Sex determination in mammalian germ cells: extrinsic versus intrinsic factors

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Abstract

Mammalian germ cells do not determine their sexual fate based on their XX or XY chromosomal constitution. Instead, sexual fate is dependent on the gonadal environment in which they develop. In a fetal testis, germ cells commit to the spermatogenic programme of development during fetal life, although they do not enter meiosis until puberty. In a fetal ovary, germ cells commit to oogenesis by entering prophase of meiosis I. Although it was believed previously that germ cells are pre-programmed to enter meiosis unless they are actively prevented from doing so, recent results indicate that meiosis is triggered by a signaling molecule, retinoic acid (RA). Meiosis is avoided in the fetal testis because a male-specifically expressed enzyme actively degrades RA during the critical time period. Additional extrinsic factors are likely to influence sexual fate of the germ cells, and in particular, we postulate that an additional male-specific fate-determining factor or factors is involved. The full complement of intrinsic factors that underlie the competence of gonadal germ cells to respond to RA and other extrinsic factors is yet to be defined.

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Introduction

Germ cells are the special cells of the embryo that ultimately give rise to the spermatozoa and oocytes. They are unique in their ability to undergo meiosis, a specialized form of cell division that allows them to halve their genetic material, producing haploid cells. Hence, germ cells are critical for the transfer of genetic material from one generation to the next.

A fundamental question in biology relates to how mammalian germ cell sexual fate is determined – how germ cells come to commit to either spermatogenesis or oogenesis. An answer may seem obvious to the non-specialist – surely XY germ cells will become sperm and XX germ cells will become oocytes. But in mammals, perhaps surprisingly, ‘the sex of a germ cell is not an autonomous property but is determined by the nature of the gonad in which it finds itself’ (Evans *et al.* 1977). In other words, whether a germ cell will ultimately form a sperm or an oocyte is not simply a matter of whether it is chromosomally XX or XY. Instead, it seems that the immediate somatic environment acts to push germ cells toward one or other fate (McLaren 1975, 1981, Palmer & Burgoyne 1991, Adams & McLaren 2002). So, germ cells of either XX or XY chromosomal constitution will take the first step toward oogenesis if they find themselves in the environment of a fetal ovary, while XX or XY germ cells will commit to spermatogenesis if they are placed in the fetal testis (reviewed by McLaren (2003)).

A commitment to oogenesis involves pre-meiotic DNA replication and entry into and progression through prophase of the first meiotic division during fetal life. On the other hand, a commitment to spermatogenesis involves avoidance of meiotic initiation during fetal life, mitotic arrest and the expression of certain cell fate markers that appear to lock in the male programme of development. Although we are making rapid progress in understanding mammalian germ cell sex determination, it is not clear to what extent germ cell sexual fate is governed by intrinsic rather than extrinsic factors. In particular, it is not clear whether both meiosis and mitotic arrest are actively triggered by the ovary and testis respectively, or whether one or both of these fates is pre-programmed and will ensue cell autonomously unless it is actively prevented. Current evidence suggests that, in this system, non-sex-specific intrinsic factors (such as transcription factors and epigenetic state) determine competence to respond to environment-specific extrinsic factors (such as growth factors and other signaling molecules).

Making, moving, and maturing the germ cells

In the mouse embryo, germ cells are specified from about 6.25 days post coitum (dpc); from about 8.5 dpc, they migrate through the embryo as it forms; and finally, they lodge in the gonad as it is forming at about

10.5 dpc. At the time of entering the gonads, germ cells are bipotential but over the next few days, under the influence of the gonadal environment, germ cells commit to one or other sexual fate. In this review, therefore, we discuss specification and migration of germ cells as a precursor trying to understand how these cells mature to the point of being competent to commit to one or other sexual fate.

Specification and transcriptional identity of the germ line

In many species, including *Drosophila melanogaster*, *Danio rerio*, and *Caenorhabditis elegans*, the germ cell lineage is set aside from the somatic lineage very early in development. This is achieved by a system where pre-formed germ line determinants in 'germ plasm' are inherited from the oocyte. In mammals including the mouse, the model system we focus on here, the germ cell lineage is not pre-determined but is induced by external signals at the egg cylinder stage of development, prior to the onset of gastrulation (Ginsburg *et al.* 1990, Lawson & Hage 1994, Tam & Zhou 1996). The main features of mammalian germ cell specification are summarized in Fig. 1. For reference, a list of genes involved in germ cell development is provided in Table 1. For a more comprehensive overview of the topic, see recent reviews (Surani *et al.* 2007, Saitou 2009b).

It is thought that bone morphogenetic protein (BMP) signals emanate from the extraembryonic ectoderm (BMP4 and BMP8B) and visceral endoderm (BMP2) as early as 5.5 dpc, and that based on a signaling gradient set up as a result, a region permissive for the formation of germ cell precursors arises in the proximal epiblast (Lawson *et al.* 1999, Ying *et al.* 2000, Ying & Zhao 2001, de Sousa Lopes *et al.* 2004). Competence to respond to the BMP signaling seems to exist only during the 5.5–6.25 dpc period and to be related to expression of *Wnt3* (Ohinata *et al.* 2009). From about 6.25 dpc the cells in this region express *Ifitm3*, a member of an interferon-inducible gene family and a key marker gene that has nonetheless proven non-essential during primordial germ cell (PGC) formation (Lange *et al.* 2008). These cells are not founder PGCs, since not all of their living descendants go on to become gametes. A subset of the *Ifitm3*-positive cells go on to express PRDM1 (also known as BLIMP1) and PRDM14, PR-domain proteins that are transcriptional repressors of a histone methyltransferase subfamily and are considered the key PGC determinants (Ohinata *et al.* 2005, Saitou *et al.* 2005, Vincent *et al.* 2005, Yamaji *et al.* 2008). Likely functions of PRDM1 in repression of the somatic genes, reactivation of potential pluripotency, induction of germ cell lineage genes, and control of epigenetic reprogramming have been reviewed (Saitou 2009a). PRDM14 does not appear to repress somatic

Figure 1 Specification of mouse PGCs. Extrinsic factors BMP4 and BMP8B (from the extraembryonic ectoderm, ExE) and BMP2 (from the visceral endoderm, VE) act on the epiblast to define a region permissive for the formation of PGC precursors. WNT3 appears to make epiblast cells competent to respond to BMPs. Intrinsic factor LIN28 is necessary for *Prdm1* expression, and PRDM1 and PRDM14 seem to repress expression of somatic genes and trigger reacquisition of potential pluripotency respectively. Ultimately, germ cell-specific chromatin modifications occur, and expression of definitive PGC marker genes *Dppa3* and *Alpl*, and PGC lineage-specific genes *Nanos3*, *Kit*, and *Dnd1* is induced.

genes but is necessary for the reactivation of the pluripotency gene *Sox2* and for successful epigenetic reprogramming (Yamaji *et al.* 2008). Expression of *Prdm1* requires the presence of the RNA-binding protein LIN28, which inhibits maturation of *Prdm1*-repressive microRNA (miRNA) let-7 family members (West *et al.* 2009). It is believed that PRDM1 represses the somatic mesodermal programme, plays a role in the induction of germ cell lineage genes, and triggers genome-wide epigenetic reprogramming. By 7.25 dpc, about 40 cells expressing *Dppa3* and *Alpl* are considered to represent the founder PGC population (Saitou *et al.* 2002, Hayashi *et al.* 2007). Despite their status as markers of the germ cell lineage, neither *Dppa3* nor *Alpl* are necessary for germ cell specification (MacGregor *et al.* 1995, Bortvin *et al.* 2004).

The pluripotency-associated genes *Pou5f1* and *Nanog* continue to be expressed in the PGC precursors and PGCs, but are downregulated in the surrounding somatic cells from about 6.75 dpc. Expression of the third member of the 'core pluripotency regulatory complex', *Sox2*, is detected in the PGC precursors only

Table 1 Symbols, names and common aliases for genes involved in mouse germ cell development and differentiation.

Gene symbol	Gene name	Common aliases
<i>Alpl</i>	Alkaline phosphatase, liver/bone/kidney	<i>Akp2</i> , <i>Tnap</i>
<i>Bax</i>	BCL2-associated X protein	
<i>Cdkn1b</i>	Cyclin-dependent kinase inhibitor 1B	<i>p27</i> , <i>p27Kip1</i>
<i>Cdkn2b</i>	Cyclin-dependent kinase inhibitor 2B	<i>p15</i> , <i>p15INK4b</i>
<i>Cyp26b1</i>	Cytochrome P450, family 26, subfamily b, polypeptide 1	<i>P450RAI-2</i>
<i>Dazl</i>	Deleted in azoospermia-like	<i>Dazla</i>
<i>Ddx4</i>	DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	<i>Mvh</i> (mouse homolog of vasa <i>Drosophila</i>)
<i>Dmc1</i>	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	<i>Dmc1h</i>
<i>Dnd1</i>	Dead end homolog 1, zebrafish	
<i>Dnmt3l</i>	DNA (cytosine-5-)-methyltransferase 3-like	
<i>Dppa3</i>	Developmental pluripotency-associated 3	<i>Stella</i> , <i>PGC7</i>
<i>Evx1</i>	Even-skipped homeotic gene 1 homolog	
<i>Fgf8</i>	Fibroblast growth factor 8	
<i>Fut4</i>	Fucosyltransferase 4	<i>Ssea-1</i> , stage specific mouse embryonic antigen
<i>Hoxb1</i>	Homeobox B1	
<i>Ifitm3</i>	Interferon-induced transmembrane protein 3	<i>fragilis</i>
<i>Kit</i>	Kit oncogene	<i>c-kit</i>
<i>Myc</i>	Myelocytomatosis oncogene	<i>c-myc</i>
<i>Nanog</i>	Nanog homeobox	
<i>Nanos3</i>	Nanos homolog 3, <i>Drosophila</i>	
<i>Pou5f1</i>	POU domain, class5, transcription factor 1	<i>Oct4</i> , <i>Oct3/4</i>
<i>Prdm1</i>	PR domain-containing 1, with ZNF domain	<i>Blimp1</i>
<i>Prdm14</i>	PR domain-containing 14	
<i>Rarb</i>	Retinoic acid receptor β	
<i>Rb1</i>	Retinoblastoma 1	<i>Rb</i>
<i>Snai1</i>	Snail homolog 1 (<i>Drosophila</i>)	<i>Snail</i>
<i>Sox2</i>	Sry-box containing gene 2	
<i>Sry</i>	Sex determining region of chromosome Y	
<i>Stra8</i>	Stimulated by retinoic acid gene 8	
<i>Sycp3</i>	Synaptonemal complex protein 3	<i>Scp3</i> , <i>Cor1</i>
<i>T</i>	Brachyury	
<i>Tdrd1</i>	Tudor domain-containing 1	
<i>Trp53</i>	Transformation-related protein 53	<i>p53</i>
<i>Trp63</i>	Transformation-related protein 63	<i>p63</i>
<i>Wnt3</i>	Wingless-related MMTV integration site 3	
<i>Wnt5a</i>	Wingless-related MMTV integration site 5A	

from about 6.25 dpc. The specific expression of *Sox2* at this time completes the pluripotency triad and may mark the time of re-acquisition of pluripotency in this lineage (Yabuta *et al.* 2006).

Germ line-specific markers *Nanos3* and *Dnd1* are first expressed in PGCs around 7.25 dpc (Yabuta *et al.* 2006). *Nanos3* encodes an evolutionarily conserved RNA-binding protein and is essential for germ cell survival due to a role in suppressing apoptosis (Tsuda *et al.* 2003, Suzuki & Saga 2008). *Dnd1* encodes the mouse ortholog of the zebrafish RNA-binding protein Dead end that acts to displace miRNAs from 3'-UTRs of mRNAs, thus preventing miRNA-mediated repression (Kedde *et al.* 2007). A natural mouse mutation of *Dnd1* (*Ter* mutation) exists: a single base change introduces a stop codon in the coding region of the gene (Stevens 1973, Youngren *et al.* 2005). Most *Ter* germ cells are removed by apoptosis during the migratory period, and DND1 seems to be involved in maintaining germ line specificity and preventing germ cells becoming tumorigenic (Youngren *et al.* 2005, Cook *et al.* 2009). Although Dead end is necessary for germ cell migration in fish (Weidinger *et al.* 2003), the few remaining

germ cells in *Ter* mice do reach the genital ridge (Youngren *et al.* 2005): this discrepancy suggests the possibility that the *Ter* mutant of *Dnd1* retains some function.

Kit, encoding the KIT tyrosine kinase receptor, is specifically upregulated in PGCs at 7.25 dpc (Yabuta *et al.* 2006). Homeobox genes are downregulated in PGC precursors starting with homeobox B1 (*Hoxb1*) at 6.75 dpc (Saitou *et al.* 2002). Posterior mesoderm markers *Evx1*, *T*, *Fgf8*, and *Wnt5a* as well as the epithelial to mesenchymal transition marker gene *Snai1* and the cell proliferation marker *Myc* are downregulated in PGCs from 7.25 dpc (Yabuta *et al.* 2006). Transcriptional profiling of genes involved in cellular signal transduction pathways indicates that PGCs are likely to decrease in sensitivity to BMP signaling and increase in sensitivity to transforming growth factor- β signaling between 7.25 and 7.75 dpc (Yabuta *et al.* 2006).

From 8.0 dpc, a transient loss of the maintenance DNA methyltransferase DNMT1 and the *de novo* methyltransferases DNMT3A and DNMT3B from germ cell nuclei occurs, followed by rapid erasure of

DNA methylation and reduction of repressive H3K9 dimethylation specifically in germ cells (Seki *et al.* 2005). At about 8.75 dpc, there is a large increase in repressive H3K27 trimethylation, a mark generally associated with pluripotent cell lineages and which in this case may be associated with reacquisition of totipotency (Seki *et al.* 2005).

An important point with respect to specification of the germ cells lineage is that no cells of the epiblast are pre-determined as PGC precursors. Cells from any part of the epiblast can be induced to give rise to PGCs if present in the correct location and at the correct time for signals to be received (Tam & Zhou 1996). Thus, all of the transcriptional and epigenetic changes necessary for the generation of the germ cell lineage are triggered by extrinsic signals from somatic cells.

Migration of the PGCs

By 7.5–8.5 dpc, the PGCs are proliferating rapidly and begin to migrate as a group from the base of the allantois, through the posterior primitive streak and into the developing hindgut. It is not clear how this mesoderm to endoderm migration is initiated; IFITM1, a member of the IFITM family of cell surface proteins was implicated (Tanaka *et al.* 2005); however, deletion of the entire IFITM gene family did not result in PGC migration defects (Lange *et al.* 2008). From about 10.5 dpc, the PGCs move into the midline dorsal body wall and then laterally into the genital ridges, the precursors of the gonads (Yeom *et al.* 1996, Molyneaux *et al.* 2001). It is believed that PGC migration is directed by somatic cell-produced chemoattractants including the chemokine stromal-derived factor 1 (SDF1; CXCL12; Ara *et al.* 2003) and the growth factor kit ligand KITL (SCF, steel factor; Mahakali Zama *et al.* 2005). Corresponding receptors CXCL4 and C-KIT (KIT oncoprotein) are expressed by the PGCs (Molyneaux *et al.* 2001, 2003, Stebler *et al.* 2004, Farini *et al.* 2007). The adherens junction component E-cadherin and β 1-integrin, expressed by PGCs as they migrate out of the hindgut, are necessary for their colonization of the genital ridges; E-cadherin is responsible for germ cell–germ cell interactions, while β 1-integrin is likely to facilitate interactions of germ cells with the extracellular matrix (Anderson *et al.* 1999, Bendel-Stenzel *et al.* 2000, Di Carlo & De Felici 2000). SDF1 and KITL are thought to act also as survival factors during migration (Wylie 1999, Molyneaux *et al.* 2003, Runyan *et al.* 2006). Since the same factors seem to be involved in directing migration and promoting survival, it has proved difficult to dissect the specific *in vivo* roles of the various factors involved. Fibroblast growth factor (FGF) and BMP signaling molecules are thought to be involved in migration, survival, and proliferation (Takeuchi *et al.* 2005, Dudley *et al.* 2007). A lipid-based system is thought to guide PGC migration in flies (reviewed by Richardson &

Lehmann (2010)), and there is evidence for a role of both isoprenoids and cholesterol in PGC migration in vertebrates (Thorpe *et al.* 2004, Ding *et al.* 2008).

It has been noted that pro-apoptotic genes are upregulated in germ cells during their migration (Runyan *et al.* 2006). Possibly germ cells at this stage are programmed to die in the absence of survival factors. Any PGCs that do not move in to the genital ridges but remain in the midline normally die by apoptosis, because midline somatic cells downregulate expression of KITL, which is presumably acting as a survival factor, between 9.5 and 10.5 dpc (Runyan *et al.* 2006). A small proportion of the migrating PGCs lodge in the nearby adrenal and are not removed by apoptosis, so presumably, their survival is supported in that environment.

During their migration, it is likely that PGCs are exposed to the signaling molecule retinoic acid (RA), which is widely produced in the embryo (Rossant *et al.* 1991). However, RA does not induce germ cells to enter meiosis during migration. It is likely that germ cells do not become competent to respond to RA until after their entry into the gonad, or into a similar tissue, such as the adrenal (as discussed below).

Bipotential germ cells in the genital ridges

The genital ridges develop along the medial surface of each of the mesonephroi, two temporary nephric organs that lie either side of the dorsal mesentery. Arising about 10.0 dpc, they are initially bipotential but, at about 10.5 dpc and under the influence of the Y-linked gene *Sry*, the XY genital ridges begin to develop as testes and, in the absence of *SRY*, the XX genital ridges begin to develop as ovaries (Koopman *et al.* 1991). *SRY* is expressed in cells of the pre-supporting lineage that then begins to differentiate into Sertoli cells; in the absence of appropriate expression of *SRY*, pre-supporting cells develop as granulosa cells. Once specified, Sertoli cells drive the male-specific development of other cell types of the testis (Palmer & Burgoyne 1991). Steroidogenic cells of the developing gonad are initially bipotential; in a testis they are triggered to develop as Leydig cells, while in the ovary they become theca cells. A male-specific migration of mesonephric cells, particularly endothelial cells, into the developing testis contributes to formation of the testis cords that eventually enclose the germ cells (Cool *et al.* 2008, Combes *et al.* 2009). Various observations indicate that the male and female somatic pathways of development are finely balanced in the mouse gonad and that, if *SRY* is not expressed early enough or at high enough levels, it is unable to overcome the default pathway of ovarian development (Eicher *et al.* 1982, Washburn *et al.* 2001, Albrecht *et al.* 2003, Bullejos & Koopman 2005, Wilhelm *et al.* 2009). It is into this dynamic environment of a bipotential primordial organ reorganizing as either a testis or an ovary that the germ cells arrive.

Changes in morphology and expression profile

As the germ cells enter the genital ridge, major morphological and biological changes are observed, irrespective of whether germ cells are XX or XY in chromosomal constitution. Post-migratory germ cells are less motile and less invasive when placed in culture, compared with migratory germ cells (Donovan *et al.* 1986). Moreover, germ cells isolated at 12.5 dpc from the gonads have lost the ability to migrate to the gonads when transplanted back into 10.5 dpc embryo slice cultures (Molyneaux *et al.* 2004). There is also some loss of potency associated with entry into the gonad; this is evidenced by that fact that it becomes progressively more difficult to generate embryonic germ cell (EGC) lines at this stage (Labosky *et al.* 1994, Tada *et al.* 1998).

There are also many changes in gene and protein expression as germ cells enter the gonad (Fig. 2). In particular, the germ cell nuclear antigen GCNA1 is first expressed at this time (Enders & May 1994). Two key germ cell lineage genes that are expressed only after entry into the gonadal ridge are *Ddx4* and *Dazl*. *Ddx4* encodes a PGC-specific RNA helicase (Tanaka *et al.* 2000, Toyooka *et al.* 2000), while *Dazl* encodes a cytoplasmic RNA-binding protein (Seligman & Page 1998, Lin & Page 2005). GCNA1, DAZL and DDX4 are discussed in more detail below. In contrast, some PGC

markers, including *Fut4* (*Ssea-1*), *Akpl* (*Tnap*), *Kit*, and *Prdm1*, decline or disappear upon entry into the gonad (Maatouk & Resnick 2003).

Sycp3 and *Dmc1*, genes associated with meiosis, are slightly upregulated in XX and XY germ cells upon entry into the gonad, and some SYCP3 protein aggregates are seen in XY as well as in XX germ cells at 13.5 dpc (Di Carlo *et al.* 2000, Chuma & Nakatsuji 2001). These observations suggest that germ cells newly resident in the gonad 'prime' for a possible entry into meiosis. Although this has been interpreted as meaning that germ cells are at this stage on the brink of a cell-autonomous entry into meiosis timed by an internal clock mechanism (Donovan *et al.* 1986, Ohkubo *et al.* 1996, McLaren & Southee 1997), sex-independent signals from the gonadal somatic cells – or similar somatic cells in ectopic locations such as the adrenal (Upadhyay & Zamboni 1982) – may trigger these expression changes.

Global demethylation and chromatin remodeling

Upon entry into the gonad, germ cells also undergo remarkable changes in genome methylation status and chromatin architecture. Some of these changes are persistent, while others are transient. Before entry into the gonads, germ cells are already hypomethylated when compared with somatic cells (Seki *et al.* 2005), but upon entry, they undergo the most widespread and complete demethylation event known during development. Both XX and XY germ cells undergo genome-wide erasure of existing methylation at imprinted and non-imprinted single copy sequences and some repetitive elements (Monk *et al.* 1987, Szabo & Mann 1995, Hajkova *et al.* 2002, Lee *et al.* 2002, Sato *et al.* 2003). The demethylation is rapid and probably active rather than passive. Moreover, the effect is persistent as remethylation does not occur until later during fetal life when XY germ cells have committed to the spermatogenic fate or, for each XX germ cell, just prior to ovulation. Reactivation of the inactive X chromosome in XX germ cells occurs upon entering the genital ridge (Monk & McLaren 1981, Tam *et al.* 1994), although it has recently been reported that this process may begin earlier, at 7.0 dpc in nascent PGCs (Sugimoto & Abe 2007).

Probably in response to these massive changes in methylation, major changes in chromatin architecture are also observed. At 11.5 dpc, germ cell nuclei swell and lose chromocentres, suggesting that the gonadal germ cells have taken on a looser chromatin organization. Heterochromatin relocates toward the nuclear periphery, and some repressive histone modifications (such as H3K27me3 and H3K9me3) are lost, while some marks associated with permissive or active chromatin (H3K4me and H3K9ac) are gained. These changes are likely to correspond to the distinctive 'pre-leptotene' morphology that has been described for XX and XY germ cells resident in the gonad and at the completion of

Figure 2 Intrinsic changes observed in germ cells upon colonization of the gonad. When germ cells enter the gonadal environment (at about 10.5–11.5 dpc), PGC markers such as *FUT4*, *ALPL*, *KIT*, and *PRDM1* are lost or downregulated, while germ cell markers *GCNA1*, *DDX4*, and *DAZL* are expressed for the first time. Germ cells lose migratory characteristics and genome-wide demethylation, and transient chromatin loosening is observed. Histone variants H1 and H2A.Z are lost. It is not known whether these changes are induced by signals from the gonadal soma or whether they arise cell autonomously.

their final mitotic division (Hilscher *et al.* 1974, McLaren 1984, 2003, McLaren & Southee 1997). Remarkably, these changes are transient, and are no longer observed at 12.5 dpc (Seki *et al.* 2005, Hajkova *et al.* 2008). This temporary alteration suggests the intriguing possibility that a window of competence exists at this critical time in germ cell biology. Chromatin modifications that persist in the germ cells are also observed, including the loss of histone variants H1 and H2A.Z (Hajkova *et al.* 2008). It is likely that these changes are triggered by signals from gonadal somatic cells, but the nature of such signals is not yet known, and it remains possible that the changes arise cell autonomously.

Sex determination in the germ line

Upon entry into the genital ridges, the bipotential germ cells still continue to divide mitotically for 2–3 days. At about 12.5–13.5 dpc, germ cells in a developing ovary cease mitosis and enter prophase of the first meiotic division, thereby committing to the female programme of development. They progress through the various stages of leptotema, zygotema, pachytoma, and diplotema until they reach dictyate arrest at around the time of birth (Monk & McLaren 1981, Speed 1982). The oocytes remain in this arrested state until just before ovulation when they complete the first meiotic division, begin the second, and arrest again. Meiosis is completed only after fertilization.

Germ cells in a developing testis do not enter meiosis during fetal life but instead arrest in G₀/G₁ phase of the cell cycle at about 12.5–13.5 dpc (Hilscher *et al.* 1974, McLaren 1984). The germ cells (or ‘gonocytes’) remain in this quiescent state until after birth when they resume proliferation, and some migrate to the peripheral basement membrane of the seminiferous tubules of the testis. They transform into germ line stem cells (spermatogonial stem cells) that can both self-renew and produce daughter cells that differentiate into spermatozoa. Daughter spermatogonia enter meiosis beginning at around 5 days post natum and complete both meiotic divisions to produce haploid cells known as spermatids. Spermatids differentiate and mature further to eventually produce functional gametes, the spermatozoa.

Sex determination in the germ line, therefore, comes down to the choice between entering meiosis, and thereby locking in a female fate, versus avoiding meiosis, entering mitotic arrest and locking in a male fate.

Choosing meiosis: do germ cells jump or are they pushed?

Although it is well accepted that the fetal testis produces a factor or factors to ensure that germ cells do not enter meiosis, there has been considerable uncertainty as to whether germ cells spontaneously enter meiotic prophase or whether they are induced to do so by the

surrounding tissue. Over several decades, evidence supporting both propositions has been presented (Bowles & Koopman 2007). Briefly, active induction of meiosis is supported by evidence that an influence from the adjacent mesonephric tissue is necessary (Byskov 1974, Byskov & Saxen 1976) and also by recent studies implicating the signaling molecule RA, discussed below. Evidence that germ cells are not triggered to enter meiosis, but that they do so cell autonomously, includes the observation that ectopic germ cells in the adrenal tend to enter meiosis (Upadhyay & Zamboni 1982, Zamboni & Upadhyay 1983, Francavilla & Zamboni 1985, Runyan *et al.* 2008), as do isolated germ cells in culture (Chuma & Nakatsuji 2001, Farini *et al.* 2005) or in co-culture with embryonic lung cells (McLaren & Southee 1997).

It is now clear that meiosis is induced. In particular, it appears that the signaling molecule RA is responsible for inducing meiosis in the developing mouse ovary and that meiosis in the developing mouse testis is avoided at least largely because of the RA-degrading action of a P450 enzyme, CYP26B1 (Baltus *et al.* 2006, Bowles *et al.* 2006, Koubova *et al.* 2006, MacLean *et al.* 2007, Anderson *et al.* 2008). *Cyp26b1* is initially expressed in developing mouse gonads of both sexes, but is then greatly upregulated in the testis and downregulated in the ovary (Menke & Page 2002, Bowles *et al.* 2006, Koubova *et al.* 2006). Treatment of fetal testes with RA (or RA agonists) in culture induces expression of the pre-meiotic marker *Stra8* (Bowles *et al.* 2006, Koubova *et al.* 2006, Trautmann *et al.* 2008), and upregulation of meiotic markers *Sycp3* and *Dmc1* (Bowles *et al.* 2006). Moreover, in two independent *Cyp26b1* knockout (KO) mouse models, XY embryos form an apparently normal testis, but RA levels are raised, the pre-meiotic marker *Stra8* and the meiotic marker *Sycp3* are greatly upregulated, and XY germ cells enter and progress through meiosis (Bowles *et al.* 2006, MacLean *et al.* 2007). Hence, if RA that is endogenously present in the developing gonad is not degraded, XY germ cells in a testis are induced to enter meiosis. These meiotic XY germ cells undergo apoptosis by birth, as would be expected given evidence that the testis somatic environment is hostile to meiotic germ cells (O & Baker 1976, Vigier *et al.* 1987). These results indicate that CYP26B1 is a male-specific meiosis-preventing factor.

Although XY germ cells enter meiosis very convincingly in the *Cyp26b1* KO, it is possible that other factors, in addition to the absence of RA, contribute to the avoidance of meiosis in the testis. One clue that this may be the case comes from mouse gonad *ex vivo* organ culture studies, where it is reported that some XY germ cells, located at a particular end of the gonad, do enter meiosis if all cellular secretion is blocked (Best *et al.* 2008). This result would suggest that testicular somatic cells secrete a factor that at least helps germ cells avoid entry into meiosis. Another clue comes from very recent

work showing that epigenetic modification, particularly histone deacetylation, may play some role in the avoidance of entry into meiosis (Wang & Tilly 2010).

So RA can trigger germ cells, including those resident in a developing testis, to enter meiosis, but is RA required for germ cells to enter meiosis? In *ex vivo* culture studies, chemical inhibition of RA receptor (RAR) function in embryonic ovaries leads to inhibition of expression of *Stra8* and of entry into meiosis (Bowles *et al.* 2006, Koubova *et al.* 2006). A recent study using a variation of the vitamin A-deficient (VAD) rat model (late VAD) is enlightening on this point also. RA is a metabolite of vitamin A, so in VAD rats, endogenous levels of RA would be expected to be low. In 'late VAD' fetal ovaries, germ cells form and migrate, and gonadal numbers are normal; however, most fail to enter meiosis and remain in an undifferentiated state (Li & Clagett-Dame 2009). This study therefore complements the mouse KO studies (Bowles *et al.* 2006, MacLean *et al.* 2007); the mouse work demonstrated that RA alone can induce meiosis in competent germ cells, while the rat study demonstrates that, *in vivo*, vitamin A (the precursor of RA) is required for the normal onset of meiosis in ovarian germ cells.

The key role of STRA8 as a gatekeeper for meiosis initiation was demonstrated by analysis of *Stra8* KO mice. In XX *Stra8* KO mice, there is no evidence whatsoever that germ cells have entered meiosis since there are no leptotene, zygotene, or pachytene chromosomal configurations and no REC8 or SYCP3 protein loaded onto chromosomes (Baltus *et al.* 2006). It seems that, in the male mouse, postnatal entry into meiosis also requires RA and *Stra8* expression (Baltus *et al.* 2006, Anderson *et al.* 2008), although some contention remains (Mark *et al.* 2008). It is reported that, in *Stra8*-deficient males, spermatogenesis ceases at the preleptotene/leptotene spermatocyte stage, and that cells then undergo apoptosis (Baltus *et al.* 2006, Anderson *et al.* 2008). In another study, in which the mutation was studied on a mixed background, it was reported that there is pre-meiotic DNA replication, partial condensation of chromosomes, and initiation of meiotic recombination, although there is no progress through the leptotene stage of prophase I (Mark *et al.* 2008). Evidence that both RA and STRA8 are involved in initiating meiosis in both sexes suggests that the molecular control of pre-meiosis S-phase entry is conserved and gender-neutral.

How does RA work its magic?

RA is a key molecule in developmental biology, regulating various events in cellular growth and differentiation (Duester 2008, Mark *et al.* 2009). In particular, RA is a known differentiation factor, particularly with respect to stem cells (Soprano *et al.* 2007). Although RA is generally widespread in the embryo during development, levels of RA are strictly regulated

in a spatiotemporal manner by the balanced actions of synthesis and catabolism enzymes. RA acts through RARs, of which there are many, to modify gene expression in a target cell. It is true that RAR KO mice do not show any defects in ovarian histology, but this is likely to be due to compensation by remaining RARs (Mark *et al.* 2009). The idea of likely redundancy correlates well with the finding that agonists of each of the three RARs (α , β , and γ) induce *Stra8* expression in cultured fetal testes (Koubova *et al.* 2006).

In the context of mouse germ cells, RA has previously been shown to act as a potent proliferation factor (Koshimizu *et al.* 1995, Morita & Tilly 1999). In the mouse gonad, RA seems to act by inducing expression of *Stra8* (Bowles *et al.* 2006, Koubova *et al.* 2006), a gene considered to be a pre-meiotic marker (Menke *et al.* 2003), although this point needs to be explored further. Since germ cells in the gonad express RARs (Dolle *et al.* 1990, Cupp *et al.* 1999, Bowles *et al.* 2006) and the *Stra8* promoter has two putative RA-response element sequences (Koubova *et al.* 2006), RA may act directly on germ cells to bring about *Stra8* expression. *Stra8* was first identified as a RA-responsive gene in P19 embryonic carcinoma (EC) cells (Bouillet *et al.* 1995). Like *Rarb* (a classical RA-response gene), *Stra8* is considered a type II immediate early response gene. The STRA8 protein was originally described as cytoplasmic in P19 EC cells (Oulad-Abdelghani *et al.* 1996), but recent evidence suggests that it shuttles between nucleus and cytoplasm, and may bind DNA (Tedesco *et al.* 2009). Differentially phosphorylated forms have been demonstrated but any significance of these has not been shown (Oulad-Abdelghani *et al.* 1996). The STRA8 protein has little homology with known proteins making elucidation of its mechanism of action difficult.

It is believed that RA flows or diffuses into the genital ridges from the adjacent mesonephric tubules (also known as the extraovarian rete; Bowles *et al.* 2006). In accord with this theory, the first known molecular evidence that XX and XY germ cells are diverging in their developmental paths is the expression of the pre-meiotic marker *Stra8* in germ cells resident in the anterior end of the ovary, beginning at about 12.5 dpc (Menke *et al.* 2003, Koubova *et al.* 2006). In parallel with an anterior-to-posterior wave of *Stra8* expression, anterior-to-posterior waves of meiotic marker (*Sycp3*, *Dmc1*, *H2AX*, and *SYN/COR*) upregulation and *Pou5f1* downregulation have been described (Menke *et al.* 2003, Yao *et al.* 2003, Bullejos & Koopman 2004). Interestingly, at about 14.5 dpc, a small population of meiotic germ cells are present transiently at the anterior end of the testis in the gonad/mesonephros junction area (McLaren 1984, Yao *et al.* 2003). These are removed by apoptosis by 15.5 dpc, but presumably they arise because there is some exposure to RA in this area. Although these observations tie RA presence and *Stra8* expression to the initiation of meiosis, we still do not know how STRA8

engages the meiotic machinery. The results of various studies indicate that STRA8 is necessary but not sufficient to push cells into meiosis, so presumably STRA8 protein works to induce meiosis only in the context of cells in a competent state (as discussed below).

Stra8 is found only in vertebrates (Baltus *et al.* 2006), so any RA-triggered meiosis is likely to be confined at least to that group. Evidence is mounting that RA may trigger entry into meiosis not just in mouse and rat, as detailed above, but also in chickens (Smith *et al.* 2008) and amphibians (Wallacides *et al.* 2009). Although a role for RA in initiating meiosis in humans has not been demonstrated so far, an anterior site of meiosis initiation – originating near the rete ovarii which connects the gonad and mesonephros – is observed in many mammalian species including humans (Byskov 1975, Bendsen *et al.* 2006).

Choosing meiosis in an ectopic context

One of the main pieces of evidence that has been used to support the theory that germ cells are endogenously programmed to enter meiosis, and do not need to be triggered, comes from examination of rare ectopic germ cells in the mouse. Germ cells that do not colonize the gonads, but instead lodge in the nearby adrenal glands or mesonephros, tend to enter meiosis at much the same time as those in the developing ovary (Upadhyay & Zamboni 1982, Zamboni & Upadhyay 1983, McLaren 1984, Francavilla & Zamboni 1985), although some in the mesonephros developed as T-spermatogonia (McLaren 1984).

A recent study, using *Bax*-null mice (in which apoptosis is suppressed to some extent), confirmed that germ cells in the adrenal gland do indeed enter meiosis (Runyan *et al.* 2008). Specifically, these germ cells downregulate early germ cell markers (FUT4 and POU5F1), begin to express DDX4, and upregulate the meiotic marker SYCP3. The newer study shows also that ectopic germ cells in other locations are heterogeneous in their propensity to enter meiosis or to remain undifferentiated (Runyan *et al.* 2008). In general, other than in the adrenal and peri-gonadal locations, POU5F1 and FUT4 remain on longer in ectopic germ cells than those resident in the fetal ovary, and activation of DDX4 and SYCP3 is delayed. Germ cells that migrated to more cranial locations in the embryo seemed more likely to enter meiosis. A population that seems to be spared from entry into meiosis was located in the tail region where the gene encoding CYP26A1 is expressed; hence, it may be that those germ cells do not enter meiosis because of the absence of RA in that area (Runyan *et al.* 2008).

Whether ectopic germ cells, *in vivo*, enter meiosis or not seems to correlate with whether they do or do not 'see' the inducing factor RA. They seem particularly prone to enter meiosis if they colonize the adrenal cortex where RA is known to be present (Haselbeck *et al.* 1997,

Haselbeck & Duester 1998). This suggests that germ cells at this time, whether or not they have entered the gonad, are competent not only to respond to RA by upregulating *Stra8*, but also to enter meiosis. Overall, the heterogeneity observed among ectopic germ cells studied *in vivo* confirms that germ cell sexual fate is very much dependent on signals from the soma. The status of ectopic germ cells is presumably dictated by the combination of all extrinsic signals encountered in a particular location. One such signal is RA, but it is possible that the other as yet unidentified signaling molecules also have some bearing on germ cell differentiation.

PGCs and germ cells, isolated from mesenteries or gonadal ridges respectively, can enter meiosis when cultured *in vitro* (Chuma & Nakatsuji 2001, Farini *et al.* 2005). In these experiments, cultures were carried out using feeder cells, serum-containing media, and additives necessary to maintain proliferation and to prevent apoptosis. It is possible that RA, and indeed other factors that were present in the system, influenced development and differentiation of the cells. We find that as little as 1×10^{-8} M RA is sufficient to trigger isolated 11.5 dpc germ cells to express *Stra8* (J Bowles & P Koopman, unpublished data), while media supplemented with 10% fetal bovine serum has been estimated to contain 3.6×10^{-8} M RA (Fuchs & Green 1981).

XY germ cell meiosis was also observed when XY urogenital ridges were dissociated and reassociated, leading to a theory that the meiosis-preventing factor is active only in an intact gonadal structure (McLaren & Southey 1997). The interpretation of this classic experiment is confounded now that the role of RA is known, since RA-producing mesonephric cells would have been included in the reaggregation cultures. Similarly, dissociation and reaggregation of germ cells with lung tissue was found to allow germ cells to progress into meiosis (McLaren & Southey 1997) – this may reflect the fact that RA is present in embryonic lung tissue (Malpel *et al.* 2000).

If not meiosis, then what?

Germ cells in the developing testis do not enter meiosis but instead stop proliferating, enter G_0/G_1 arrest, and commit to the spermatogenic pathway (Hilscher *et al.* 1974, McLaren 1984, Adams & McLaren 2002, Western *et al.* 2008). It is not clear whether the events of mitotic arrest and commitment to spermatogenesis occur cell autonomously in the absence of entry into meiosis, or whether they are triggered by extrinsic signals from the testicular soma.

It has been postulated that cessation of proliferation results from a cell-intrinsic programme regulated stochastically (McLaren 1984, Ohkubo *et al.* 1996). More recent studies suggest that the absence of RA is important (Trautmann *et al.* 2008), and this makes

sense considering that RA appears to have a potent mitotic effect on germ cells (Koshimizu *et al.* 1995). This conclusion was reached by studies of testes at 13.5 dpc or later, when the resident XY germ cells are no longer susceptible to the meiosis-inducing effects of RA. It was shown that if 13.5 dpc XY germ cells in a testis are treated with RA, they upregulate *Stra8*, but they do not enter meiosis, presumably because the window of opportunity has closed. Instead, they avoid mitotic arrest, the effect working through a phosphatidylinositol 3-kinase signaling mechanism. RA also delayed extinction of FUT4 and remethylation of the genome, suggesting that both mitotic arrest and commitment to the male programme of development had been prevented by the presence of RA (Trautmann *et al.* 2008). This study therefore suggests that mitotic arrest and male fate selection both follow from the degradation of RA by CYP26B1.

Other studies suggest that it is not simply the absence of meiosis or the absence of RA that leads to mitotic arrest and commitment to the male programme. Early studies demonstrated that germ cells in a fetal ovary do not enter meiosis if the mesonephros is removed, but instead become atretic and disappear (Byskov 1974, O & Baker 1976). In the light of our understanding of the role of RA produced by the mesonephric duct and tubules, this observation suggests that meiosis fails to occur because the source of RA has been removed. Such XX germ cells do not follow the spermatogenic path, however, suggesting that the male fate choice does not occur by default if meiosis is avoided and therefore that it must be triggered by the testis soma. The observation that XY germ cells seem to be irreversibly committed by the male pathway by 12.5 dpc, while XX germ cells are not committed to the female pathway until 13.5 dpc (McLaren & Southee 1997, Chuma & Nakatsuji 2001, Adams & McLaren 2002) is also in accordance with the theory that XY germ cells are actively triggered to commit to the male pathway and that it is not simply their failure to enter meiosis that ensures their spermatogenic fate. The testicular factor or factors responsible for positively inducing male germ cell fate have not been identified.

Intrinsic changes in germ cells in the testis environment

Although we do not know what triggers germ cells in a testis to stop proliferating and commit to spermatogenesis, we do have some knowledge of the molecular changes that occur in germ cells at the time of these events. When XY germ cells in a testis arrest, there are transcriptional changes in some genes encoding cell cycle regulators (Western *et al.* 2008) as well as an upregulation of transcription of the γ isoform of *Trp63*, a relative of *Trp53* (Petre-Lazar *et al.* 2007). In addition, the P27 protein appears as germ cells enter mitotic quiescence (Western *et al.* 2008). RB1 is a major cell

cycle regulator, and it has recently been demonstrated that the active non-phosphorylated form is present in the fetal testis, whereas only the inactive phosphorylated form is present in the fetal ovary (Western *et al.* 2008, Spiller *et al.* 2010). Complete absence of the RB1 protein is associated with a modest delay in mitotic arrest at 14.5 dpc, but this is resolved by 16.5 dpc (Spiller *et al.* 2010). The concomitant upregulation of genes encoding cell cycle inhibitors *Cdkn1b* and *Cdkn2b* in the *Rb1*-null testes suggests that the testis is able to compensate for the absence of this key cell cycle regulator, and, therefore, that cell cycle arrest in the XY germ cells is likely to involve redundant mechanisms (Spiller *et al.* 2010).

In addition to stopping their proliferation, XY germ cells commit to the male programme of development during fetal life. A marker of undifferentiated germ cells, FUT4, is lost. *Nanos2*, which encodes an RNA-binding protein, seems to lock in spermatogenic fate since in its absence XY germ cells are still susceptible to the meiosis-inducing effects of RA as late as 15.5 dpc (Suzuki & Saga 2008). *Nanos2* is male-specifically expressed in germ cells beginning at about 13.5 dpc (Tsuda *et al.* 2003). *Nanos2*-null XY germ cells undergo mitotic arrest normally at 14.5 dpc but revert to proliferation at 15.5 dpc. Moreover, *Nanos2*-null XY germ cells initiate meiosis at 15.5 dpc before being rapidly removed by apoptosis (Suzuki & Saga 2008). If apoptosis is prevented (by ablation of the intrinsic pro-apoptotic factor BAX), XY germ cells start to express low levels of *Stra8* at around 14.5 dpc (2 days later than *Stra8* expression is first observed in the developing ovary; Menke *et al.* 2003, Suzuki & Saga 2008). Presumably these meiotic germ cells are responding to increasing levels of RA because *Cyp26b1* expression falls at this stage of development (Bowles *et al.* 2006, Koubova *et al.* 2006). Overexpression of NANOS2 in XX germ cells in a developing ovary induces markers of male germ cell fate including *Dnmt3l* and *Tdrd1*. DNMT3L seems to act as a general activator of *de novo* methyltransferases and is involved in progressive remethylation of imprinted sequences in the fetal prospermatogonia from 14.5 dpc (Bourc'his *et al.* 2001, Bourc'his & Bestor 2004, Sakai *et al.* 2004, Kato *et al.* 2007). TDRD1 is a component of nuage/germinal granules, an evolutionarily conserved cytoplasmic structure in the germ line, in prospermatogonia (Chuma *et al.* 2003, 2006).

What makes a 'meiosis-competent' germ cell?

The evidence, overall, suggests that germ cells in the gonad are induced to enter meiosis by RA and also, possibly, inhibited from doing so by one or more as yet unidentified testis-specific factors. However, it is obvious that the mere presence of RA is not sufficient to instruct a cell to embark on meiotic division. RA is widespread in the embryo and is involved in many differentiation events during development (Mark *et al.* 2009), but only

germ cells at particular times and in particular locations enter meiosis. Specifically, such germ cells would need to be competent to respond to RA by upregulating *Stra8*, and they would need to be competent to allow STRA8 to trigger pre-meiotic replication. Presumably, such cells have an intrinsic competence, marked by a set of proteins that are yet to be identified and endowed on them because, first, they are germ cells and, secondly, they have been influenced by extrinsic factors as they traveled through the embryo and entered the environment of the gonad. Extrinsic factors that induce meiotic competence and intrinsic molecular characteristics that define meiotic competence are not yet known, although some clues can be pieced together, as discussed below.

It is likely that epigenetic factors play a major role in the establishment of germ cell competence. A limited range of cell types, including non-germ cells, is able to respond to RA by upregulating *Stra8*, albeit in artificial culture conditions. Known responders are stem cells, including embryonic stem cells (ESCs), EGCs (Oulad-Abdelghani *et al.* 1996), and embryonal carcinoma cells (Bouillet *et al.* 1995), all of which would be expected to be expressing the core pluripotency transcription factors POU5F1, SOX2, and NANOG. It is likely that competence to respond to RA with the expression of *Stra8* relates to a particular epigenetic state or chromatin conformation. Stem cells tend to have an 'open' or accessible chromatin conformation, unlike lineage-specific somatic cells (Niwa 2007). The loose open nuclear architecture noted in germ cells shortly after their arrival in the gonad may prove significant in terms of meiotic competence. Intriguingly, these chromatin changes are transient and most obvious at 11.5 dpc (Seki *et al.* 2005, Hajkova *et al.* 2008), precisely the time when germ cells would be subject to sex-specific cues from the somatic cells of the developing ovary or testis.

Given this apparent correlation between 'open conformation' pluripotent cell types and the ability to respond to RA with the upregulation of *Stra8*, it is not surprising that treatment of gonads with the potent histone deacetylase inhibitor, trichostatin A (TSA), leads to an increased propensity to express *Stra8* (Wang & Tilly 2010). TSA alters the epigenetic status of a cell directly, increasing histone acetylation and thus making regulatory regions more 'open' or accessible to transcription factors (Toth *et al.* 2004, Durcova-Hills *et al.* 2008). More specifically, treatment of gonadal germ cells with TSA has been shown to cause rapid downregulation of *Prdm1* (the repressor of an overt pluripotency phenotype in germ cells) and to trigger germ cells to dedifferentiate or reprogramme into EGCs (Ancelin *et al.* 2006, Durcova-Hills *et al.* 2008).

So, in general, cells with an 'open' chromatin configuration express *Stra8* when subjected to RA. However, most such cells do not subsequently embark on meiosis. During fetal life, meiotic competence

appears to be limited to XX and XY germ cells during the period shortly after their arrival in the gonad. The period of competence begins earlier than is obvious in nature, a fact revealed when the RA-degrading enzyme CYP26B1 is ablated (Bowles *et al.* 2006). The end of the period of competence is difficult to ascertain based on published work. Some studies have indicated that by 12.5 dpc, XY germ cells have already become less able to enter meiosis, presumably because they have already received male fate instructions from somatic cells (McLaren & Southee 1997, Chuma & Nakatsuji 2001, Adams & McLaren 2002). Consistent with this, a more recent work demonstrated that the period of competence must be closed by 13.5 dpc at the latest, because at that time XY germ cells are able to respond to RA by upregulating *Stra8* but are not able to enter meiosis (Trautmann *et al.* 2008). On the other hand, XY germ cells that upregulate *Stra8* around 14.5 dpc, in the NANOS2-null; BAX-null background, do enter meiosis (Suzuki & Saga 2008). Possibly this relatively late entry into meiosis, at 14.5 dpc, indicates that under normal circumstances NANOS2 acts to close the period of competence by committing germ cells to the spermatogenic pathway. A very recent study suggests that XY germ cells remain meiosis-competent up to at least 16.5 dpc (Li *et al.* 2009). Apparent discrepancies with respect to the end of the period of meiotic competence are likely to reflect the fact that germ cells do not tend to act in concert and processes such as meiosis entry and mitotic arrest occur over several days. Hence, a sub-population of XY germ cells may be refractory to meiosis induction by 12.5 dpc, but a different sub-population may remain competent for several days after 12.5 dpc.

Intrinsic factors that underlie meiotic competence are yet to be defined, but some candidates have emerged in recent years. Germ cells normally upregulate *Ddx4* only after reaching the gonads, and it is not known what triggers this change in expression (Tanaka *et al.* 2000, Toyooka *et al.* 2000). DDX4 continues to be expressed throughout embryogenesis in germ cells of the fetal testis and ovary and in spermatocytes, postmeiotic spermatids, and primary oocytes postnatally (Fujiwara *et al.* 1994, Tanaka *et al.* 2000, Toyooka *et al.* 2000). DDX4 is not expressed by pluripotent ES, EG, or inner cell mass (ICM) cells (Fujiwara *et al.* 1994), but its expression can be induced when mouse EGCs (mEGCs; but not mESCs) are cultured together with fetal gonadal cells (Toyooka *et al.* 2000). These results suggest that *Ddx4* expression is induced when germ cells come into contact with gonadal somatic cells, and that ESCs are less competent to respond than EGCs. Interestingly, germ cells in the adrenal not only enter meiosis but also express *Ddx4* (Runyan *et al.* 2008). It may be that those germ cells are encountering competence-inducing signals that are the same or similar to signals produced by fetal gonadal cells. It is not likely, however, that expression of *Ddx4* alone ensures competence to enter meiosis since

Figure 3 Key intrinsic and extrinsic factors involved in sex determination in murine fetal germ cells. Upon entry into the gonad, germ cells express GCNA1, DDX4, and DAZL, undergo epigenetic modifications, and are presumably competent to enter meiosis. In the presence of RA, produced by somatic cells, germ cells begin to express *Stra8* at about 12.5 dpc and go on to initiate pre-meiotic DNA replication. Ultimately, meiotic marker genes are upregulated, and the germ cells enter prophase I of meiosis thereby committing to the female fate. If RA is degraded by CYP26B1, germ cells do not express *Stra8*. Mitotic arrest, possibly due to the absence of RA, begins in some germ cells as early as 12.5 dpc, although this is not synchronous in the population. A secreted testis-specific factor or factors, as yet unidentified, are likely to signal to the germ cells. At about 13.5 dpc, germ cells in the testicular environment begin to express *Nanos2*. By 14.5 dpc, quiescent spermatogonia, in G₀/G₁ mitotic arrest, are observed. These spermatogonia express markers of male fate such as *Dnmt3l* and *Tdrd1*. Timing of various processes is approximate.

ablation of *Ddx4* does not affect normal oogenesis. In the male, DDX4 is not required for entry into meiosis but is required for the continued proliferation and differentiation of germ cells in the testis (Tanaka *et al.* 2000).

Another possible intrinsic competence factor is the germ cell antigen GCNA1 which, like DDX4, is expressed as germ cells and enter the gonad (Enders & May 1994). Since this marker is also observed when 8.5 dpc migratory PGCs are placed in culture (Richards *et al.* 1999), in germ cells located in the adrenal at 14.5 dpc, and in SF1 mutant embryos (in which gonad formation is aborted; Wang *et al.* 1997), it is possible that GCNA1 expression reflects a 'cell-intrinsic programme' of development rather than being an induced event. It is also possible, however, that the necessary inducing signals are present in cell culture media and in the adrenal and peri-gonadal environments. Interestingly, both pre-migratory (8.5 dpc) and post-migratory germ cells (10.5 dpc) expressed GCNA1 when treated in culture with the demethylation agent, 5-azacytidine, or the histone deacetylase inhibitor, trichostatin A

(Maatouk & Resnick 2003). Possibly GCNA1 expression upon entry into the gonad is related to the significant changes in demethylation and chromatin structure that occur at that time. No GCNA1 KO is available since the gene encoding this antigen is not yet known.

Another gene expressed by XX and XY germ cells shortly after their arrival in the genital ridge is *Dazl*, which encodes a cytoplasmic RNA-binding protein (Ruggiu *et al.* 1997, Seligman & Page 1998, Lin & Page 2005). Recent results suggest that DAZL may be a 'meiosis competence' factor (Lin *et al.* 2008). In the *Dazl* KO, on a C57BL/6 background, RA is unable to trigger upregulation of *Stra8*, increased *Sycp3* transcription and translation are not observed, and the double strand breaks characteristic of meiosis do not form. The strict requirement for DAZL in 'meiosis-competent' germ cells is presumably an oversimplification since in the complete absence of DAZL on a non-C57BL/6 background, it allows germ cells in the developing ovary to enter prophase I and progress through to pachytene (15 dpc) before meiosis is arrested and germ cells

degenerate (Ruggiu *et al.* 1997, Saunders *et al.* 2003, Haston *et al.* 2009). Nonetheless, DAZL is extremely important for the continued development of germ cells once they colonize the gonad. Expression of *Dazl* seems to be necessary for correct methylation erasure of at least the H19 imprinted locus and for normal levels of expression of *Ddx4* upon entry into the gonad (Reynolds *et al.* 2005, Haston *et al.* 2009). DAZL is known to bind the 3'-UTR of the *Sycp3* transcript and positively regulate its translation (Reynolds *et al.* 2007), so it would seem to make sense that DAZL-negative germ cells would have difficulty in progressing through meiosis. It is possible that DAZL works to affect translation of a number of germ line- and meiosis-specific transcripts. *In vitro*, DAZL is necessary for the generation of EGCs from germ cells and important for the generation of germ cells from mESCs (Haston *et al.* 2009). A recent report demonstrates that, when *Dazl* is overexpressed in mESCs, germ cells can be derived directly, without the need for embryoid body formation (Yu *et al.* 2009). It is possible, therefore, that DAZL will prove to be a 'gonadal germ cell integrity' factor rather than just a 'meiosis competence' factor.

Conclusions

Sex determination of the germ cell lineage, in the mammal, occurs in the gonad during fetal development. Germ cells either enter meiosis, and thereby commit to the oogenic pathway, or avoid entry into meiosis, enter a state of quiescence and commit to the spermatogenic pathway. A thorough understanding of germ cell sex determination is important because aberrations in the process underlie infertility and the development of germ cell tumors. Knowledge of the intricacies of the *in vivo* mechanisms may also aid future attempts to generate gametes *in vitro* (Feng *et al.* 2002, Hubner *et al.* 2003, Toyooka *et al.* 2003, Geijsen *et al.* 2004, Nayernia *et al.* 2006).

The specification of PGCs from a field of pluripotent epiblast cells is dependent on BMP and WNT3 signaling molecules, which seem to induce competence to become the germ cell lineage (Surani *et al.* 2007, Ohinata *et al.* 2009, Saitou 2009b). Various intrinsic markers such as PRDM1, PRDM14, DPPA3, POU5F1, NANOG, and SOX2, which correlate with the commitment and potency state of the cells, are known. In addition, germ cells at various stages of the process are characterized by particular epigenetic signatures and chromatin architectures.

The migration, proliferation, and survival of germ cells as they journey through the embryo seem to be dependent on a number of extrinsic factors. The nature of germ cell development is such that the cells are at different times in different somatic environments – this may ensure the characteristic genetic and epigenetic states observed at various times.

By the time the germ cells enter the gonad they appear to have matured to the point where they are able to respond to an extrinsic cue to enter meiosis (Fig. 3). The extrinsic cue is now firmly established as RA (Baltus *et al.* 2006, Bowles *et al.* 2006, Koubova *et al.* 2006, MacLean *et al.* 2007, Li & Clagett-Dame 2009), and the first evidence of its effect is the expression of *Stra8* around 12.5 dpc (Menke *et al.* 2003, Koubova *et al.* 2006). Proteins that mark germ cells competent to enter meiosis include DDX4, GCNA1, and DAZL, although it is not clear that any of these are critical 'competence factors' since, at least for DDX4 and DAZL, meiosis can occur even in their complete absence (Ruggiu *et al.* 1997, Tanaka *et al.* 2000, Saunders *et al.* 2003, Haston *et al.* 2009). XY and XX germ cells are competent to be pushed into meiotic prophase even earlier than XX germ cells do *in vivo* (Bowles *et al.* 2006). Presumably XY germ cells remain meiosis-competent until they commit to the male programme at around 12.5 dpc (McLaren & Southee 1997, Adams & McLaren 2002).

Commitment to the male programme, as delineated by both mitotic arrest and the expression of male germ cell fate markers such as *Nanos2* and *Dnmt3l*, is likely to be prompted by a male-specific gonadal signaling factor (or factors), as yet unidentified (Fig. 3). A key protein involved in the male fate commitment is the RNA-binding protein NANOS2, but neither the means by which its expression is initiated nor its molecular mode of action are known (Suzuki & Saga 2008). Mitotic arrest in the developing testis appears to occur only in the absence of RA (Trautmann *et al.* 2008), but it is likely that it is actually triggered by a male-specific factor or factors, the same or differing from those involved in inducing the expression of male germ cell fate markers.

Although we are making progress in understanding mammalian germ cell sex determination, a great deal remains mysterious. It is not clear to what extent germ cells are pre-programmed to develop and differentiate and to what extent their intrinsic nature at each step reflects the extrinsic signals they have been exposed to. Future studies will focus on key genes *Stra8*, *Dazl*, and *Nanos2* and on the identity of postulated extrinsic signaling molecules that are relevant to the process. RNA-binding proteins are likely to be important, as they are in other species during the sex determination phase of germ cell development. In addition, the presence and actions of Piwi-interacting RNAs (Aravin & Bourc'his 2008, Kuramochi-Miyagawa *et al.* 2008) and miRNAs (Hayashi *et al.* 2008), in the environment of the developing gonad, are areas of study that are likely to prove illuminating.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this work.

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