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Retinoic acid, meiosis and germ cell fate in mammals

Josephine Bowles and Peter Koopman*

Although mammalian sex is determined genetically, the sex-specific development of germ cells as sperm or oocytes is initiated by cues provided by the gonadal environment. During embryogenesis, germ cells in an ovary enter meiosis, thereby committing to oogenesis. By contrast, germ cells in a testicular environment do not enter meiosis until puberty. Recent findings indicate that the key to this sex-specific timing of meiosis entry is the presence or absence of the signaling molecule retinoic acid. Although this knowledge clarifies a long-standing mystery in reproductive biology, it also poses many new questions, which we discuss in this review.

Introduction

As cells go, germ cells are undeniably idiosyncratic. The founder population of primordial germ cells (PGCs) is established remarkably far in advance of the functional differentiation of gametes, and the colonization of the gonads by PGCs involves a long and perilous journey. During this time, PGCs must retain the capacity to differentiate into either oocytes or sperm, depending on whether they end up in an ovary or a testis. Germ cells clearly play a unique role as the carriers of genetic information between generations. To fulfill this role, germ cells are able to partition their genetic material in such a way as to generate haploid cells via a unique type of cell division known as meiosis (see Box 1). Not surprisingly, the origins, properties and behavior of germ cells continue to fascinate developmental and reproductive biologists.

This review focuses on how mammalian germ cells are directed towards the alternative pathways of oogenesis or spermatogenesis, and the role of retinoic acid (RA), the active derivative of vitamin A, in this process. The sex differentiation of germ cells is determined not by their chromosomal constitution but by cues from their environment (McLaren, 1995; McLaren, 2003). Recent studies have shown that the initial choice of male or female identity is governed in mice by exposure to RA in the fetal gonad (Bowles et al., 2006; Koubova et al., 2006). These studies reported that, although RA induces germ cells to enter meiosis in the ovary at around 13.5 days post coitum (dpc), its degradation protects germ cells from entering meiosis in males at that time. We argue here that these findings reconcile two substantial bodies of data that have led to opposing theories about how germ cell sexual fate is specified. We also review evidence that suggests that the role of RA might be reprised during the regulation of entry of male germ cells into meiosis in the postnatal testis.

Germ cell specification, proliferation, migration and maturation

Much of our knowledge regarding germ cell behavior in mammals has been gleaned from studies in mice. At around 7.2 dpc in mice, somatic signals earmark a small cohort of proximal epiblast cells as potential germ cell precursors (Ginsburg et al., 1990; Lawson et al., 1999; Lawson and Hage, 1994; Tam and Zhou, 1996; Ying et al., 2000). This group of cells moves into the extraembryonic tissues at the base of the allantois, where a second round of molecular selection occurs, resulting in a group of about 45 cells that are identifiable as germ cell precursors or PGCs (Lawson and Hage, 1994; Ohinata et al., 2005; Saitou et al., 2002; Tanaka and Matsui, 2002). PGCs proliferate rapidly and migrate anteriorly through the elongating hindgut towards the future site of the primitive gonads, the genital ridges. The PGCs colonize the genital ridges at around 10.5 to 11.5 dpc, just after these structures arise from the intermediate mesoderm and somatic sex determination is underway. The movements of PGCs suggest that they respond to an attractant that emanates from the genital ridges, possibly the chemokine SDF1 (also known as CXCL12 – Mouse Genome Informatics) and/or the growth factor kit ligand (KITL) (Farini et al., 2007; Molyneaux et al., 2001; Molyneaux et al., 2003). PGCs then change their shape, become less motile and continue to proliferate for 1-2 days, so that about 26,000 germ cells reside in the gonads by 13.5 dpc (Donovan et al., 1986; Tam and Snow, 1981).

Box 1. Meiosis

Gametes are haploid, and so are produced by reductive nuclear division of a diploid germ cell in a specialized process known as meiosis. Meiosis includes two cell divisions (meiosis I and meiosis II), the first with, and the second without, DNA replication.

During prophase of meiosis I, each pair of homologous chromosomes aligns and is held together tightly by a meiosis-specific nucleoprotein structure known as the synaptonemal complex. As is the case for mitotic division, each chromosome duplicates by DNA replication, producing two sister chromatids. The pairing of chromosome homologs allows genetic recombination to occur, because non-sister chromatids of homologs can cross-over (forming 'chiasmata') and exchange analogous fragments of DNA. Such a four-chromatid structure is called a bivalent.

During metaphase of meiosis I, bivalents line up perpendicular to the spindle and at anaphase the pair of chromosomal homologs (each composed of two sister chromatids) break apart and move to opposite poles of the spindle, and division I of meiosis occurs. The two cells produced are still diploid in terms of the amount of DNA they contain, but they differ from normal diploid cells in that they have two near-identical copies of each chromosome – differing only where genetic recombination has occurred – rather than a paternal and a maternal copy of each chromosome. A second round of cell division, without further DNA replication, is necessary before haploid gametes are produced. In division II of meiosis (meiosis II), duplicated chromosomes align along a second spindle and sister chromatids separate to produce two cells with haploid DNA content. To summarize, each diploid cell entering meiosis undergoes one round of DNA replication followed by two rounds of cell division, potentially producing four genetically different haploid cells. In the male, this amounts to four spermatozoa but, in the female, only one functional gamete is ultimately produced, the other genetic material being lost in the form of polar bodies. The time required for each stage of meiosis differs greatly among species and between the sexes of a single species. In general, meiotic prophase I takes the longest time, ranging from days (e.g. in human males) to decades (e.g. in human females).

Division of Molecular Genetics and Development, and ARC Centre of Excellence in Biotechnology and Development, Institute for Molecular Bioscience, The University of Queensland, Brisbane, QLD 4072, Australia.

*Author for correspondence (e-mail: p.koopman@imb.uq.edu.au)

The first morphological evidence of sex-specific germ cell development is seen at around 13.5 dpc (Fig. 1). At that time, in a developing mouse ovary, germ cells stop proliferating and enter prophase of the first meiotic division, progressing through leptonema, zygonema, pachynema and diplonema. At around the time of birth, they then enter a specialized, prolonged arrest stage known as dictyate (Speed, 1982). The oocytes remain in this state until just before ovulation, at which point they complete the first meiotic division, begin the second and arrest again. Meiosis is completed only after fertilization.

In males, germ cell behavior is markedly different. In mouse, at around 13.5 dpc male germ cells arrest in G_0 or G_1 of the mitotic cycle, resuming mitosis after birth (Hilscher et al., 1974; McLaren, 1984). Primary spermatocytes are seen as early as 5 days post natum (dpn), signifying that some spermatogonia have entered meiosis. In terms of germ cell differentiation, this stage in mice is equivalent to puberty in humans. The primary spermatocytes then complete both meiotic divisions promptly to generate spermatids, which mature further into functional gametes called spermatozoa or sperm. In the male, waves of meiosis continue throughout life, providing a continuous supply of sperm from spermatogonial stem cells at the periphery of the testis cords.

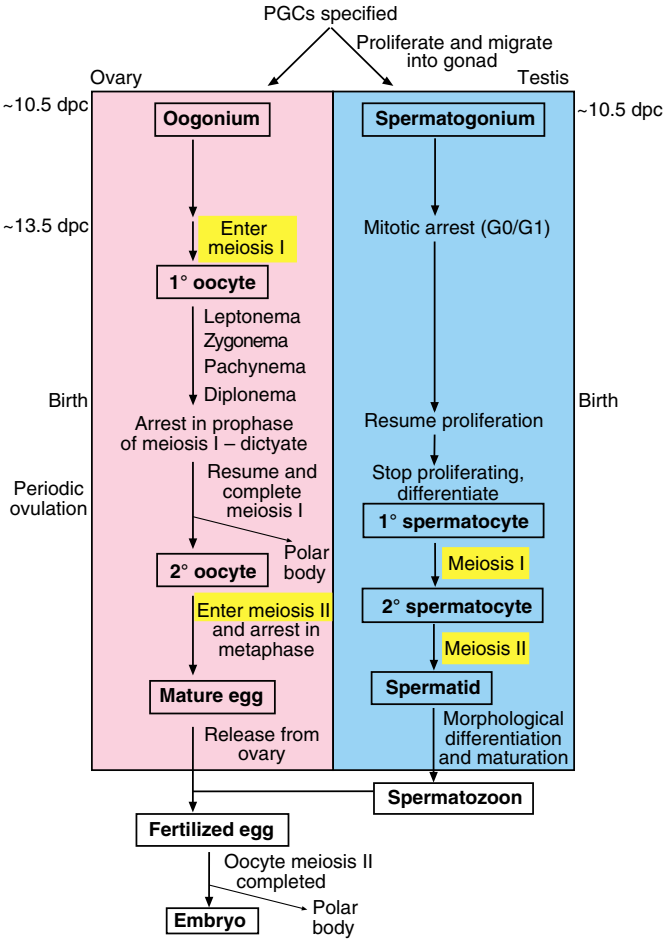

Fig. 1. The timing of mature gamete production from mouse primordial germ cells. Primordial germ cells (PGCs) are specified at ~7.2 dpc and subsequently proliferate and migrate towards the bipotential gonads, which they occupy at ~10.5 dpc. In the ovary (pink), at ~13.5 dpc, PGCs begin to enter meiosis I (the first meiotic division). Primary oocytes pass through the leptotene, zygotene and pachytene stages before entering diplonema/dictyate arrest at around birth. Once sexual maturity is reached, at each ovulation a cohort of arrested oocytes is stimulated to resume and complete meiosis I and enter meiosis II. The released mature egg is arrested in metaphase II, completing meiosis II after fertilization. In the testis (blue), germ cells do not enter meiosis at 13.5 dpc, but arrest mitotically. Immediately after birth, germ cells re-enter the mitotic cycle and meiosis I is initiated several days later. Both divisions are completed rapidly to produce spermatozoa (sperm). The process is repeated many times throughout life to ensure a continuous supply of mature sperm.

Somatic influences on sex-specific germ cell fate

What causes the sex-specific behavior of germ cells? Studies involving XX↔XY chimeras have shown that germ cells, whether XX or XY, enter meiosis if they find themselves in a developing ovary, but avoid doing so in the environment of a developing testis (Evans et al., 1977; Palmer and Burgoyne, 1991). Therefore, the initial sex differentiation of a germ cell is determined not by its chromosomal constitution, but by its environment (McLaren, 1995; McLaren, 2003). However, the completion of meiosis and the formation of functional gametes are influenced by the sex-chromosome composition of the germ cell (Amleh et al., 2000; Bradbury, 1983; Burgoyne, 1987; McLaren, 1981; Park and Taketo, 2003; Taketo-Hosotani et al., 1989).

A well-accepted hypothesis to explain the effect of tissue environment on germ cell fate has been that the developing testicular tissue produces a meiosis-inhibiting factor (Buehr et al., 1993; Francavilla and Zamboni, 1985; McLaren, 1984). This factor was supposedly produced at around 12.0 dpc by immature Sertoli cells, the supporting somatic cells in the developing testis, and considered likely to be a short-range diffusible factor (McLaren and Buehr, 1990; McLaren and Southee, 1997). The mechanism by which meiosis is triggered in the ovary has been a subject of considerable debate. The point at issue has been whether, in the developing ovary, initial entry into meiosis occurs spontaneously and cell-autonomously, perhaps regulated by a molecular clock (Donovan et al., 1986; McLaren and Southee, 1997; Ohkubo et al., 1996), or whether this step is induced by surrounding somatic tissue (Byskov, 1974; Byskov, 1975; McLaren, 1984). These two opposing theories will be discussed later, in the light of recent findings.

Exposure to RA regulates entry into meiosis

Two recent studies (Bowles et al., 2006; Koubova et al., 2006) indicate that exposure to RA controls whether mouse fetal germ cells enter meiosis or not. RA is a small, polar molecule that easily diffuses through tissues and acts by binding to nuclear RA receptors (RARs, which include the RARα, β and γ isotypes), which heterodimerize with nuclear retinoid X receptors (RXRα, β and γ) (Chambon, 1996; Mark et al., 2006). RAR-RXR dimers bind to RA-response elements (RAREs) and thereby control the expression of RA-responsive genes (Chambon, 1996). RA has many known roles in morphogenesis, growth and differentiation during vertebrate embryogenesis and organogenesis (Mark et al., 2006).

The first clue that RA might play a role in female-specific gonadal development came from male versus female gonad gene expression screens in mice. In some screens, the gene *Cyp26b1* was more highly expressed in male than in female gonads, particularly at 13.5 dpc, the critical time at which female, but not male, germ cells enter meiosis (Bowles et al., 2000; Menke and Page, 2002). *Cyp26b1* encodes a P450 enzyme, CYP26B1 (also known as P450RAI-2), that acts by catabolizing all-trans RA into inactive metabolites (White et al., 2000; Yashiro et al., 2004). CYP26 enzymes act to regulate local levels of RA in several developmental contexts, such

Table 1. Details of genes involved in the meiotic division of germ cells in the mouse

Gene (common aliases)	Encoded protein and function	Mouse mutant phenotype (male)	Mouse mutant phenotype (female)	Expression pattern in mouse fetal gonads	References
Cyp26b1 (*CP26*, *P450RAI-2*)	Cytochrome P450, family 26, subfamily b, polypeptide 1; P450 enzyme, degrades RA to inactive metabolites	Die at birth, various developmental defects, germ cells express *Stra8* and *Scp3* ectopically and enter meiosis during fetal life	Die at birth, various developmental defects, germ cells express *Stra8* prematurely	Expressed in somatic cells of both sexes from at least 11.5 dpc then upregulated in testis and down-regulated in ovary	(MacLean et al., 2001; Menke and Page, 2002; Bowles et al., 2006; White et al., 2000; Yashiro et al., 2004; MacLean et al., 2007)
Dmc1 (*Dmc1h*)	Homolog of yeast Disrupted meiotic cDNA 1, dosage suppressor of mck1 homolog, meiosis-specific homolog of bacterial RecA; ssDNA and dsDNA binding protein, repairs DSBs	Sterile, germ cells arrest at zygotene-like stage of meiosis I and then undergo apoptosis. Chromosomal synapsis is not initiated	Sterile, germ cells arrested at pre-pachytene stage, germ cells apoptose and the ovary degenerates	Expressed pre-meiotically in germ cells of both sexes at a low level, then upregulated in ovary in an A/P wave from ~13.5 dpc	(Chuma and Nakatsuji, 2001; Menke et al., 2003; Nakatsuji and Chuma, 2001; Pittman et al., 1998; Yoshida et al., 1998)
Pou5f1 (*Oct4*, *Oct3/4*)	POU domain, class 5, transcription factor; considered a master regulator of pluri-potency, maintains viability of mammalian germ line	Male and female null develop to blasto-cyst stage and die at implantation. In germ cell-specific nulls, XY and XX germ cells undergo apoptosis between 9.5 and 10.5 dpc, before colonization of the gonad		In female PGCs: repressed during meiotic prophase I. In male PGCs: expressed throughout fetal life	(Bullejos and Koopman, 2004; Kehler et al., 2004; Nichols et al., 1998; Pesce et al., 1998; Wang et al., 2006)
Stra8	Stimulated by retinoic acid, gene 8; cytoplasmic protein, required for pre-meiotic DNA replication and the subsequent events of meiotic prophase	Infertile, pre-meiotic spermatocytes appear and show chromosomal condensation but do not enter prophase of meiosis I. Germ cell number reduced	Infertile, germ cells develop pre-meiotic morphology but do not enter prophase of meiosis I. By birth, ovaries are severely depleted of germ cells	Expressed exclusively in pre-meiotic germ cells (spermatogonia and possibly preleptotene spermatocytes). In embryonic ovary an A/P wave initiates at ~12.5 dpc	(Baltus et al., 2006; Menke et al., 2003; Oulad-Abdelghani et al., 1996)
Sycp3 (*Scp3*, *Cor1*)	Synaptonemal complex protein; structural protein, main constituent of the axial elements of the synaptonemal complex	Sterile, axial elements fail to form and germ cells die by apoptosis	Sub-fertile, germ cell aneuploidy (trisomy or monosomy) due to defective meiotic chromosome segregation	Expressed pre-meiotically in germ cells of both sexes at a low level, then upregulated in ovary in an A/P wave from ~13.5 dpc	(Bullejos and Koopman, 2004; Chuma and Nakatsuji, 2001; Nakatsuji and Chuma, 2001; Yao et al., 2003; Yuan et al., 2000; Yuan et al., 2002)

A/P; anterior/posterior; dpc, days post coitum; DSB, double-strand break; ss, single-stranded; ds, double-stranded.

as during central nervous system, inner ear, eye and limb patterning (Hernandez et al., 2007; Romand et al., 2006; Sakai et al., 2004; Yashiro et al., 2004). *Cyp26b1* transcripts are detectable in mouse gonads at 11.5 dpc, at higher expression levels in males than females (Bowles et al., 2006). After 11.5 dpc, *Cyp26b1* expression is undetectable in female gonads but is very high in male gonads. Expression is associated with Sertoli cells and some interstitial cells, and peaks at around 13.5 dpc (Bowles et al., 2006; Menke and Page, 2002). This profile of expression suggests that CYP26B1 might protect male gonads from the actions of RA between 12.5 and 14.5 dpc, and matches that expected of the postulated meiosis-inhibiting factor (McLaren and Southee, 1997).

It is now clear that RA acts to initiate meiosis (Bowles et al., 2006; Koubova et al., 2006). First, RA levels are higher in the female than the male mouse gonad at 13.5 dpc (Bowles et al., 2006). Furthermore, exogenous RA can induce XY germ cells in a cultured mouse fetal testis to enter meiotic prophase (Bowles et al., 2006; Koubova et al., 2006), as judged by the histological detection of condensed meiotic nuclei and the expression of three meiotic markers, named: stimulated by retinoic acid gene 8 (*Stra8*), synaptonemal complex protein 3 (*Sycp3*) and dosage suppressor of mck1 homolog (*Dmc1*) (Chuma and Nakatsuji, 2001; Nakatsuji and Chuma, 2001; Oulad-Abdelghani et al., 1996) (see Table 1). Untreated XY germ cells do not normally express *Stra8*, and express *Sycp3* and *Dmc1* weakly only (Chuma and Nakatsuji, 2001; Menke et al., 2003; Nakatsuji and Chuma, 2001). Finally, chemically antagonizing RARs in cultured mouse fetal ovaries prevents XX germ cells from entering meiosis (Bowles et al., 2006; Koubova et al., 2006). These findings complement earlier studies that showed that exogenous RA added to rat XX germ cells in ex vivo organ culture accelerates their entry into meiosis (Livera et al., 2000).

As mentioned above, the male-specific expression of *Cyp26b1* in developing gonads had suggested that RA is actively degraded in the fetal mouse testis. In support of this theory, treatment of cultured male mouse fetal gonads with broad-spectrum cytochrome P450 inhibitors, or with more-specific CYP26 inhibitors, caused a strong upregulation of the RA-responsive gene *Stra8*, indicative of RA levels having increased in the male gonad (Bowles et al., 2006;

Koubova et al., 2006). *Sycp3* and *Dmc1* expression also increased, indicating that these XY germ cells in the treated gonads are in the early stages of meiosis I (Bowles et al., 2006; Koubova et al., 2006). When male gonads were cultured with both a CYP26 inhibitor and an RAR panantagonist, *Stra8* expression was not induced, indicating that, in a developing male gonad, CYP26B1 functions to degrade RA, which would otherwise act via RARs to upregulate *Stra8* expression (Koubova et al., 2006).

To prove that CYP26B1 functions as a meiosis inhibitor endogenously, *Cyp26b1*-knockout mice were also examined (Bowles et al., 2006). *Cyp26b1*-null mice die immediately after birth with multiple abnormalities, including limb defects (Yashiro et al., 2004). XY gonads from *Cyp26b1*-null embryos showed upregulation of *Stra8* and *Sycp3* expression at 13.5 dpc, demonstrating that germ cells in the XY mutant embryos are entering meiotic prophase I at this early stage, as if in a normal XX embryo (Bowles et al., 2006). These findings were recently extended by the analysis of a second *Cyp26b1*-null line of mice (MacLean et al., 2007); it was found that, in the absence of CYP26B1, XY germ cells enter meiosis by 13.5 dpc and proceed through meiotic prophase, with some reaching zygotene/pachytene, as judged by histological staining and chromosome spread analysis. By birth, virtually all germ cells were lost in *Cyp26b1*-null testes because of progressive apoptosis (MacLean et al., 2007). Importantly, these studies confirmed independently that RA levels are increased in embryonic testes when CYP26B1 is not present. Using a synthetic retinoid that cannot be degraded by CYP26B1, it was demonstrated that the role of CYP26B1 in this system is to degrade RA, hence preventing it from signaling, rather than to produce an active RA metabolite.

The role of CYP26B1 in germ cell development might not be limited to preventing meiosis in the fetal testis. In *Cyp26b1*-null ovaries, the wave of *Stra8* expression appears to initiate earlier than is normal (Bowles et al., 2006). Because *Cyp26b1* is expressed initially in the gonads of both sexes (Bowles et al., 2006) (Fig. 2), we surmise that, in *Cyp26b1*-null XX gonads, complete absence of CYP26B1 might allow RA to accumulate to a meiosis-inducing level earlier than in wild-type female gonads. Therefore, *Cyp26b1* expression might be relevant to the timing of meiotic entry in germ cells of both sexes. It will be interesting to see whether early entry into meiosis has any detrimental effect on *Cyp26b1*-null XX germ cells.

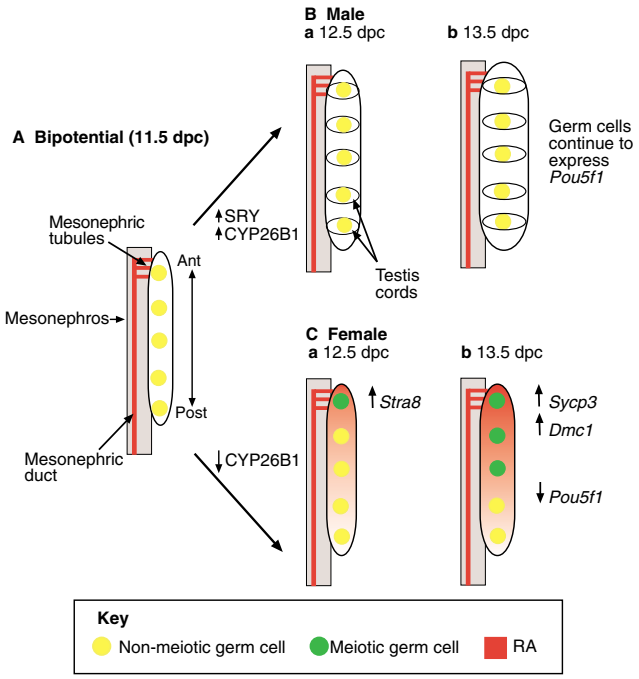

Fig. 2. Regulation of germ cell entry into meiosis in the developing gonads. (**A**) In the bipotential (11.5 dpc) mouse gonad, germ cells are present and retinoic acid (RA) is produced in the mesonephric duct and tubules. *Cyp26b1* is expressed at low levels in the gonad of both sexes. The mesonephric tubules, which produce RA, are physically connected with the anterior (Ant) end of the gonad during this time. (**Ba**) Once *Sry* is expressed in the male gonad (at 11.5 dpc), *Cyp26b1* expression is upregulated, probably in both Sertoli and interstitial cells. The testis cords, which form around germ cell clusters, might concentrate the enzyme in these regions, thereby protecting germ cells from the actions of RA. (**Bb**) Germ cells in the male gonad do not enter meiosis at 13.5 dpc, and continue to express the pluripotency marker *Pou5f1*. (**Ca**) In the female gonad, *Cyp26b1* expression is detectable at 11.5 dpc, but disappears by 12.5 dpc. Germ cells at the anterior end of the gonad begin to express *Stra8* at 12.5 dpc; (**Cb**) by 13.5 dpc, later markers of meiosis, such as *Sycp3* and *Dmc1*, are expressed strongly. Meiosis markers are upregulated in an anterior-to-posterior (Post) wave over ~3 days. *Pou5f1* is no longer expressed by germ cells once they enter meiosis. Germ cells at the anterior end of the gonad might be exposed to RA earlier than those at the posterior end, or the RA concentration might be greater at the anterior end than the posterior end. See text for more details.

What is the source of RA during urogenital development?

One question raised by these observations is whether RA is produced in the developing fetal gonads or whether it comes from elsewhere in the developing urogenital system. Bowles et al. have provided several lines of evidence indicating that RA is not synthesized in the gonads themselves, but rather by the mesonephroi to which the gonads are attached (Bowles et al., 2006). The gonad begins to grow out from the mesonephros at around 10 dpc, and the two organs remain in contact for some time during development. Using reporter mice and cells in which the reporter gene *lacZ* is driven by an RARE (RARE-*lacZ* mice), it was demonstrated that the ducts and tubules of the mesonephroi of both sexes produce RA (Bowles et al., 2006). In concordance with this, *Aldh1a2*, which encodes the enzyme RALDH2 and is most commonly associated with RA production in the embryo, is highly expressed in the mesonephros from at least 10.5 dpc (Bowles et al., 2006). The complementary patterns of an RA-synthesizing enzyme in the mesonephros and an RA-catabolyzing enzyme in the attached male gonad indicates that an RA source/sink system exists, analogous to that in the developing retina and inner ear (McCaffery et al., 1999; Romand et al., 2006).

Several studies have shown that germ cells in the developing ovary enter meiosis in an anterior-to-posterior wave and not, as might have been expected, simultaneously or stochastically (Bullejos and Koopman, 2004; Menke et al., 2003; Yao et al., 2003). This wave is evident from *Stra8* expression, which begins at ~12.5 dpc in the anterior part of the gonad and extinguishes at ~16.5 dpc at the posterior end (Menke et al., 2003) (Fig. 3A). This pattern is also seen, about 1 day later, with the upregulation of the meiosis marker *Sycp3* and the downregulation of the pluripotency marker POU domain, class 5, transcription factor 1 (*Pou5f1*, *Oct3/4*) (Bullejos and Koopman, 2004; Yao et al., 2003) (Fig. 3B). *Sycp3* encodes a major structural protein of the synaptonemal complex (see

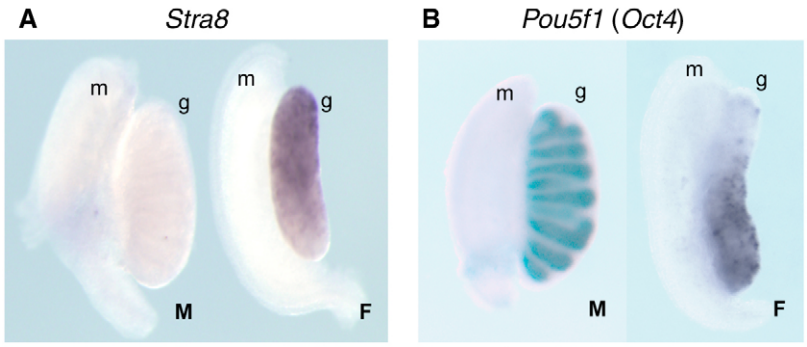

Fig. 3. Marker gene expression in 13.5 dpc mouse gonads. Urogenital ridge tissue samples were dissected from wild-type mouse embryos. (**A**) Expression of the pre-meiotic marker *Stra8* is absent in male (M, left) but present in female (F, right) 13.5 dpc mouse urogenital ridge samples. Only germ cells, just prior to entry into meiosis, express this marker gene and higher expression is seen in germ cells located at the anterior (top) end of the gonad. (**B**) Expression of the pluripotency marker *Pou5f1* (*Oct4*) in male and female 13.5 dpc mouse urogenital ridge samples. Only pre-meiotic germ cells express this gene at this stage of development. Staining highlights the clustering of germ cells in the male sample in testis cord structures and the dispersal of germ cells in the female sample. Germ cells are present throughout the length of the female gonad, but *Pou5f1* expression has been downregulated at the anterior (top) end at this stage (see text). g, gonad; m, mesonephros.

Box 1) and is considered to be a specific marker of meiotic germ cells (Chuma and Nakatsuji, 2001; Nakatsuji and Chuma, 2001), whereas *Pou5f1* encodes a transcription factor that represses differentiation genes (Pesce et al., 1998). In addition, *Pou5f1* promotes germ cell survival (Kehler et al., 2004). Because there is no evidence that germ cells colonize the anterior end of the gonad first (Molyneaux et al., 2001), this slow anterior-to-posterior wave of entry into meiosis is consistent with meiosis being induced, rather than being automatic, in germ cells.

What causes the wave of entry into meiosis in the fetal ovary? We propose that RA enters the gonad predominantly through open mesonephric tubules that connect the mesonephros and gonad at their anterior ends (Byskov, 1978b; Karl and Capel, 1995) (Fig. 2). If so, the anterior germ cells would be the first to be exposed to RA. Alternatively, cells might migrate from the mesonephric tubules into the anterior tip of the gonad and emit RA, producing a gradient that might lead to the observed wave of expression of meiosis markers.

When gonads and mesonephroi were explanted from RARE-*lacZ* mouse embryos, β-galactosidase staining confirmed that RA is more concentrated at the anterior than at the posterior end of the gonads at 12.5-14.5 dpc (Bowles et al., 2006). It remains to be seen whether the developing ovary can be separated experimentally from the mesonephros early and cleanly enough to test the importance of the mesonephros in regulating meiosis. Proof that the mesonephros is the source of RA would require the production and analysis of mesonephros-specific *Aldh1a2*-deleted mice (Vermot et al., 2006); total ablation of *Aldh1a2* is lethal by 10.5 dpc (Niederreither et al., 1999).

A model summarizing our current understanding of the regulation of germ cell entry into meiosis, and the role of RA in this process in mouse fetal gonads, is shown in Fig. 2.

Reconciling new findings with established theories

The issue of whether meiosis is induced or whether it initiates spontaneously and cell-autonomously has been contentious, because both arguments are supported by convincing experimental data. Here, we examine the evidence supporting each of the two main theories and then re-evaluate them in the light of recent findings.

The 'intrinsic clock' theory

Germ cells that accidentally colonize non-gonadal locations, such as the adrenal gland, enter meiosis as they would in the developing ovary (Upadhyay and Zamboni, 1982; Zamboni and Upadhyay, 1983). Similarly, XY germ cells isolated at 10.5 or 11.5 dpc, combined with dissociated embryonic lung cells and cultured for several days, also commence meiosis (McLaren and Southee, 1997). In the ectopic location of the mesonephros, XY germ cells tend to enter meiosis (Byskov, 1978c; McLaren, 1983; McLaren, 1984). Because it appeared that meiosis could occur in almost any tissue environment, it was hypothesized that this pathway might be automatic in germ cells of a certain age. Thus, timing of entry into meiosis might be determined by the number of mitoses occurring after the germ cell lineage was established or had started to migrate (McLaren, 2003; McLaren and Southee, 1997; Ohkubo et al., 1996).

This 'intrinsic clock' theory (Donovan et al., 1986) was supported by studies of cultured germ cells (Chuma and Nakatsuji, 2001; Nakatsuji and Chuma, 2001). When 10.5 dpc mouse gonad-mesonephros complexes, or 11.5 dpc gonads, are dissociated and cultured on feeder cells with 20% fetal calf serum, germ cells of either sex can reach the leptotene stage of the first meiotic division (Chuma and Nakatsuji, 2001). Germ cells isolated prior to entry into the gonads also enter meiosis when cultured, supporting the theory that a signal from the gonadal cells is not required to initiate meiosis (Chuma and Nakatsuji, 2001).

A corollary of this hypothesis is that an overriding mechanism must operate in the fetal testis to actively block entry into meiosis (Buehr et al., 1993; Francavilla and Zamboni, 1985; McLaren, 1984). The production of this meiosis-inhibiting factor evidently depended on the structural integrity of the testis cords, because dissociation and re-aggregation of fetal urogenital ridges allows germ cells to enter into meiosis (McLaren and Southee, 1997).

The meiosis-inducing-substance theory

Despite the obvious attractions of the intrinsic clock theory, a large and long-standing body of experimental evidence has supported the existence of a somatically derived, meiosis-inducing substance. For example, meiosis in XY germ cells can be induced by culturing male fetal gonads for 6 days with conditioned media from cultured ovaries or testes in which meiosis is occurring (Byskov et al., 1993; Byskov and Saxen, 1976) or in follicular fluid taken from pre-ovulatory

follicles (Westergaard et al., 1984). Similarly, conditioned media from cultured fetal epididymides of fetal mice (Byskov, 1978a), pubertal mice or bulls (Grinsted et al., 1979), or fetal and adult humans (Grinsted and Byskov, 1981), can also induce mouse XY fetal germ cells to undergo meiosis.

In particular, meiosis appeared to be induced by a factor secreted from a region known as the rete ovarii (Byskov, 1974). The rete ovarii develops from mesonephric cells that migrate into the developing gonad, and includes the mesonephric tubules and their connections with the anterior end of the gonad (Byskov, 1978b). When the cranial half of 12.0 dpc fetal mouse ovaries, with the extra-ovarian rete tubules and mesonephros still attached, is grafted into nude mice, the germ cells enter meiosis and continue to develop normally. By contrast, when the caudal part, without associated rete tubules, is grafted, germ cells do not enter meiosis and remain as oogonia (Byskov, 1974). In other species, such as cats, minks and ferrets, meiosis begins in the part of the ovary that is in closest contact with mesonephric tissue (Byskov, 1975). Fetal testicular germ cells that happen to have been excluded from testis cords and instead end up located near the connecting rete cords can enter meiosis, leading to the conclusion that male, as well as female, rete can induce meiosis but that germ cells are normally protected from this effect in the developing testes (Byskov, 1978c; Byskov, 1986).

Byskov et al. attempted to characterize meiosis-inducing substances from human follicular fluid and bull testes (Byskov et al., 1995). Perhaps because they assayed resumption rather than the initiation of meiosis, their experiments led them to isolate naturally occurring sterols that are intermediates of the cholesterol biosynthetic pathway: these appear not to trigger initial entry into meiosis (Byskov et al., 1998).

In view of the considerable evidence supporting the intrinsic clock theory, and given that no meiosis-inducing substance was identified in recent decades, the existence of a meiosis-inducing substance was largely discredited. Researchers instead concentrated on finding the male-specific meiosis-inhibiting substance.

A model for sex-specific regulation of germ cell fate

So, which theory is correct – active induction of meiosis, or spontaneous entry of fetal germ cells into meiosis unless prevented by a meiosis-inhibiting factor? The answer is neither and both: the recent identification of the RA-CYP26B1 system suggests a model that includes elements of both theories (Fig. 2). RA can, and normally does, induce fetal germ cell meiosis (Bowles et al., 2006; Koubova et al., 2006), indicating that meiosis is actively induced rather than occurring spontaneously via an intrinsic clock mechanism. When *Cyp26b1* is absent from the fetal testis, germ cells enter meiosis (Bowles et al., 2006; MacLean et al., 2007). Hence, CYP26B1 is a meiosis-inhibiting factor, if not the meiosis-inhibiting factor. The new model also includes a feature not previously predicted – that the meiosis-inhibiting factor controls not just the avoidance of meiosis in the fetal testis, but also the timing of entry into meiosis in the ovary.

How does this model explain the apparently spontaneous entry of ectopic or cultured fetal germ cells into meiosis, which formed the basis of the intrinsic clock theory? It seems likely that those germ cells enter meiosis because of exposure to RA. First, RA is present in many embryonic tissues, including the lungs, adrenals and mesonephroi (Horton and Maden, 1995; Rossant et al., 1991). The concentration of all-trans RA is around 4×10^{-8} M in visceral tissues at 10.5 and 13.5 dpc (Horton and Maden, 1995), and we find that as little as 1×10^{-8} M RA can induce *Stra8* expression in isolated 11.5 dpc germ cells in vitro (J.B. and P.K., unpublished data). Secondly, in experiments in which dissociated gonadal cells have been cultured in isolation, or co-cultured with dissociated embryonic lung cells, the bovine serum in the media probably provides sufficient RA to drive meiotic induction. Media supplemented with 10% fetal bovine serum is estimated to contain 3.6×10^{-8} M RA (Fuchs and Green, 1981). It remains to be seen whether 'spontaneous' entry into meiosis in cell culture can be inhibited by blocking RARs.

The observation that dissociation/re-aggregation of fetal male urogenital ridges is permissive for meiosis (McLaren and Southee, 1997) is most likely to be explained by the juxtaposition of germ cells with RA-producing mesonephric cells (Bowles et al., 2006), and not by the loss of a meiosis-inhibiting factor, as was originally hypothesized. In the intact fetal testis, germ cells are surrounded by Sertoli cells in cords, and this arrangement might protect germ cells from RA (Fig. 2). CYP26B1 is produced by Sertoli cells (Bowles et al., 2006) and, because this enzyme acts within the cytoplasm of the cells in which it is produced, the cord structure could physically shield germ cells from any RA in the interstitium.

A pillar of the intrinsic clock theory is that a short-range signaling molecule emanates from somatic (probably Sertoli) cells of the developing testis and prevents meiosis. Although we now know that a testis-derived meiosis inhibitor acts in fetal mice, this molecule is not a secreted signal but rather the cytosolic enzyme CYP26B1 (Bowles et al., 2006; Koubova et al., 2006). So, the new paradigm of regulation of germ cell fate in mice by a system of RA induction of meiosis, combined with the testis-specific expression of CYP26B1 to prevent this induction in males, combines elements of both of the previous theories on germ cell-fate specification. It also raises several new questions.

Why might a meiosis-inducing substance, such as RA, be produced in male and female reproductive tissue? RA is widespread and regulates many developmental processes (Rossant et al., 1991), and it would seem imprudent to rely on sex-specific exposure of germ cells to RA to ensure that entry into meiosis occurs only in the ovary. Instead, a pathway of gene regulation set in motion by SRY and leading to *Cyp26b1* expression provides a robust, male-specific system of eliminating RA only in male gonads.

Does RA provide the only signal that stimulates germ cell entry into meiosis in the mouse fetus? By chemically antagonizing RARs in cultured mouse fetal ovaries, germ cells are prevented from entering meiosis (Bowles et al., 2006; Koubova et al., 2006), suggesting that RA is essential and that other pathways either do not exist, or are insufficient to induce meiosis. It remains possible that male developing gonads have a supporting or back-up mechanism, in addition to RA degradation by CYP26B1, to prevent germ cells from entering meiosis. Certainly, the developing male gonads have mechanisms for dealing with germ cells that inadvertently enter and progress through meiosis: in *Cyp26b1*-null fetal testes, meiotic markers are upregulated in germ cells, but meiotic germ cells subsequently undergo apoptosis (MacLean et al., 2007).

Is RA instructive or merely permissive for germ cell entry into meiosis? The data suggest that it is instructive, because the blocking of RARs results in downregulation of meiosis markers, especially of *Stra8* (Bowles et al., 2006; Koubova et al., 2006), which encodes a protein known to be essential for meiotic initiation (Baltus et al., 2006). If RA is instructive, then it should first be absent (before meiotic induction) and then present (when meiosis is induced). Direct measurements of RA levels at various times of development in the ovary have not been made. However, it is possible to infer that RA levels increase with time, because *Cyp26b1* expression in the fetal ovary disappears between 11.5 dpc and 12.5 dpc (Bowles et al., 2006). Moreover, the pattern of entry into meiosis of XX germ cells

is consistent with an instructive signal coming predominantly from the anterior end of the gonad-mesonephros complex. A further distinction between induction and permission might be found in the molecular details of the relationship between RA and the engagement of the meiotic machinery, to which we now turn.

How does RA induce meiosis?

Because the upregulation of meiotic markers and the downregulation of *Pou5f1* are inhibited by RAR antagonists in fetal ovary culture, RA must signal through RARs to induce meiosis (Bowles et al., 2006; Koubova et al., 2006). Moreover, RAR agonists selective for each of the three RAR isotypes can induce *Stra8* expression in cultured ex vivo testes, suggesting that RA can signal through any RAR in this system (Koubova et al., 2006). At least some RARs and RXRs are expressed by germ cells at the relevant stage of development, but somatic cells also express these receptors (Boulogne et al., 1999; Bowles et al., 2006; Li and Kim, 2004; Morita and Tilly, 1999; Vernet et al., 2006) so it is theoretically possible that RA acts on somatic cells, which then send a secondary signal to germ cells to induce meiosis.

Assuming that RA acts directly on germ cells, what are the molecular steps between the reception of the RA ligand and the orchestration of a meiotic response? Germ cells do not initiate meiosis in *Stra8*-null mice (Baltus et al., 2006), so RA must trigger meiosis by inducing the cytoplasmic protein STRA8 (Oulad-Abdelghani et al., 1996). *Stra8* was first identified as an RA target gene (Bouillet et al., 1995; Oulad-Abdelghani et al., 1996), although whether it is regulated directly or indirectly by RA is not known. Because treatment of embryonal carcinoma (EC) cells (stem cells of teratocarcinomas) with RA upregulates *Stra8* expression in less than 2 hours (Oulad-Abdelghani et al., 1996), RA might bind as a ligand to an RAR-RXR heterodimer that then recognizes and binds an RARE sequence upstream of *Stra8*. The RXR-RARγ isotype combination plays the major role in mediating *Stra8* induction (Chiba et al., 1997). Nonetheless, an indirect pathway involving some intermediate transcription factor(s) remains possible. In some circumstances, unliganded RAR-RXR complexes bind and repress the transcription of targets until an RA ligand becomes available (Weston et al., 2003). This mechanism might also underlie the regulation of *Stra8* and of any other target genes in this system.

In germ cells of the *Stra8*-null mouse fetal ovary, cohesion proteins are not produced, the synaptonemal complex (see Box 1) does not form and DNA double-strand breaks appear not to form or repair. However, pre-meiotic chromosome condensations are observed (Baltus et al., 2006). The presence of such chromosome condensation supports the idea that germ cells in a developing gonad of either sex are poised to respond to signals from the environment, whether they be inductive or preventative (McLaren and Southee, 1997). Although XY germ cells do not normally enter meiosis during fetal development, the low level expression of meiosis-associated genes, such as *Sycp3* and *Dmc1*, may indicate that all germ cells prepare to enter meiosis after their arrival in the genital ridge (Byskov, 1978c; Chuma and Nakatsuji, 2001; Di Carlo et al., 2000; Nakatsuji and Chuma, 2001). This preparation must be independent of any RA effect because *Cyp26b1* is expressed in the gonads of both sexes from an early time-point and because there is no reported expression of *Stra8* in the male developing gonad at 12.5 dpc or earlier. RA might directly or indirectly upregulate *Stra8* expression, leading to the upregulation of other meiosis-associated genes. Although we now have good evidence that STRA8 is necessary for entry into meiotic prophase (Baltus et al., 2006), the cellular function of the protein is unknown.

Even though RA is widespread throughout the embryo, it seems that *Stra8* is never expressed in somatic cells (Oulad-Abdelghani et al., 1996). Possibly, RA is only able to upregulate *Stra8* expression if a cell is in a specific epigenetic configuration (Seki et al., 2005). In addition, male and female patterns of DNA methylation or histone modification might be mediated by additional sex-specific factors (Matsui and Hayashi, 2007) underlying, for example, the failure of XY germ cells to survive and progress past pachytene in an XX gonadal environment.

When germ cells in a fetal mouse ovary begin to express *Stra8*, they downregulate the pluripotency marker *Pou5f1* (Menke et al., 2003). Perhaps RA has a direct or indirect effect on *Pou5f1* transcription; various studies have linked RA-induced differentiation with the downregulation of *Pou5f1* in ES and EC cells (Okamoto et al., 1990; Rosner et al., 1990; Scholer et al., 1989). It appears that RA, directly via RARs or indirectly via molecules such as germ-cell nuclear factor (GCNF or NR6A1, an orphan nuclear receptor), can affect *Pou5f1* transcription (Fuhrmann et al., 2001; Gu et al., 2005; Minucci et al., 1996; Pikarsky et al., 1994). The further study of the distal and proximal enhancers (DE/PE) and the TATA-less proximal promoter of *Pou5f1* might clarify this point; in particular, the DE site is thought to regulate *Pou5f1* expression in migratory and post-migratory germ cells (Yeom et al., 1996).

Does RA induce meiosis in the pubertal testis?

Parsimony suggests that meiosis might be induced by similar mechanisms in prenatal XX germ cells and postnatal XY germ cells. This might indeed be the case, because genetic deletion of *Stra8* precludes germ cells of either sex progressing into prophase of meiosis I (Baltus et al., 2006). Also, the observation that media conditioned for 24 hours with minced adult mouse testes can induce XY germ cells in a fetal testis to enter meiosis (Byskov et al., 1993) further supports idea of a common meiosis inducer in both sexes.

In male mice, the first meiotic entry at male puberty is followed by a self-perpetuating cycle of meiotic initiation in germline stem cells. It is not known how the initial round of meiosis, or later rounds, are triggered. The expression profile of *Stra8* in postnatal and adult mouse testes is consistent with RA being involved in both events in males: in both pre-pubertal and adult male mice, *Stra8* is expressed by spermatogonia and, possibly, by preleptotene-stage spermatocytes immediately prior to entry into meiosis (Oulad-Abdelghani et al., 1996). Furthermore, in sections of adult mouse testes, *Stra8* is highly expressed in basal non-somatic cells of some tubules, and weakly or not at all in others, suggesting that its expression is restricted to a pre-meiotic stage of the spermatogenic cycle (Oulad-Abdelghani et al., 1996).

In mice (McCarthy and Cerecedo, 1952) and rats (Thompson et al., 1964), an association between vitamin A deficiency (VAD) and male infertility is well-known. In the seminiferous tubules of VAD mice, germ cells degenerate and only type A spermatogonia (male germline stem or early differentiating pre-meiotic cells) remain (van Pelt and de Rooij, 1990a). The situation is similar in VAD rats, but, in addition to type A spermatogonia, the slightly more differentiated forms – type B spermatogonia and preleptotene spermatocytes – are also found (Huang and Hembree, 1979; van Pelt and de Rooij, 1990b), and when retinol or large doses of RA are provided to VAD rats and mice, meiosis resumes promptly and synchronously (Morales and Griswold, 1987; van Pelt and de Rooij, 1990a; van Pelt and de Rooij, 1991). Recently, it was noted that the type A spermatogonia that remain in VAD adult male mice differ from their wild-type counterparts in that they do not express *Stra8*

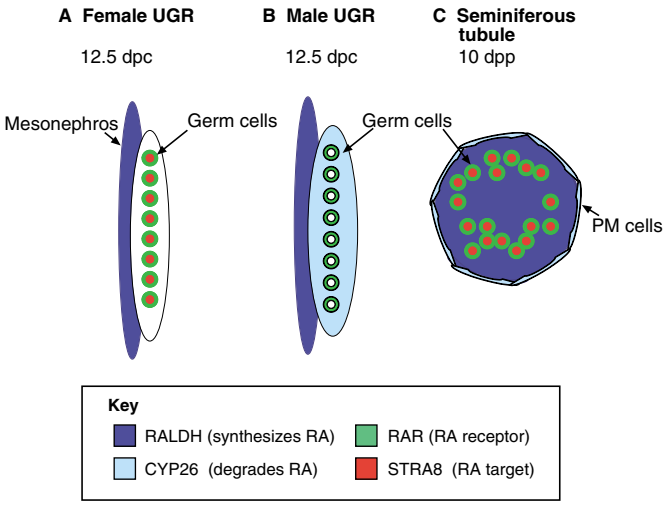

Fig. 4. A model of RA regulation during prenatal and postnatal meiosis. (**A**) Schematic of a 12.5 dpc female urogenital ridge (UGR). The mesonephros produces an RA-synthesizing enzyme (RALDH2). We postulate that retinoic acid (RA) moves from the mesonephros into the adjacent gonad through open mesonephric tubules. Germ cells resident in the female gonad express RA receptors (RARs) and respond to RA by expressing STRA8. (**B**) Schematic of a 12.5 dpc male UGR. The RA-degrading enzyme CYP26B1 is produced by somatic cells. We postulate that, although all germ cells express RARs and thus can respond to RA, they are not exposed to sufficient amounts of RA to induce STRA8 production. (**C**) Schematized cross-section of a seminiferous tubule at 10 days post partum (10 dpp). CYP26 enzymes (CYP26A1, CYP26B1 and CYP26C1) are produced by peritubular myoid (PM) cells, which surround and isolate seminiferous tubules from the rest of the body. Within the seminiferous tubules, Sertoli cells produce RALDH enzymes (RALDH1 and RALDH2) and germ cells express RARs. As germ cells enter meiosis they produce STRA8. We postulate that RA, produced locally within the seminiferous tubules, triggers *Stra8* expression in germ cells and, hence, triggers entry into meiosis.

(Ghyselinck et al., 2006). Expression of *Stra8* is restored when VAD mice are given RA (Ghyselinck et al., 2006; Koubova et al., 2006).

A recent study has examined the expression in testes of RA-synthesizing and RA-degrading enzymes, and of RARs and RXRs of various isotypes (Vernet et al., 2006). In the early postnatal period (1-20 dpn), RA-synthesizing enzymes are expressed in Sertoli cells, and RARs and RA-binding proteins are expressed by germ cells, consistent with a role for RA in driving meiosis postnatally (Fig. 4). RA-degrading enzymes (CYP26A1, CYP26B1 and CYP26C1) are produced in peritubular myoid cells that line the outer surface of the seminiferous tubules, separating them from the circulation (Vernet et al., 2006). Undifferentiated spermatogonia that are found closest to the Sertoli cells were found to express the RA target gene *Stra8* (Vernet et al., 2006).

These observations lead us to propose a model in which RA acts to induce meiosis in the pre-pubertal male testis (Fig. 4). We postulate that retinol (but not RA, which is degraded by CYP26 enzymes) passes freely from the circulation through the barrier of peritubular myoid cells into the Sertoli cells. There, the final two steps of RA production could be carried out under the control of alcohol dehydrogenases (retinol to retinal) (Deltour et al., 1997) and RALDH1/RALDH2 (retinal to RA). RA produced by the Sertoli cells could act in a paracrine manner on adjacent pre-meiotic germ cells, ranging in stages of maturity from type A spermatogonia through to preleptotene spermatocytes, all of which express RARs (Vernet et al., 2006). The pre-meiotic germ cells could then respond to RA by expressing *Stra8*, thereby triggering the first round of meiosis. The observation of robust expression of the cellular RA-binding protein CRABP1 in all spermatogonia and in no other cell type of the testis (Vernet et al., 2006) supports the theory that RA has a function in one or more of the transitions up to and including meiotic prophase. By contrast, expression of cellular retinol-binding protein (CRBP1) in Sertoli cells suggests that they have the capacity to store retinol (Zhai et al., 1997). The catabolic barrier of CYP26 enzymes produced by peritubular myoid cells, which surround and isolate the seminiferous tubule (see Fig. 4G), might explain the observation that the treatment of VAD mice with retinol restores fertility, but treatment with RA is far less effective (van Pelt and de Rooij, 1991).

In summary, we suggest that RA is probably produced by Sertoli cells in the postnatal mouse testis, and delivered in a juxtacrine fashion to spermatogonia, which then enter meiosis. If this is correct, the timing, site of production and means of delivery of RA to the germ cells differs in males and females, even though the effect – entry into meiosis via the upregulation of *Stra8* expression – is conserved.

Conclusions

Available evidence indicates that RA stimulates germ cells to enter meiosis in the developing mouse ovary, and that it might also regulate meiotic progression in the pubertal testis. During male embryogenesis, CYP26B1 acts to remove RA and so functions as a meiosis-inhibiting factor. Experimental observations involving the addition or functional blockade of RA function in mouse fetal gonads indicate that RA is both necessary and sufficient to trigger the initial transition from mitosis to meiosis in germ cells of appropriate maturity. Evidently, RA induces meiosis via direct or indirect upregulation of *Stra8* expression in fetal germ cells.

Mouse fetal testes and ovaries are remarkably different in structure; in the male gonad, germ cells are enclosed in testis cords, whereas there is far less organization in the ovary. Hence, it is possible that the system of preventing meiosis in fetal testes by RA degradation is assisted by the physical shielding provided by this compartmentalization. If and when germ cells do enter meiosis in a developing testis, whether occasionally in nature or induced in experimental situations, these cells are eliminated by apoptosis (MacLean et al., 2007; Yao et al., 2003). A multi-level system that guards against meiosis in fetal male germ cells might be important for ensuring the normal somatic development of the testes, because meiotic germ cells probably produce signaling factors appropriate for ovarian but not testicular development (McLaren, 1991). Also, because meiotic germ cells apoptose in the fetal testis, avoidance of meiosis would maximize fertility.

The recent identification of a role for RA in the initiation of meiosis adds much to our understanding of sex-specific germ cell development in mammals, and will influence the approaches used to study the mechanism of meiosis and its somatic control. Many new questions have arisen based on these findings, opening up new lines of investigation. As yet, there are no clear answers to the questions of why is meiotic division specific to germ cells, what defines the window of competence of germ cells to respond to RA and how is RA signaling used to engage the meiotic machinery? With the current intense interest in the regulation of germ cell behavior among reproductive and developmental biologists, answers to these questions will be keenly sought.

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