.* 2002). In *Drosophila*, cyclin B3 is expressed in both mitotic and meiotic cells (Jacobs *et al.* 1998) and was shown to be essential for fertility, but only in the female. There have not been any reports of the consequences of loss of cyclin B3 function in the mouse model; however, in transgenic mice that express cyclin B3 throughout meiosis, there are severe defects in spermatogenesis and reduced sperm production (Refik-Rogers *et al.* 2006). It thus appears that cyclin B3 is present in a relatively narrow window of meiotic prophase in which the cells are in fact not progressing in the cell cycle but are rather undergoing the business of recombination, and that it is turned over in order to resume the cell cycle in preparation for the first meiotic division. If cyclin B3–Cdk complexes are also poorly functional *in vivo*, one might speculate that its function is to interact with the non-cycling Cdks that are present, essentially preventing them from forming active complexes that would drive division.

6. D-TYPE CYCLINS

The D-type cyclins, D1, D2 and D3, play crucial roles for cells to be able to enter into G1/S of the mitotic cell cycle (Sherr 1994; Kozar *et al.* 2004). Although best known (and studied) for their role in mitosis, they have been implicated in diverse cellular events, including differentiation (Kato *et al.* 1993; Sicinski *et al.* 1995; Bartkova *et al.* 1998), apoptosis (Freeman *et al.* 1994) and as mentioned above, in non-cell cycle-related functions in activating transcription factors (Zwijsen *et al.* 1998). Each of the D-type cyclins has been characterized for their expression patterns in the adult mouse testis (figure 1) and each has been knocked out in mouse models (Sicinski *et al.* 1995, 1996; Sicinska *et al.* 2003; Kozar *et al.* 2004; table 1). Not surprisingly, all three D-type cyclins are expressed in proliferating spermatogonia. However, the expression of cyclins D2 and D3 was also detected in spermatocytes, low-level expression in round spermatids (Ravnik *et al.* 1995; Zhang *et al.* 1999; Beumer *et al.* 2000) and quite clear expression of cyclin D3 in elongating spermatids (Zhang *et al.* 1999). There is also expression of the D cyclins in the somatic testicular cells, with cyclin D3 appearing to be the preferred cyclin D in Sertoli cells (Zhang *et al.* 1999) and D1 and D3 being more abundant in Leydig cells (Beumer *et al.* 2000). Interestingly, cyclin D3 expression persists even after the Sertoli cells are no longer dividing (as in the adult testis)

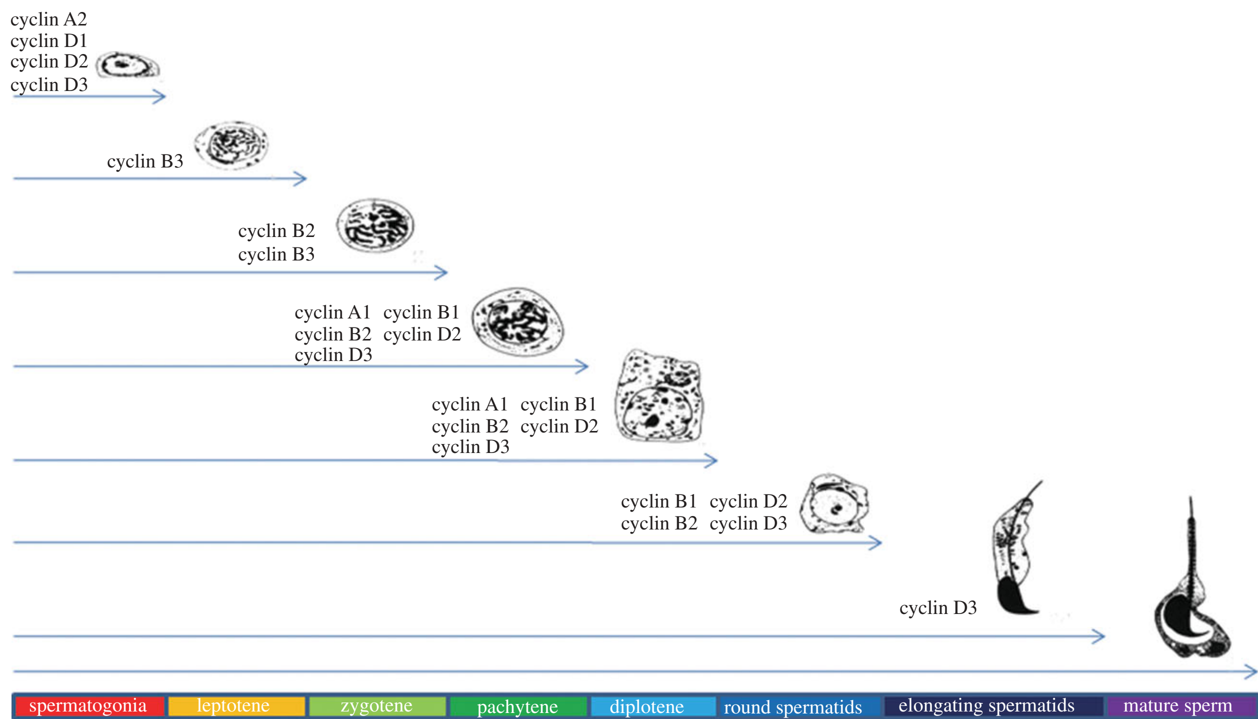

Figure 1. Cartoon summarizing the meiotic stages of spermatogenesis in which cyclin expression has been documented. The arrows represent cellular progression through spermatogenesis. Cyclins listed are expressed as protein in that specific stage. The coloured bars at the bottom denote the cell type shown directly above.

and it is readily detected in the slowly dividing Leydig cells (Beumer *et al.* 2000).

As noted above, each of the D-type cyclins has been studied by targeted mutagenesis and, as in other tissues in which their function would have been predicted to be very important, their individual loss of function had little effect on testis development and spermatogenesis in general (Sherr & Roberts 2004; table 1). Whether there is functional redundancy among the D-type cyclins in testicular cells that express all three cyclin Ds, such as spermatogonia, or whether they are simply not critical in the male germ line remains to be determined. There was mention that although cyclin D2-deficient males are fertile, their testes are hypoplastic (Sicinski *et al.* 1996). Loss of function of all three D-type cyclins resulted in embryonic lethality, although much later in development than for loss of function of cyclin B1 and cyclin A2 (Kozar *et al.* 2004).

7. CYCLINS IN ONCOGENESIS AND INFERTILITY

It is evident that the cyclins play a critical role in regulation and control of spermatogenesis. Therefore, deregulation of the cell cycle that causes testicular cancer might be expected to involve some of the same cyclins dictating spermatogenic control. Cyclins A1, A2 and D2 have been shown to have a role in various types of germ cell tumours (GCTs). We have shown that cyclin A2 protein levels are elevated in the majority of GCTs and expression levels were strongly correlated with the severity of the tumour (Liao *et al.* 2004). In contrast, cyclin A1 was virtually undetectable in carcinoma *in situ*, and seminomas, but aberrantly expressed in all non-seminomatous GCTs. Histone kinase activities of both cyclin A1/Cdks and cyclin A2/Cdks were found to be elevated in the tumours. One of the D-type cyclins, cyclin D2, has also been implicated in testicular cancer. The role of cyclin D2 in testicular cancer has focused on mutations in chromosome 12 and the effects on the G1/S-phase transition. In GCT cell lines, the levels of expression of cyclin D2 were inversely correlated with the extent of differentiation properties characteristic of the cell line (Houldsworth *et al.* 1997). In human germ cells cyclin D2 protein levels were not detectable; however, cyclin D2 was expressed in abnormal germ cells of all of the human GCTs examined. In another study examining the role of cyclin D2 in GCTs, there was increased expression of cyclin D2 that interacts with p27, consistent with its known ability to sequester and block the cyclin E inhibitory function of p27 (Kukoski *et al.* 2003). In mice lacking inhibin alpha, cyclin D2 apparently promoted gonadal growth and tumour development, and male mice died as early as 12 weeks of age (Burns *et al.* 2003). Interestingly, male mice that are doubly deficient for both inhibin alpha and cyclin D2 were markedly less susceptible to tumour development, with a 50 per cent survival at 40–41 weeks of age and almost one-third of the mice were still alive at 1 year of age.

Given the clear essential role for cyclin A1 in the male germ line, and the likely important function for several of the others, including cyclins E2, B1 and A2, it is of interest to ask whether mutations in these genes might be implicated in human infertility. This is particularly true for cyclin A1, since male sterility is the only phenotype observed; that is, men harbouring mutations in the human *CCNA1* gene would be predicted to be otherwise healthy with infertility as

the only phenotype. As the only phenotype observed in Cdk2-deficient mice was male (and female) sterility, human CDK2 might also be a candidate gene for screening in cases of unexplained infertility in healthy men. A similar phenotype might be predicted for defects in other genes uniquely involved in meiosis. Indeed, the examination of such candidate genes in unexplained cases of human infertility has been the subject of some interest (Mandon-Pepin *et al.* 2002) and warrants further attention.

8. SUMMARY

Understanding the genetic programme controlling the mitotic and meiotic divisions of the germ line presents a unique opportunity for providing insight into cell cycle control *in vivo*, during development and differentiation. Elucidating the key control points and proteins involved in their regulation may also enhance our understanding of the etiology of human infertility and, ultimately, provide new directions for contraception.

This work was supported in part by a grant from the NIH to D.J.W., HD034915.

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