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Review

Germ cell sex and cell cycle

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Summary. Germ cells are the only cells in the body capable of transferring an individual's genetic and epigenetic information to the next generation. However, the developmental processes that provide the foundation for male and female germ line development and later gamete production are complex and poorly understood. In mice the primordial germ cells enter the bipotential gonad at E10.5 and, in response to the testicular or ovarian micro-environment, commit to spermatogenesis or oogenesis. This paper reviews progress in understanding the molecular processes underlying the early stages of male and female germ line development.

Key words: Germ cell, Meiosis, Mitotic arrest, Sex determination, Cell cycle

Introduction

Germ cells are required for the transmission of genetic and epigenetic information from one generation to the next. The germ line is derived from cells of the epiblast, the portion of the embryo that gives rise to all other embryonic lineages. Less than ten cells are instructed to commit to germ cell development and retain the unique ability to initiate development of all lineages in the following generation, a quality known as totipotency. The processes that underpin the differentiation of these few cells to ultimately form male and female gametes is highly complex and the subject of many research fields. Here we focus on fetal germ cell development, with particular reference to the earliest steps of the sex specific developmental pathways.

After specification, the primordial germ cells migrate to and enter the gonad at around embryonic day (E)10.5, at which time they are highly proliferative and have the ability to form either the male (spermatogenic) or female (oogenic) germ lines (reviewed by Saitou, 2009). Whether these bipotential primordial germ cells enter the male or female pathway depends on the signals they receive from the somatic cells of the developing gonad. In a developing testis the germ cells commit to spermatogenesis and enter mitotic arrest between E12.5 and E14.5. In contrast, germ cells resident in the developing ovary enter meiosis from E13.5-E15.5. A number of experiments over recent decades clearly demonstrate that male-female germ cell fate is strongly influenced by the surrounding cellular environment (McLaren, 1995; Ewen and Koopman, 2009; Kocer et al., 2009). For example, mouse germ cells exposed to a testicular developmental environment commit to male germ cell development by E12.5, while germ cells exposed to an ovarian environment commit to female germ cell development by E13.5 (Adams and McLaren, 2002). Although these and other elegant experiments have told us much about the cell biology of germ cells, the molecular pathways that regulate early male and female germ cell differentiation are relatively poorly understood. This review concentrates on recent advances in understanding the initiation of sex specific germ cell development, with a focus on the cell cycle regulators involved in female germ cell entry into meiosis and male germ cell entry into mitotic arrest. The consequences of disrupted germ cell differentiation will also be discussed.

Cell cycle and bipotential germ cells

In the mouse, proximal epiblast cells are recruited and committed to germ line development from E6.25 (Ohinata et al., 2005). Around six cells are thought to found the germ line and these cells proliferate to approximately 1,000 by E10.5, at which time they enter the gonad. The germ cells continue to rapidly proliferate for another 2-3 days, with an approximate cell cycle rate of one division every 14-16 hours. By the time germ cells begin entering mitotic arrest in the male, or meiosis in the female, they number 25,000 (Tam and Snow, 1981). How the germ cells exit this rapid proliferative

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phase into either mitotic arrest or meiosis remains poorly understood.

Progression through the cell cycle is characterized by the phases G1, S, G2 and M, with restriction points controlling progression though G1-S and G2-M. Each phase is controlled by restricted expression of the Cyclins and their corresponding Cyclin Dependent Kinases (CDKs), which control passage through the G1-S and G2-M restriction points. These restriction points are of particular consequence in developing germ cells as male germ cells exit the cycle to enter G0 arrest prior to passage from G1 into S, while female germ cells exit the mitotic cycle into meiosis at G2/M.

The Retinoblastoma (RB) family of proteins controls progression of cells from G1 into the DNA synthesis phase, S. When active, RB binds to and inhibits E2F proteins that ultimately promote proliferation. In a proliferative environment the CYCLIN-E/CDK2 or CYCLIN-D/CDK4/6 complexes phosphorylate RB, resulting in its inactivation and allowing E2F to promote transcription of genes required for DNA synthesis. By contrast, an anti-proliferative environment results in up regulation of the CIP/KIP (p21^{CIP1}, p27 ^{KIP1}, p57^{KIP2}) and/or the INK4 (p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}) family members. The products of these genes then bind to their respective CDK targets and consequently block the CDK-CYCLIN interaction, preventing inactivation of RB, which in its active hypo-phosphorylated form promotes exit from the cell cycle into G0 at the G1/S checkpoint (Richardson et al., 2006).

The G2/M checkpoint is activated in response to DNA damage. When single and/or double strand breaks are detected, Ataxia telangiectasia mutated (ATM) and/or ataxia telangiectasia and Rad3-related protein (ATR) are activated and phosphorylate Checkpoint Kinase 1 (CHK1) and 2 (CHK2). These then phosphorylate and inhibit CDC25 A, B or C, which is required for activation of the mitosis promoting factor, a complex of CYCLIN B and CDC2 (Zhou and Elledge, 2000).

Though male germ cells commit to spermatogenesis by E12.5, at this time they are highly proliferative and express similar cell cycle regulators as their female counterparts (Western et al., 2008; Miles et al., 2010). KI-67, which is absent in cells that have entered G1/G0 arrest, is expressed throughout the nucleus in both male and female germ cells at E12.5. By E15.5 KI-67 is no longer expressed in male germ cells, indicating that these cells have entered G1/G0 arrest (Western et al., 2008). In female germ cells KI-67 expression becomes localized to the periphery of the meiotic nucleus in 2-3 spots, possibly marking heterochromatin, as these spots are also DAPI rich (Fig. 1B) (Miles et al., 2010). Other cell cycle proteins expressed in E12.5 male and female germ cells include phosphorylated RB, ATR-P and CHK1-P (Fig. 1A) (Western et al., 2008; Miles et al., 2010). It has been speculated that expression of ATR-P and CHK1-P in early germ cells is related to an evolutionarily conserved mechanism involved in maintaining the genomic integrity of the germ cell population (Miles et al., 2010).

Although the cell cycle profiles of E12.5 male and female germ cells are similar, there are some differences in the regulation of their proliferation by this stage. For example, although the germ line marker, mouse vasa homologue (MVH) is expressed in both male and female germ cells from E10.5, it appears to be required for male germ cell proliferation only (Tanaka et al., 2000; van den Bergen et al., 2009). From E11.5 loss of this gene results in decreased mitosis without increased germ cell death in male germ cells (Tanaka et al., 2000).

Female germ cell sex and differentiation

One of the earliest known characteristics of ovary formation is the entry of germ cells into meiosis, marking the onset of oogenesis. It has long been speculated that meiosis is induced by the presence of a meiosis inducing factor (Byskov and Saxén, 1976). A number of studies have shown that deficiency in dietary vitamin A (retinol) leads to a failure of spermatogenesis and that administration of retinol or retinoic acid can re-initiate spermatogenesis in these animals (Griswold et al., 1989; van Pelt and de Rooij, 1991). Additional work indicates that induction of meiosis in female germ cells is mediated by RA, which in mice is thought to pass into the developing ovary from the adjacent mesonephros (Bowles et al., 2006; Koubova et al., 2006). Incubating fetal ovaries with an RA receptor antagonist in *ex-vivo* culture prevented entry of XX germ cells into meiosis as assessed by histological identification of meiotic germ cells and reduced expression of the RA responsive gene, stimulated by retinoic acid gene 8 (*Stra8*) (Bowles et al., 2006; Koubova et al., 2006). When E11.5 male gonads were cultured in the presence of high levels of exogenous RA, transcription of *Stra8*, synaptonemal complex protein 3 (*Sycp3*) and dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (*Dmc1*) increased in XY germ cells. In addition, histological analysis indicated that these cells had entered meiosis (Bowles et al., 2006; Koubova et al., 2006; Trautmann et al., 2008). Consistent with this, treatment of XX gonads with retinoic acid receptor inhibitors reduced transcription of *Stra8*, *Scp3* and *Dmc1* (Bowles et al., 2006; Koubova et al., 2006). Furthermore, *Cyp26b1*, which encodes an RA metabolising enzyme, was strongly expressed in the developing somatic cells of the testis and not the ovary, leading to the hypothesis that RA acts as a meiosis inducing factor, while CYP26B1 inhibits the meiosis inducing action of RA in the testis (Bowles et al., 2006; Koubova et al., 2006). Supporting this, the germ cells of male *Cyp26b1* null mice activated transcription of *Stra8* and *Sycp3* and engaged in meiosis, prior to many of the germ cells entering apoptosis (Bowles et al., 2006; MacLean et al., 2007). Moreover, deletion of *Stra8* leads to a failure of meiosis in males and females and *Stra8* fails to activate in animals deficient for vitamin A and RA synthesis. Combined these experiments have led to

the conclusion that germ cell meiosis is induced by RA in the fetal ovary and inhibited by CYP26B1 in the developing testis.

However, other observations question the role of RA in inducing meiosis (Best et al., 2008; Kocer et al., 2009; Kumar et al., 2011). Germ cells that aberrantly locate to the mesonephros tend to enter mitotic arrest despite their exposure to RA (McLaren, 1984; Francavilla and Zamboni, 1985; Best et al., 2008). Consistent with this, when E12.5 male germ cells were aggregated with E12.5 female urogenital cells (including mesonephros) and cultured for three or four days, the male germ cells did not enter meiosis, despite their association with RA producing tissue (Adams and McLaren, 2002). In similar experiments, treatment of male germ cells with RA led to high levels of germ cell death, although data published by Trautmann et al., 2008 indicate that in some cases RA induced cell death may follow initial entry into meiosis (Petre-Lazar et al., 2006; Best et al., 2008; Trautmann et al., 2008). RA is synthesised in the embryo largely through aldehyde dehydrogenase family 1, subfamily A2 (*Raldh2*) and *Raldh3*. In mice *Raldh2* is highly expressed in the mesonephros of males and females, while *Raldh3* is expressed at low levels in both the developing testis and ovary (Bowles et al., 2006). Interestingly, transcription of the related gene *Raldh1* is robustly upregulated in the developing testis at E12.5 and E13.5, but is detected at relatively low levels in the E12.5 ovary and is 50-100 fold lower in the E13.5 ovary than the testis (Bowles et al., 2009) and its potential to

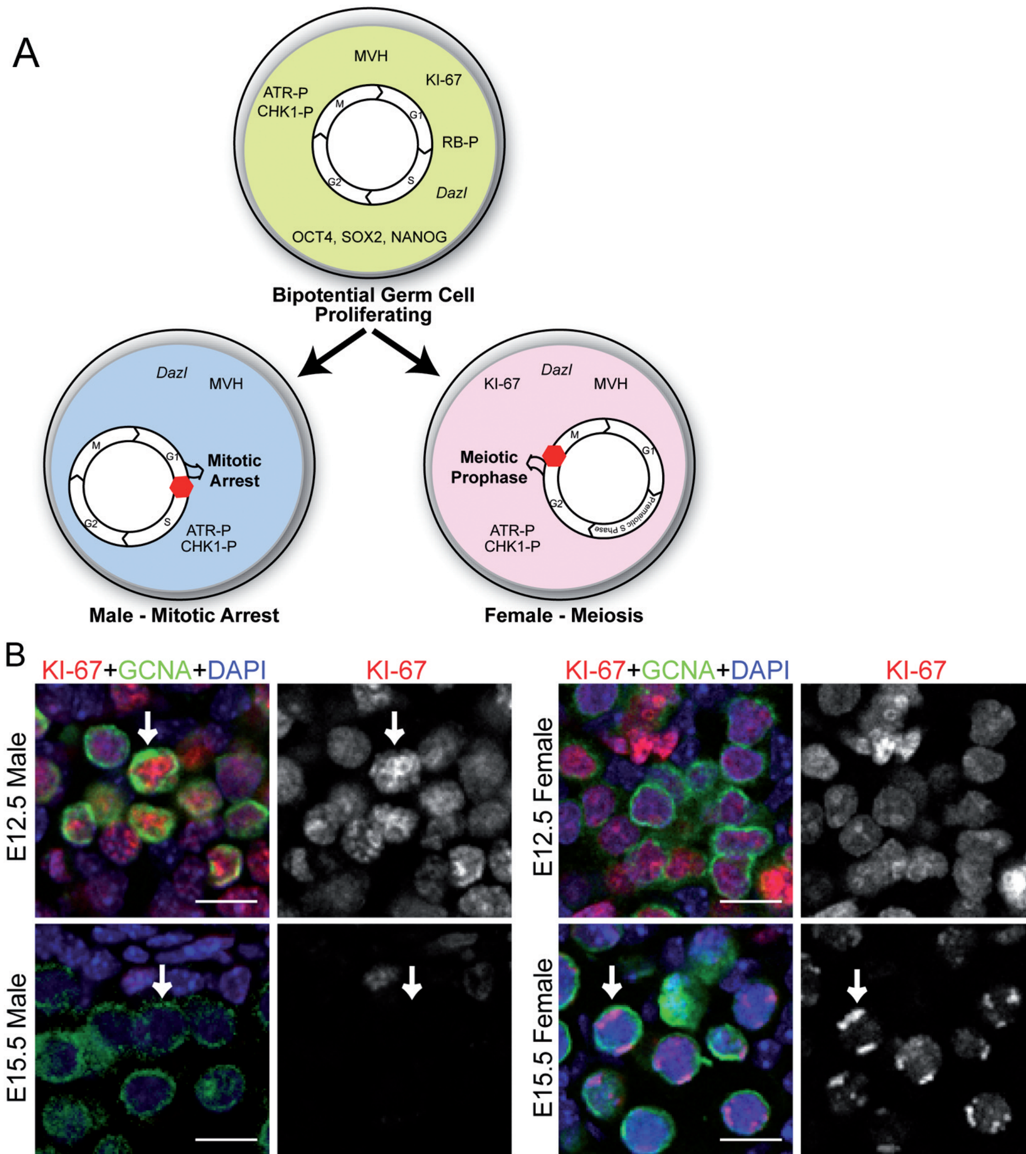

Fig. 1. A. Mitotic arrest and meiosis – early events in male and female germ cell development. At E12.5 germ cells in the developing testis and ovary are rapidly proliferating and express the cell cycle markers KI-67, phosphorylated (inactive) RB, phosphorylated ATR and CHK1, the pluripotency genes OCT4, SOX2 and NANOG and the germ cell markers MVH, GCNA and *Dazl*. *Mvh* is required for male germ cell proliferation, while *Dazl* is required for differentiation of male and female germ cells. It has been proposed that ATR and CHK1 regulate genome integrity in the male and female germ lines. Between E12.5 and E14.5 germ cells resident in the developing testis enter mitotic arrest, while germ cells in the ovary enter meiotic prophase between E13.5 and E15.5. **B.** Changes in KI-67 expression mark the entry of male and female germ cells into mitotic arrest and meiosis, respectively. As male germ cells enter mitotic arrest they become KI-67 negative. However, in females KI-67 becomes localized to 2-3 discreet foci as the germ cells enter meiotic prophase. Expression of KI-67 is shown in red, with GCNA marking the germ cells in green. DAPI marks the nucleus in blue. Arrows indicate germ cells. Scale bar: 6.25 μm.

produce RA in this context remains obscure. Analysis of *Raldh2 -/-* null and *Raldh2 -/- Raldh3 -/-* double null mice showed that in the absence of detectable RA, expression of *Stra8* and entry into meiosis was induced in female germ cells indicating that RA is not required to induce *Stra8* or for germ cell entry into meiosis *in-vivo* (Kumar et al., 2011). Supporting this, germ cells within E11.5 ovaries cultured without the mesonephros (the proposed source of RA) express *Stra8* and enter meiosis (Guerquin et al., 2010). The role of CYP26B1 in clearing the developing testis of RA was also re-examined (Kumar et al., 2011). In line with previous studies, ketoconozole inhibition of CYP26B1 function led to *Stra8* expression in wild type testes. However, this result was also observed in cultured testes from *Raldh2 -/-* mice, which lacked detectable RA, indicating that CYP2B1 inhibits *Stra8* expression through an RA independent mechanism (Kumar et al., 2011).

Further studies have examined the role of RA in inducing meiosis in other species, including chickens and humans (Bowles et al., 2009; Le Bouffant et al., 2010). The authors conclude that although RA is likely involved in inducing meiosis, a number of important differences were observed between the mouse and the human and chick systems. Firstly, RA synthesis is thought to originate in the mesonephros in the mouse but is apparently intra-ovarian in the chick and human ovary (Bowles et al., 2006, 2009; Le Bouffant et al., 2010). Moreover, treatment of human fetal ovaries with RA resulted in relatively limited initiation and completion of meiosis, with half the germ cells not initiating meiosis and the vast majority not completing meiosis. Finally, the genes thought to regulate gonadal levels of RA are regulated in quite different patterns in the mouse and human, indicating that differences exist in the mechanisms involved in these species (Le Bouffant et al., 2010).

Though the molecular mechanisms inducing endogenous *Stra8* expression require further clarification, genetic evidence indicates that *Stra8* is required for female germ cells to enter the final DNA synthesis phase prior to meiosis, referred to as “pre-meiotic” S-phase for the purposes of this discussion (Baltus et al., 2006). *Stra8* null germ cells accumulate at the beginning of S-phase, but fail to progress further. Moreover, although the meiotic recombination proteins REC8 and SYCP3 are produced in E15.5 *Stra8* null oocytes, these proteins fail to load onto the chromosomes, indicating that *Stra8* null cells fail to enter prophase 1. In addition, *Stra8* null germ cells fail to form DNA double strand breaks (DSBs), a hallmark of meiotic recombination (Baltus et al., 2006; Anderson et al., 2008). Combined these data support the conclusion that although initiation of some components of the meiotic machinery, including expression of SYCP3 and REC8, are *Stra8* independent, *Stra8* has a significant role in controlling cell cycle progression through pre-meiotic S-phase. Since the phenotype of female *Stra8* null germ cells appears limited to germ cells entering meiosis, these data also indicate that pre-meiotic S-phase is qualitatively different from mitotic S-phase, which occurs in a *Stra8* independent manner. The role of *Stra8* in pre-meiotic S-phase is yet to be understood. However, recently it was shown that essentially all RB remains inactive in female germ cells during their progression through pre-meiotic S-phase (Western et al., 2008). This presumably occurs to permit female germ cells free passage through pre-meiotic S phase. One possibility is that the STRA8 protein indirectly controls the activity of the key the G1-S phase check point regulator RB, maintaining it in an inactive form and therefore promoting passage through the G1-S check-point (Western, 2009).

Recently it was reported that germ cell entry into meiosis also involves the germ cell intrinsic factor, deleted in azoospermia-like (*Dazl*), which does not require induction by RA (Saunders et al., 2003; Lin and Page, 2005). *Dazl* is expressed in both male and female germ cells from E11 (Lin and Page, 2005). Analysis of *Dazl -/-* mice revealed that deletion of *Dazl* results in a failure of female germ cells to express multiple genes (and proteins) required for meiosis, including *Sycp3* and *Stra8*, and a failure to initiate meiosis (Ruggiu et al., 1997; Lin et al., 2008). Although *Sycp3* is commonly used as an indicator of entry into meiosis, it is also expressed in male germ cells at the equivalent stage (Di Carlo et al., 2000). Consistent with this, and the phenotypes of *Dazl* and *Stra8* null female germ cells, it has been argued that *Dazl* regulates the ability of germ cells in both male and female gonads to enter a state of meiotic competence, with meiosis only promoted in female germ cells following the action of STRA8 (Lin et al., 2008). However, *Dazl* is also required for early male germ cell survival (Lin and Page, 2005), indicating that it regulates additional aspects of germ cell differentiation that are unrelated to induction of meiosis.

Another gene that is required for proper initiation of meiosis is *Dmrt1*, which encodes a transcription factor required for multiple facets of germ and somatic cell development in the differentiating testis (Krentz et al., 2009b, 2011; Matson et al., 2011). A recent publication has shown that loss of *Dmrt1* in female germ cells results in poor activation of *Stra8* and a quite severe reduction in the number of germ cells loading SYCP3 and γH2AX (phosphorylated H2A histone family, member X) to the chromosomes during prophase 1 (Krentz et al., 2011). Despite the aberrant activation of these key regulators of early meiosis and recombination, *Dmrt1-/-* females are fertile. Given that *Stra8-/-* animals of both sexes are sterile, the apparently normal fertility of *Dmrt1-/-* females is quite surprising. However, a possibility raised by the authors is that sufficient remaining *Stra8* expression in the developing oocyte population sustains oogenesis, albeit at levels that produce around half the normal number of follicles (Krentz et al., 2011). Since *Stra8* has an apparent role in controlling progression of female germ cells through premeoitic S-phase, it would be informative to

comparatively analyse progression of *Dmrt1* and *Stra8* null female germ cells into and through this process. This could be achieved using flow cytometry, or a similar technology, that could identify the proportions and progress of germ cells passing from G1 to S and into G2/M.

Another striking feature of *Dmrt1*-/- oocytes is their almost complete lack of γH2AX. H2afx is abundantly transcribed in E12.5-E15.5 female germ cells and phosphorylation of its protein product is an exquisite sensor of DNA double strand breaks (DSBs) (J. van den Bergen, D. Miles and P. Western unpublished data, (Fernandez-Capetillo et al., 2003). An almost immediate response to DSBs involves the phosphorylation of large amounts of H2AX and recruitment of the resulting γH2AX to megabases of DNA surrounding the DSBs. This is a conspicuous feature of meiotic germ cells, which become strongly positive for γH2AX from E14.5. Loss of *H2afx* (which encodes H2AX) in female mice results in aneuploidy and reduced litter size (Celeste et al., 2002). The almost complete lack of γH2AX in *Dmrt1* null female germ cells at E15.5 is surprising considering the moderate reduction in follicle number and normal fertility in the adult animals. However, a number of possibilities may account for this disparity. Onset of γH2AX may be delayed in *Dmrt* -/- germ cells, or sufficient germ cells may express γH2AX through oogenesis and are maintained, while γH2AX negative cells are lost, or γH2AX may not be required for fertility in these mice.

Cell cycle and meiosis

As discussed above, in order to enter meiosis female germ cells must change their cell cycle profile, exiting a phase of rapid mitotic division and entering prophase. This occurs from E13.5 and involves the cessation of DNA synthesis and entry into prophase I at the G2/M checkpoint. By E15.5 the female germ cell population is comprised of cells in leptotene, zygotene or pachytene with 95% of the female germ cell population containing a 4N DNA content (Borum, 1961; Bakken and McClanahan, 1978; Miles et al., 2010). The cell cycle genes involved in passage of female germ cells through G1-S and entry into meiosis from G2 remain poorly understood. Early work showed that *Cyclin B3* is highly expressed as female germ cells enter meiosis (Nguyen et al., 2002) and over-expression of a stabilised form of *Cyclin B3* in cultured somatic cells promoted G2/M arrest (Nguyen et al., 2002). Furthermore, prolonged expression of *Cyclin B3* in spermatocytes during zygotene prevented the transition into pachytene indicating that degradation of *Cyclin B3* is required for meiosis (Refik-Rogers et al., 2006). The striking up regulation of *Cyclin B3* in female meiotic germ cells occurs concomitantly with repression of the cell cycle promoting *Cyclin B1*, perhaps indicating that *Cyclin B3* is involved in the switch from mitosis to meiosis or exit from pre-meiotic S-phase into prophase at the G2/M check-point (Miles et al., 2010).

In female germ cells the G2/M checkpoint is presumably activated in response to double strand breaks generated by homologous recombination. Expression of several G2/M DNA damage pathway members during male meiosis and recruitment of γH2AX supports this view (Mahadevaiah et al., 2001; Hamer et al., 2003, 2004; Bellani et al., 2005; Burgoyne et al., 2007). Consistent with this, it was recently demonstrated that the ATM/CHK2 pathway is activated during entry of female germ cells into meiosis (Fig. 2) (Miles et al., 2010). These observations, combined with evidence in *Atm* null mice, in which infertility occurs due to an inability of the germ cells to complete meiosis, indicate an essential role for this pathway in female germ cell meiosis (Barlow et al., 1996, 1998; Xu et al., 1996). Deletion of *H2afx* in female mice resulted in reduced litter size and aneuploidy, while male mice lacking *H2afx* were infertile due to a block at pachytene of meiosis 1 (Celeste et al., 2002). Functional analysis of *Chk2* yielded more equivocal results, as its deletion in mice had no effect on fertility (Celeste et al., 2002; Hirao et al., 2002).

Male germ cell sex and differentiation

Like female germ cells, the commitment of the male germ cell lineage is also dependent upon the surrounding somatic environment. However, rather than entering meiosis at G2/M, male germ cells exit the cell cycle from G1 into G0, prior to passage into the following S-phase. It has been hypothesised, based on *ex vivo* organ culture and aggregate experiments of male or female germ cells with male or female gonadal somatic cells or other tissues, that an unidentified male specific meiosis-inhibiting factor prevents precocious entry of male germ cells into meiosis (Byskov and Saxén, 1976; Byskov, 1986; Dolci and De Felici, 1990; McLaren and Southee, 1997). As discussed earlier, it appears that *Cyp26b1*, which encodes an intracellular enzyme produced by the Sertoli and potentially interstitial cells, functions as a meiosis-inhibiting factor (Bowles et al., 2006; Koubova et al., 2006). Supporting this conclusion, analysis of SYCP3 expression and H&E staining of testis sections suggested that male germ cells in *Cyp26b1* -/- mice initially enter meiosis from E13.5 but then undergo apoptosis (MacLean et al., 2007). However, although one does not preclude the other, additional evidence indicates that a meiosis-inhibiting factor is secreted by the Sertoli cells in the developing testis. Sexually dimorphic, expressed in male gonads 1 (*Sdmg1*; officially known as *Tmem184a*) encodes a protein involved in transmembrane trafficking that is specifically expressed in male Sertoli cells (Best et al., 2008). When transmembrane trafficking was blocked *in vitro* by Brefeldin A treatment, male germ cells entered meiosis, indicating that active secretion of a meiosis-inhibitory factor is required to prevent precocious meiosis in male germ cells (Best et al., 2008). In support

of this, Guerquin et al. (2010) provide evidence that a molecule of greater than 10 KDa secreted by the somatic cells of the developing testis inhibits the entry of female germ cells into meiosis. Interestingly, this analysis indicates that inhibition of meiosis and promotion of male germ cell development is a complex process, involving meiosis-inhibiting factor(s) secreted by both the interstitial and Sertoli cells and factor(s) secreted by the interstitial cells that inhibit germ cell proliferation (Guerquin et al., 2010).

One possibility is that fibroblast growth factor 9 (FGF9) acts as a secreted meiosis-inhibiting factor (Barrios et al., 2010; Bowles et al., 2010). Prior to E11.5 both male and female germ cells proliferate and survive in the absence of FGF9, however, from E11.5 male germ cells become reliant on FGF9 for their survival (DiNapoli et al., 2006). It has recently been argued on the basis of genetic evidence and *ex-vivo* organ culture experiments that the balance between FGF9 and RA signaling influences germ cell fate, with RA promoting female fate and meiosis, and FGF9 promoting male germ cell development (Barrios et al., 2010; Bowles et al., 2010). The FGF receptors FGFR2 and FGFR3 have been detected in developing male germ cells at the cell surface and in the nucleus respectively, indicating that FGF9 signaling may be facilitated through these receptors (Bowles et al., 2010; Western et al., 2011).

Another intriguing gene involved in male germ cell commitment is *Nanos2*, which encodes the NANOS2 RNA binding protein. NANOS proteins are expressed by the developing germ cells of species as diverse as *Drosophila melanogaster* and mice. In *Nanos2* -/- mice that also lack the pro-apoptotic gene *Bax*, male germ cells fail to enter mitotic arrest and instead enter meiosis during a period of concomitant reduction in *Cyp26b1* levels (Tsuda et al., 2003; Suzuki and Saga, 2008). When *Nanos2* is ectopically expressed in primordial germ cells of the developing ovary, they subsequently failed to enter meiosis and exhibited male germ cell characteristics, such as the expression of DNA methyltransferase DNMT3L (Suzuki and Saga, 2008). This led to the proposal that NANOS2 plays an important role in preventing *Stra8* expression and precocious induction of male meiosis (Suzuki and Saga, 2008). As discussed earlier, recent data indicate that FGF9 and RA may respectively promote and oppose *Nanos2* expression in male germ cells (Barrios et al., 2010; Bowles et al., 2010).

Although male germ cell commitment and entry into G1/G0 arrest has been well described, the signaling factors contributing to mitotic arrest remain unknown. To ascertain which cell type(s) produce mitotic arrest signal(s) Guerquin et al., (2010) cultured XX germ cells with Sertoli cells and male interstitial cells and Sertoli or male interstitial cells alone (Guerquin et al., 2010). It was observed that the interstitial cells were more effective at inducing mitotic arrest in XX germ cells than Sertoli cells. These data indicate that a mitotic arrest factor(s) may originate from the interstitial cells, rather than the Sertoli cells which until recently have been widely thought to be the source of this signal. However, as the induction of male characteristics in XX germ cells was not complete in presence of XY interstitial or Sertoli cells, it remains possible that factors from both the interstitial and Sertoli cell compartments combine to regulate male germ cell fate and mitotic arrest.

Recently, Moreno et al., (2010) analysed the importance of TGF signaling in mitotic arrest of male germ cells (Moreno et al., 2010). These authors used a combination of two approaches, a conditional *Tgfbr2* (Transforming growth factor receptor II) knockout model and *ex vivo* organ culture. In these models loss of *Tgfbr2* or addition of TGFß2 resulted in moderately higher levels of germ cell proliferation or arrest respectively, indicating that TGFß contributes to male germ cell arrest. Increased germ numbers have also been observed in E15.5 male mice lacking *Inhba*, indicating Activin signaling may also impact germ cell proliferation (Mendis et al., 2010). RA has also been demonstrated to play a role in male germ cell proliferation. When RA was added to E13.5 male gonads in culture, male germ cells were prevented from entering mitotic arrest and continued proliferating, indicating that RA levels must be controlled to allow mitotic arrest (Trautmann et al., 2008). Combined, this evidence indicates that several growth factors and morphogens interact to control the proliferation of germ cells and

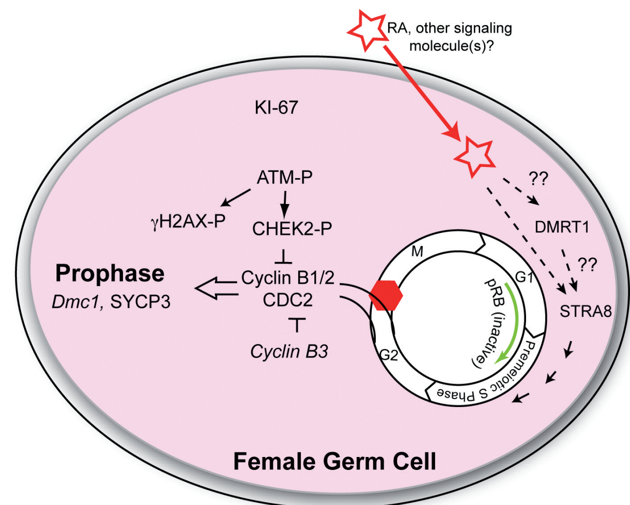

Fig. 2. Female germ cell entry into meiosis. Entry into meiosis is induced by signal(s) that promote *Stra8* expression, which is required for transition through the final S phase before meiosis. DMRT1 is required for full expression of *Stra8.* The key G1-S phase check point protein RB remains phosphorylated (inactive) allowing S phase progression. Germ cells exit the cell cycle at G2/M and progress through prophase I. Exit at G2/M involves the cyclins *CyclinB3* and *CyclinB1*, which are upregulated and repressed, respectively, and the ATM/CHK2 DNA damage pathway, which is activated. Female germ cells maintain expression of KI-67 and express genes required for meiosis such as *Dmc1* and *Sycp3.*

regulate the important decision to enter mitotic arrest or meiosis. This may also explain why mitotic arrest of fetal germ cells appears to be a relatively robust process that has been difficult to analyse in models involving deletion of single genes in signaling pathways.

Cell cycle and G1/G0 arrest

Studies using cell counting in gonad sections demonstrated that germ cells in E8.5-E13.5 male embryos are proliferative and total around 25,000 (Tam and Snow, 1981). Sapsford (1962) reported, using cell counting, that male germ cells were actively dividing until E16-E17 (Sapsford, 1962) and using [3H]thymidine labeling index, Vergouwen et al. (1991) demonstrated that male germ cells were actively proliferating at E14 but were quiescent from E16 (Vergouwen et al., 1991). Other studies showed that male germ cells entered quiescence at E13.5 (McLaren, 1995). Recently, flow cytometry was used to isolate the developing germ cell population (using MVH staining) and analyse germ cell progress into mitotic arrest by simultaneously measuring cell cycle stage using DNA content, and passage through S-phase by measuring BrdU or EdU incorporation (Western et al., 2008, 2011). These analyses allowed the entry of male germ cells to be tracked with a high level of precision and showed that mitotic arrest is initiated from E12.5 and, depending on the mouse strain, by E14.5 more than 90% of the germ cells are in G1/G0, with few still progressing through S-phase (Western et al., 2008, 2011).

There have been a number of studies aimed at identifying cell cycle proteins that regulate mitotic arrest of male germ cells. Analysis of the G1 exclusive CYCLIN D proteins in the developing testis showed that in the proliferative phase (E14) germ cells express CYCLIN D3 and during quiescence (E17) germ cells express CYCLIN D2 as well as CYCLIN D3 (Beumer et al., 2000). It has also been shown that from E12.5 to E15.5 male germ cells repress *Cyclin E1* and *CyclinE2* (Western et al., 2008). Consistent with this repression, analysis of RB in male germ cells during this time revealed that RB switches from an inactive to an active state as germ cells enter mitotic arrest. Moreover, at the initiation of mitotic arrest male germ cells up regulate several cell cycle inhibitors, including the INK4 family members $p15^{INK4B}$ and $p16^{INK4B}$ and the CIP/KIP family members $p27^{KIP1}$, $p21^{CIP1}$ and $p57^{KIP2}$ (Beumer et al., 1999; Western et al., 2008). Though not strongly up regulated at the transcriptional level, $p27^{KIP1}$ protein levels become highly expressed in the male germ cell nucleus as RB is activated and the germ cells enter mitotic arrest (Beumer et al., 1999; Western et al., 2008, 2011). These data supported a model whereby mitotic arrest of male germ cells is mediated through the activation of cell cycle inhibitors $p15^{INK4B}$, $p16^{INK4B}$ and $p27^{KIP1}$, the repression of *Cyclin E* and the activation of the G1-S phase check point protein RB (Fig. 3) (Western et al., 2008). This model has been partly substantiated in *ex vivo* cultures of gonads from mice lacking RB. The male germ cells in these mice exhibit increased proliferation at E14.5 and E15.5 relative to heterozygous or wild-type controls. However, this phenotype was transient, as by E16.5 the RB null germ cells had entered mitotic arrest and the gonads of these mice contained increased levels of $p27^{Kip1}$ and $p15^{Ink4b}$ expression (Spiller et al., 2009), indicating that mitotic arrest could be completed through an RB independent mechanism. This occurs at a stage during which normal levels of RB are low in male germ cells, indicating that RB is not required for maintaining mitotic arrest after E15.5 (Western et al., 2008).

Consequences of improper germ cell development

Disorders stemming from improper testis and/or germ cell development include infertility and the formation of testicular germ cell tumours (TGCTs). In humans TGCTs have been classified into 5 major groups (Oosterhuis and Looijenga, 2005). Type I GCTs are found in neonates and children, while Type II GCTs occur in males typically 15 to 45 years old. Type I and II TGCTs are widely considered to originate from germ cells that have become developmentally blocked at fetal developmental stages and this review will therefore focus on these two classes. Type I GCTs manifest as teratomas and yolk-sac tumours that have formed from germ cells with partially erased DNA methylation patterns at imprinted loci, indicating that these tumours arise from primordial germ cells in which epigenetic erasure is incomplete (Bussey et al., 2001; Schneider et al., 2001; Oosterhuis and Looijenga, 2005). Type II GCTs (seminomas and non-seminomas) arise from a common precursor cell known as carcinoma in situ (CIS) (Skakkebaek, 1972). It is thought that the origin of CIS is a germ cell of fetal origin (a pgc or gonocyte) that has a block in maturation. CIS have genomic imprinting patterns, telomerase activity and gene expression patterns similar to those observed in pgc's or gonocytes (van Gurp et al., 1994; Albanell et al., 1999; Almstrup et al., 2004). Expression of several proteins associated with gonocyte development has also been detected in CIS, including AP2γ, c-KIT and PLAP. CIS cells also have maintained or re-activated expression of OCT3/4 and NANOG and are typically aneuploid. (Palumbo et al., 2002; Looijenga et al., 2003a,b; Rajpert-De Meyts et al., 2004; Hart et al., 2005). As mentioned previously, CIS progresses to form either seminomas or non seminomas. Seminomas are morphologically similar to CIS and remain in a relatively stable differentiation state with cells accumulating to form uniform tumours. Although seminomas express OCT4 and NANOG, they lack expression of SOX2, which is widely considered to be a core regulator of pluripotency. Although SOX2 is not expressed in seminomas, a related gene SOX17 is expressed in these tumours (de Jong et al., 2008).

The second major class of Type II TGCTs is known as non-seminoma and these tumours are initiated through the formation embryonal carcinoma (EC) either from the CIS precursor or by progression of seminoma to EC. EC cells are considered the stem cell of non-seminomatous tumours and can exhibit overt pluripotency/totipotency, as demonstrated by their ability to give rise to cells of all three germ layers, extraembryonic tissues and the germ line. Non-seminomas show greater resistance to treatment and consequently result in higher mortality rates than seminomas. Non-seminomas occur in men with a median age of 25 years, approximately ten years younger than for seminomas, for which patients are diagnosed at a median age of 35. Around 10% of non-seminomas also contain seminomatous material, and it has been proposed that seminomas can progress to EC, an event that is associated with reprogramming, resulting in the activation of pluripotency (Looijenga et al., 1999; Oosterhuis et al., 2003). Consistent with the activation of pluripotency SOX2 is expressed in EC, but is not expressed in seminomas. The broad differentiation potential of ECs is reflected in their ability to differentiate as yolk sac tumours, teratomas or choriocarcinomas, all of which commonly occur in non-seminomatous tumours (Oosterhuis and Looijenga, 2005).

How CIS are formed in the testis is currently unknown, and the pathways that are involved in transformation of CIS cells into seminomas and non-seminomas are also poorly understood. Several chromosomal aberrations are common in seminoma and non-seminoma, however, with the exception of the SCF (KIT Ligand)-KIT signaling pathway it has proven difficult to identify functional gene mutations associated with tumour formation (McIntyre, 2005; Korkola et al., 2008; Kanetsky et al., 2009; Rapley et al., 2009; Van De Geijn et al., 2009). A marker of CIS progression to seminoma or non-seminoma is the amplification of 12p, which contains genes such as STELLAR, NANOG, KRAS and CYCLIND2 (Looijenga et al., 2003b). Notably STELLAR and NANOG are both associated with pluripotency and NANOG is over-expressed in TGCTs (Ezeh et al., 2005; Hart et al., 2005). Global expression patterns associated with TGCTs have also been examined, with studies identifying gene signatures associated with tumour types and survival outcomes for patients exhibiting resistance to chemotherapy (Korkola et al., 2009; Looijenga, 2009). More extensive discussions of TGCTs can be found in a number of excellent reviews (Oosterhuis and Looijenga, 2005; Kristensen et al., 2008; Looijenga, 2009).

Although germ cell tumours occur in mice, these tumours form benign teratomas from fetal stages and are generally considered to be representative of Type I TGCTs, rather than Type II. A number of informative mouse models exist for Type I TGCTs that have contributed to understanding germ cell derived teratoma formation in mice.

Mouse models for teratoma (Type I TGCTs)

Germ cell tumours in mice were first recognised in the 129 strain in which teratomas form spontaneously at a rate of around 3% (Stevens and Hummel, 1957). In mice of the 129 strain a mutation at the "*ter*" locus increased teratoma incidence to 94% (Noguchi and Stevens, 1982; Noguchi and Noguchi, 1985). Recently it was discovered that the mutation underlying *ter* affects dead end homologue 1 (*Dnd1*), which is expressed in male fetal germ cells as they enter mitotic arrest (Youngren et al., 2005). *Dnd1* encodes an RNA binding protein that has been shown to target the 3' UTR of p27^{Kip1} and *Nanos* transcripts in zebrafish germ cells (Kedde et al., 2007). It has been demonstrated that the *ter* mutation in *Dnd1* causes germ cell death in a variety of mouse genetic backgrounds, but in the 129Sv/J background results in teratoma formation (Cook et al.,

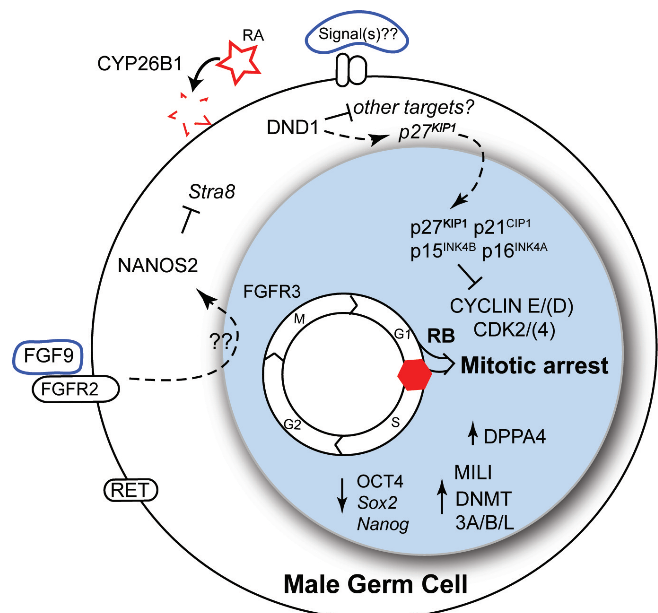

Fig. 3. Mitotic arrest and male germ cell development. CYP26B1 prevents germ cell entry into meiosis in the developing testis, while an anti-proliferative signal(s) induces mitotic arrest in male germ cells. At this time FGF9 signaling is required for male germ cell development, promoting male genes including *Nanos2*. *Nanos2* encodes an RNA binding protein with multiple roles in male germ cell differentiation, including repression of *Stra8* and aberrant entry into meiosis. FGFR3 localises to the nucleus of male germ cells as they enter mitotic arrest and RET signaling is required for male germ cell survival. In response to anti-proliferative signals, the cell cycle inhibitors p27^{KIP1}, p21^{CIP1}, p16^{INK4A} and p15^{INK4B} are upregulated in male germ cells and promote mitotic arrest by blocking the ability of the G1 cyclin/cdk complexes to phosphorylate (inactivate) RB. As a consequence active (hypo-phosphorylated) RB promotes arrest of male germ cells in G1/G0. DND1 may facilitate male differentiation and cell cycle arrest by protecting target RNAs (including *p27^{KIP1}*) from the regulatory effects of micro RNAs. Regulators of pluripotency (OCT4, *Sox2 and Nanog)* are repressed, while male germ cell differentiation and epigenetic modifications are promoted.

2009, 2011). When *Dnd1* -/- mice were bred to an apoptosis deficient *Bax* -/- background these mice formed teratomas on a 129Sv/J - C57Bl/6 mixed background but not on a C57BL/6 background. Analysis of male germ cells in *Dnd1* -/- mice on a 129Sv/J background and *Dnd1* -/- *Bax* -/- mice on the 129Sv/J - C57Bl/6 background revealed that the male fetal germ cells in these mice do not express the cell cycle regulators $p27^{KIP1}$ or $p21^{CIP1}$ and subsequently did not enter mitotic arrest (Cook et al., 2011). These data support a model whereby translation of $p27^{KIP1}$ in male germ cells is partially controlled by DND1 (Western, 2009). It should be noted that in previous experiments, loss of $p27^{Kip1}$ alone did not cause teratoma formation in mice, however, this analysis was not carried out on the teratoma susceptible 129 background (Nakayama et al., 1996).

A notable process during male germ cell commitment is the repression of key components regulating pluripotency including OCT4, SOX2 and NANOG (Yamaguchi et al., 2005; Western et al., 2010). As mentioned previously, reactivation of pluripotency occurs in germ cell derived Type I and II testis cancers in humans (Oosterhuis and Looijenga, 2005). In common with this observation, analysis of *Dnd*-/- *Bax*-/- mice on the 129Sv/J and C57BL/6 backgrounds has demonstrated that pluripotency is specifically maintained during teratoma formation in mice. Germ cells in *Dnd*-/- mice on the C57Bl/6 background repress SOX2, NANOG and OCT4 and do not form teratomas (Cook et al., 2011). However, in *Dnd*-/- mice of a 129Sv/J background, germ cells maintain expression of SOX2, NANOG and OCT4 and form teratomas. Interestingly, germ cell specific deletion of the transcription factor *Dmrt1* on a 129Sv background also resulted in maintained expression of OCT4, SOX2 and NANOG within clusters of embryonal carcinoma cells that ultimately differentiate as teratomas (Krentz et al., 2009a). Interestingly, in E13.5 testes of *Dmrt* -/- mice the Ret proto-oncogene was down-regulated 8 fold, indicating an underlying role for RET in teratoma formation. Moreover, recent analysis of *Ret* -/- mice has revealed a role for RET in male germ cell survival on a C57/BL6 background (D. Miles and P. Western unpublished data). Although RET is most widely known for its ability to mediate signaling from the ligand GDNF, we found no evidence that GDNF is required for survival of male fetal germ cells (D. Miles and P. Western unpublished data).

Another mouse model in which signaling has been associated with teratoma formation is loss of the phosphatase and tensin homolog *Pten*-/-. These mice form teratomas from 0 days postpartum. This was thought to be partly due to hyper-activation of the thymoma viral proto-oncogene, AKT (Kimura et al., 2003). However, when mice that expressed a hyper-activated form of *Akt* in their germ cells were analysed, no teratoma formation was observed, although the fetal germ cells from these mice were able to establish pluripotent embryonic germ cell cultures at a greater developmental age than those from control animals (Kimura et al., 2008).

Although there is no broadly recognised animal model for Type II TGCTs, mice that over-express GDNF form GCTs later in life, and this model has been considered by some to be representative of Type II TGCTs (Meng et al., 2001). The GCTs in these mice share some characteristics with human TGCTs in that they are made up of cells that have a round gonocytic appearance, express germ cell markers and are aneuploid. However, the bilateral appearance of tumours and differences in the precursor lesions, which appear as clusters of cells in the mouse model, indicate that this model varies in some respects from the human condition (Sariola and Meng, 2003).

Conclusions

Recent work has provided greater understanding of how mouse germ cell differentiation and cell cycle are regulated. There are many important genes and processes that are conserved between the germ cell developmental programs of mouse and man. For example, fetal male germ cells enter a phase of mitotic arrest, pluripotency is repressed in the germ cells and a range of other developmentally important genes, such as the *Nanos* family members, DMRT1, DND1 and others are conserved in both species. Some analyses have shown that disruptions in the processes regulated by these genes lead to the retention of germ cell pluripotency and teratoma formation; advances that promise to provide insight into molecular mechanisms underlying germ cell tumour formation in humans. Further understanding of how male and female germ cell development is intertwined with that of the somatic cells of testis or ovary still requires examination. Advances in understanding the fundamental processes of male fetal germ cell differentiation, in particular, promises to provide important insight into germ line formation and testicular cancer.

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