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Regulation of germ cell meiosis in the fetal ovary

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ABSTRACT Fertility depends on correct regulation of meiosis, the special form of cell division that gives rise to haploid gametes. In female mammals, germ cells enter meiosis during fetal ovarian development, while germ cells in males avoid entering meiosis until puberty. Decades of research have shown that meiotic entry, and germ cell sex determination, are not initiated intrinsically within the germ cells. Instead, meiosis is induced by signals produced by the surrounding somatic cells. More recently, retinoic acid (RA), the active derivative of vitamin A, has been implicated in meiotic induction during fetal XX and postnatal XY germ cell development. Evidence for an intricate system of RA synthesis and degradation in the fetal ovary and testis has emerged, explaining past observations of infertility in vitamin A-deficient rodents. Here we review how meiosis is triggered in fetal ovarian germ cells, paying special attention to the role of RA in this process.

KEY WORDS: *germ cell, meiosis, ovary, retinoic acid*

Introduction

Mammalian life begins with the union of two cells, spermatozoa and oocytes, the generation and maturation of which are carefully controlled throughout fetal and reproductive life. In the embryo, the specification and migration of germ cells to the developing gonads is the same in males and females. When the primordial gonads begin to differentiate as ovaries or testes, the developmental pathways of germ cells diverge, leading to either oogenesis in females or spermatogenesis in males. In a newly specified ovary, germ cells enter into the first phase of meiosis—the special form of cell division, unique to germ cells, that allows them to produce haploid cells necessary for sexual reproduction. In the fetal ovary, entry into meiosis is seen as the first indication that germ cells have embarked on oogenesis (McLaren 1984). In a newly specified testis, germ cells enter a period of mitotic quiescence and remain in that state until just prior to puberty, when meiosis commences (Hilscher 1974).

Clearly, sex differentiation represents a crucial fork in the road of germ cell development and it is important to understand how the correct path is chosen. Somewhat surprisingly, the intrinsic sex chromosome content of the germ cells (XX or XY) plays no part in this decision, but instead the germ cells are instructed by molecular signals from the testicular or ovarian environment (Evans *et al.,* 1977; Ford *et al.,* 1975; Palmer and Burgoyne 1991). In the last decade, the nature of some of these signals has been uncovered. In this review we focus on how meiosis entry is triggered in the fetal ovary and especially on the role of retinoic acid (RA) in the process.

Generating germ cells: a brief history

Germ cells originate from the primitive streak of the extra-embryonic ectoderm from 6.25 days *post coitum* (dpc) in the mouse embryo (Lawson 1999; Ying *et al.,* 2000; Ying and Zhao 2001). At this time, bone morphogenetic protein (BMP) signalling factors BMP2 and BMP4 emanate from the extra-embryonic ectoderm and visceral endoderm to induce germ cell markers within a cohort of approximately 40 cells by 7.25 dpc (Ohinata *et al.,* 2005; Saitou *et al.,* 2002). Accordingly, loss of BMP signalling results in a diminished founding germ cell population (Ying *et al.,* 2000). Consistent with the pluripotent nature of the germ cell population, their transcriptional profile is characterised by expression of pluripotency genes, including *Oct3/4* and *Sox2* (Ohinata *et al.,* 2005; Scholer *et al.,* 1990; Yabuta *et al.,* 2006), and repression of somatic marker genes under the influence of BLIMP1 (Hayashi *et al.,* 2007; Ohinata *et al.,* 2005). For a comprehensive review of this process, see (Saitou 2009).

Abbreviations used in this paper: CYP26, cytochrome P450-related hydroxylase; EC, embryonal carcinoma; ES, embryonic stem; HDAC, histone deacetylase; RA, Retinoic acid; RALDH, retinal dehydrogenase; RAR, retinoic acid receptor; RXR, retinoid-X receptor; SAHA, suberoylanilide hydroxamic acid; SC, synaptomemal complex; TSA, trichostatin-A; VAD, vitamin A deficient.

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As development progresses the germ cells proliferate and migrate out of the ectoderm and into the embryo proper. Here they become motile in response to directional cues originating from the surrounding soma, including stromal derived factor 1. This change in motility is correlated with germ cell morphological changes, such as polarisation and extension of cytoplasmic protrusions (Anderson *et al.,* 2000). From 8.5 - 10.5 dpc the germ cells migrate anteriorly through the hindgut towards the newly forming genital ridges (Lawson and Hage 1994; Molyneaux *et al.,* 2001); reviewed by (Richardson and Lehmann 2010). Once residing in the genital ridges, motility and polarisation are lost and the germ cells exhibit a large rounded shape with prominent nucleoli and surrounding ring-like cytosol (Baillie 1964; Donovan *et al.,* 1986). Changes to chromatin architecture also occur upon entry into the genital ridge; germ cells erase all genome methylation (Hajkova *et al.,* 2002; Sato *et al.,* 2003; Szabo *et al.,* 2002) and lose repressive histone modifications (Hajkova *et al.,* 2008; Seki *et al.,* 2005). The previously inactive X chromosome of XX germ cells is also re-activated (Monk and McLaren 1981; Tam *et al.,* 1994).

Cross-roads: oogenesis or spermatogenesis?

Having reached the genital ridges, the surrounding somatic cells become responsible for nurturing the germ cell lineage during all subsequent stages of development. In addition to supporting germ cell survival, coercing the germ cells towards oogenesis or spermatogenesis is arguably the most crucial role of the soma at this time. During specification, migration and genital ridge colonisation, the behaviour of XX and XY germ cells is identical; it is only when sex differentiation of the gonadal soma has occurred that germ cells commit to a male or female sexual fate (McLaren and Southee 1997). In an XY genital ridge, expression of the gene *Sry* from 10.5 dpc in pre-supporting cells triggers differentiation of Sertoli cells and the subsequent cascade of testis development (Koopman *et al.,* 1991). Germ cells in the fetal testis environment become encased within the developing testis cords and are directed to enter the G_1/G_0 phase of mitosis from 12.5 dpc, apparently marking their commitment to spermatogenesis (Hilscher 1974). In males, germ cells initiate meiosis postnatally. In an XX genital ridge, in the absence of *Sry,* expression of *Rspo1, Wnt4* and *Foxl2* in the pre-supporting cells triggers ovarian development (reviewed by (Liu *et al.,* 2010)). Germ cells in the fetal ovary are directed to enter the first phase of meiosis from 12.5 - 13.5 dpc, apparently marking their commitment to oogenesis (McLaren and Southee 1997).

Meiosis: mechanics and markers

Meiosis involves two rounds of cell division, the first (Meiosis I) with, and the second (Meiosis II) without, DNA replication. Sharing many similarities with mitosis, each division is broken into the stages prophase, metaphase, anaphase and telophase. A defining feature of meiosis during prophase I sees homologous chromosomes (one maternal and one paternal) 'cross-over' by forming double strand breaks that recombine to exchange genetic information. Crossing over requires a special cellular scaffold called the synaptonemal complex (SC) that forms along each sister chromatid, binding them together (termed synapsis) to facilitate the genetic exchange.

Because the SC is unique to meiosis, the proteins that comprise this structure are useful in recognizing meiotic onset; particularly, synaptonemal complex protein 3 (SYCP3) and 1 (SYCP1) which form part of the lateral element of the SC (extending along the length of the chromosome) and the central element, respectively (Dobson *et al.,* 1994; Heyting *et al.,* 1988; Klink *et al.,* 1997). Other markers include factors involved in double strand break formation and repair: SPOII (Spo11 meiotic protein covalently bound to DSB homolog [*S. cerevisiae*]), γH2AX (H2A histone family, member X), Rad51 (Rad51 homolog [*S. cerevisiae*]) and DMC1 (dosage suppressor of mck1 homolog, meiosis-specific homologous recombination) (Keeney *et al.,* 1997; Pittman *et al.,* 1998; Yoshida *et al.,* 1998). These proteins, in addition to chromosome structure/morphology, are considered reliable hallmarks of meiotic entry, while gene expression of *Sycp3* is considered a marker of germ cells preparing for meiosis, rather than robust meiotic entry (Novak *et al.,* 2006).

Entry into meiosis: a role for retinoic acid

For many years it was unclear whether germ cells were intrinsically programmed to enter meiosis, or if they were responding to signals from the somatic cell environment. Evidence for cell-autonomous meiosis entry was seen in several experimental situations: when germ cells migrated to ectopic locations, such as the adrenal gland or mesonephros, though they were devoid of the testicular or ovarian niche, some germ cells were observed entering meiosis (McLaren 1983; Upadhyay and Zamboni 1982; Zamboni and Upadhyay 1983). Similarly, when germ cells were co-cultured with other tissues such as embryonic lung cells, meiosis entry was also witnessed (McLaren and Southee 1997). These findings suggested that meiosis entry was intrinsic to the developing germ cells rather than a result of somatic cue(s) (Donovan *et al.,* 1986).

In contrast to this body of work, under a different set of experimental conditions it became clear that germ cells were being directed to enter meiosis by some exogenous factor. Co-culture of testis and ovary tissue revealed the ability of ovarian somatic cells to induce meiosis in XY germ cells (Byskov and Saxen 1976). Further, it was discovered that the mesonephros, and specifically the tubule network that makes up the rete ovarii/testis, was a source of meiosis-inducing signal(s) since, in its absence, meiosis was avoided (Byskov 1974; Byskov and Saxen 1976). Decades later, we now know that the factor produced from the mesonephros is retinoic acid (RA); RA is also produced in many extra-gonadal tissues (including the lung and adrenal), presumably explaining why ectopic germ cells enter meiosis in these locations. We now look at retinoid signaling in detail, discuss how it is implicated in germ cell development and ask the question: is RA necessary for meiosis?

Fine-tuning retinoid levels: synthesis vs. degradation

Retinoic acid, derived from retinol (vitamin A), is a lipophilic molecule synthesized within numerous tissues. There are two steps in RA synthesis: firstly, retinol is reversibly converted to retinal by the alcohol dehydrogenase and short chain dehydrogenase/reductase family of enzymes. Secondly, in the presence of retinal dehydrogenase (RALDH) enzymes, retinal is oxidized irreversibly to RA.

RA is the active metabolite of retinoid signalling and a ligand for nuclear receptors that control gene transcription. Retinoic acid receptors (RARs) form heterodimers with retinoid-X receptors (RXRs); in the absence of RA the RAR/RXR complex is bound to retinoic acid response elements (RAREs) found in gene promoters

and co-repressor recruitment induces transcriptional repression. Co-repressor complexes often comprise nuclear receptor co-repressor (NCOR2) and histone deacetylases (HDACs). Binding of RA to the RAR/RXR complex alters the RAR ligand-binding domain such that co-activators, including nuclear receptor co-activator (NCOA), CREB binding protein (CBP) and p300, are recruited and transcription is promoted (reviewed by (Niederreither and Dolle 2008)). More that 500 genes have been identified as being RA-responsive, though it is likely that the majority of these are secondary targets (Balmer and Blomhoff 2002).

RA is able to be catabolised by the cytochrome P450 family of enzymes that convert RA to primary metabolites including 4-oxo-RA, 4-OH-RA and 18-OH-RA. Within the P450 family there are three CYP26 isoforms: CYP26A1, B1 and C1, which share only 42-51% aa sequence homology (Taimi *et al.,* 2004; White *et al.,* 2000a). The preferred substrate for CYP26A1 and B1 is all-*trans*-RA, while CYP26C1 favours 9-*cis*-RA. CYP26A1 has the highest catalytic activity and CYP26B1 the highest affinity for all-*trans*-RA, respectively (Topletz *et al.,* 2012). There are conflicting reports as to whether the metabolites produced during RA oxidation are biologically active; *in vitro* several oxidative metabolites have been shown capable of binding RARs and possess pharmacological activity (Idres *et al.,* 2002). Further, differentiation and proliferation was stimulated by some metabolites in spermatogonia in vitamin A-deficient mice (Gaemers *et al.,* 1996) and in epithelial differentiation of intestinal cells *in vivo* (McCormick *et al.,* 1978). In contrast to these adult tissues described above, it seems that RA oxidative metabolites are superfluous to mouse embryonic development; genetic ablation of the gene encoding the RA-producing enzyme RALDH2 rescued the affects of *Cyp26a1* loss of function in mice (Niederreither *et al.,* 2002).

The amount of RA to which a particular cell or tissue is exposed is tightly regulated, being controlled at multiple levels (reviewed by (Niederreither and Dolle 2008; Pennimpede *et al.,* 2010)). First, the availability of retinol during embryonic development is dependent on the mother's diet. Secondly, RA-producing enzymes convert retinol to all-*trans*-RA at different efficiencies; therefore, RA concentration is determined by which retinal dehydrogenase enzyme (RALDH1, 2 or 3) a particular tissue expresses. Thirdly, RA-degrading enzymes degrade RA to polar metabolites with different efficiencies; therefore, RA concentration is affected by which degradation enzyme (CYP26A1, B1, C1) the tissue expresses. Fourthly, the nuclear receptors for RA (RARs α,β,γ; RXRs) each have different responsiveness for both RA and their co-factors (both repressors and activators); this determines a cell's sensitivity in the presence and absence of RA. Additionally, each RAR displays tissue-specific expression, allowing for further fine-tuning of the intracellular response to RA.

Is retinoic acid necessary for meiosis?

Even before the RA metabolism pathway was elucidated in the 1960s (Dowling and Wald 1960), vitamin A deficiency (VAD) in rats had highlighted the importance of this essential nutrient for both male and female reproduction (Evans 1928; Wolbach and Howe 1925). It was later discovered that dietary supplementation of VAD rats with either vitamin A or RA overcame the block in meiosis and restored male fertility (Morales and Griswold 1987; Van Pelt and De Rooij 1990). Studies of female VAD rats determined that 40 – 230 mcg/rat/day of RA was required for ovulation, fertilisation, implantation and subsequent embryo development: the higher doses needed from mid-gestation onwards (Kaiser *et al.,* 2003; See *et al.,* 2008; White *et al.,* 2000b; White *et al.,* 1998). Such studies have uncovered the important roles for RA during many aspects of development, reviewed by (Clagett-Dame and Knutson 2011).

With respect to fetal germ cell development, RA was first reported in this system as a pro-survival and proliferation factor for *in vitro* culture of germ cells at 8.5, 11.5 and 13.5 (Koshimizu *et al.,* 1995; Morita and Tilly 1999) and female germ cells were found to express RARs at 13.5 dpc (Morita and Tilly 1999). In rat ovaries, RA and an RARα agonist were found to accelerate germ cell progression through meiosis (Livera *et al.,* 2000). Later, expression screens of whole embryonic gonads highlighted male-specific expression of the RA-degradation enzyme *Cyp26b1* (Bowles *et al.,* 2006; Menke and Page 2002) and female-specific expression of the RA-responsive gene *Stra8* (Bowles *et al.,* 2006; Menke *et al.,* 2003). These observations provided the first hints that RA levels might be providing instructive cues for female germ cells.

Stimulated by retinoic acid, gene 8 (*Stra8*), as its name implies, was first identified as an immediate early RA-responsive gene from P19 embryonal carcinoma (EC) cells: RA induces *Stra8* expression in these cells in as little as 2 hours (Bouillet *et al.,* 1995; Oulad-Abdelghani *et al.,* 1996). Two putative RAREs have been identified within the *Stra8* promoter and, using mutational analysis, both appear to be required for RA-induced expression in F9 cells (Wang and Tilly 2010). While the structure and precise function of STRA8 remains a mystery, its expression is restricted to pre-meiotic germ cells (during oogenesis and spermatogenesis) and, upon stimulation with RA, embryonic stem (ES) cells and EC cell lines P19 and F9 (Bouillet *et al.,* 1995; Oulad-Abdelghani *et al.,* 1996). Importantly, using genetic deletion in mice, *Stra8* was shown to be essential for germ cell initiation of meiosis in both sexes (Anderson *et al.,* 2008; Baltus *et al.,* 2006); in its absence germ cells fail to undergo pre-meiotic DNA replication and subsequent meiotic chromosome condensation, cohesion, synapsis and recombination.

As mentioned, expression of *Stra8* in female germ cells, together with expression of *Cyp26b1* in the testis, suggested a mechanism for meiosis regulation in the developing gonads: RA was present in the fetal ovary to induce *Stra8* (and initiate meiosis) and this signal would be actively degraded in the testis (to avoid meiosis) (Fig. 1). This notion has subsequently been tested in many ex vivo culture experiments as well as genetic models. Treatment of fetal testes or isolated XY germ cells with exogenous RA, RAR agonists (BMS-194753, BMS-213309, BMS-270394) or CYP26B1 inhibitors (ketoconazole, R115866) induced expression of many pre-meiotic and meiotic markers in XY germ cells including *Stra8*, *Sycp3, Dmc1* and γH2AX (Bowles *et al.,* 2010; Bowles *et al.,* 2006; Koubova *et al.,* 2006; MacLean *et al.,* 2007; Naillat *et al.,* 2010; Trautmann *et al.,* 2008). Importantly, in explant cultures, pharmacological inhibition of both CYP26B1 (to increase RA levels) and RARs (BMS204493, AGN193109; to prevent cells from responding to RA) caused no increase in *Stra8* expression (Koubova *et al.,* 2006) implying that CYP26b1 must function to degrade RA in this system. Conversely, in XX gonads, addition of RAR antagonists suppressed expression *Stra8* relative to wild type controls (Bowles *et al.,* 2006; Koubova *et al.,* 2006), as did RALDH inhibition in both mouse and human ovaries using citral (Bowles *et al.,* 2006; Le Bouffant *et al.,* 2010).

In vivo models in which the RA signaling cascade is disrupted at

different levels have also shed light on the role of retinoid signaling in germ cell meiosis. Loss of *Cyp26b1* in mice led to a 3-fold increase in RA levels within fetal testes; subsequently XY germ cells expressed markers of meiosis (*Stra8*, *Scp3, SpoII, Dmc1,* γH2AX) and some progressed through to pachytene of meiosis I by 16.5 dpc (Bowles *et al.,* 2010; Bowles *et al.,* 2006; MacLean *et al.,* 2007). Interestingly, XX germ cells in *Cyp26b1-/-* ovaries also expressed higher levels of *Stra8* relative to wildtype germ cells (Bowles *et al.,* 2010). This finding is likely explained by the observation that, before its upregulation in testes at 12.5 dpc, *Cyp26b1* is expressed at low levels in both XX and XY gonads at 11.5 dpc. *Cyp26b1* expression at this time would presumably ensure that germ cells arriving at the genital ridges are not exposed to RA immediately. In the *Cyp26b1* knockout, exposure to RA at this early stage was found to induce premature meiotic entry (Bowles *et al.,* 2010).

While experiments involving the *Cyp26b* knockout mouse model confirmed that XY germ cells are capable of responding to RA (just like XX germ cells) in this artificial situation, more direct evidence for RA's instructive role during natural XX germ cell meiosis came from analysis of VAD ovarian development. In rat embryos severely deficient for vitamin A (mothers fed just 1.5μg all-*trans* RA per gram of diet) the majority of XX germ cells remained undifferentiated and did not express *Stra8* or enter meiosis (Li and Clagett-Dame 2009). This effect was dose-dependent: numbers of meiotic germ cells were decreased in moderately VAD embryos (fed 12μg all-*trans* RA per gram of diet) compared to controls (30% compared to 75%), and so too was the level of *Stra8* expression in these ovaries (Li and Clagett-Dame 2009). In that study, germ cell numbers remained unchanged between VAD and control ovaries, demonstrating that dietary intake of Vitamin A positively correlates with meiosis entry in XX fetal germ cells in rodents.

Notably, induction of meiosis by RA does not appear to be limited to fetal rodent ovarian development. Recently, expression of RA synthesising enzymes (RALDH1, 2 and 3), along with RAR expression was detected within human fetal ovaries (Childs *et al.,* 2011; Le Bouffant *et al.,* 2010). Using a human fetal organ culture system, RALDH1 was found to be expressed at highest levels during meiosis initiation and its inhibition using citral reduced numbers of meiotic germ cells. Conversely, addition of exogenous RA to this system increased numbers of meiotic germ cells relative to controls (Childs *et al.,* 2011). RA metabolism has also been studied in chickens: here RALDH2 appears the predominant RA synthesising enzyme that triggers expression of *Stra8* prior to meiotic entry (Nekrasova *et al.,* 2011; Smith *et al.,* 2008; Yu *et al.,* 2012). Ovarian and testicular cultures of an amphibian species, *Pleurodeles waltl,* with RA have also highlighted a conserved role for RA-dependent meiosis in vertebrates (Wallacides *et al.,* 2009).

Unlike the situation in the developing ovary, testicular germ cells initiate meiosis just prior to puberty, which in rodents occurs within 1-2 weeks postnatally. In VAD mice modulation of RA signaling, both by the germ cells and supporting Sertoli cells, was shown to be responsible for the synchronous cycling of germ cell differentiation (Sugimoto *et al.,* 2012). Data on whether human testes, or testes in other vertebrates, similarly utilise RA regulation for this purpose is currently lacking, reviewed by (Hogarth *et al.,* 2011).

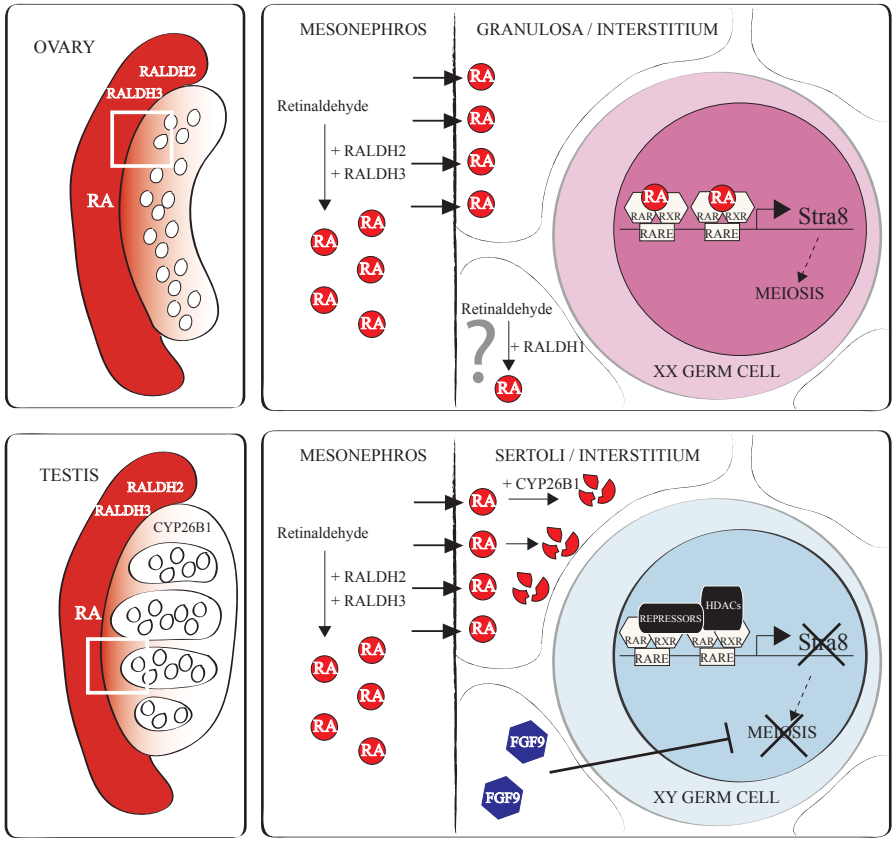

Fig. 1. Regulation of germ cell meiosis by retinoic acid. *The mesonephros attached to the ovary or the testis expresses the retinoic acid (RA) synthesizing enzymes RALDH2 and RALDH3, resulting in the production of high levels of RA. RA produced in the mesonephros diffuses into the ovary where it acts directly on germ cells. RA binds RAR/RXRs on both identified RAREs of the* Stra8 *promoter to induce transcription. Meiosis then progresses in ovarian germ cells. The granulosa and interstitial cells express RALDH1 at low levels, perhaps also contributing to the local production of RA within the ovary. In the testis, Sertoli and interstitial cells express the RA-degrading enzyme CYP26B1, preventing RA from stimulating* Stra8 *transcription and subsequent meiosis in testicular germ cells. In the absence of RA, repressor co-factors including HDACs bind the RAREs of the* Stra8 *promoter to repress transcription. Additionally, FGF9 produced by the Sertoli cells acts directly on testicular germ cells to inhibit meiosis.*

Where does the retinoic acid come from?

While the initial clue that RA was involved in germ cell meiosis was ovarian expression of *Stra8* and testicular expression of *Cyp2b1*, a more complete picture of which cells in the developing gonads respond to RA and how, when, and where it is degraded has now been formed. But what is the source of the RA? As mentioned, very early work revealed that the mesonephros was required for inducing meiosis in XX germ cells (Byskov 1974; Byskov and Saxen 1976); this ability was evidently not restricted to the XX mesonephros, since mesonephroi from both sexes could equally induce meiosis

in XY germ cells (Byskov and Saxen 1976; O and Baker 1976). It was not surprising, then, to find that *Raldh2* is expressed at high levels in the mesonephric duct and tubules, which are physically connected to the anterior end of the gonads of both sexes (Bowles *et al.,* 2006; Byskov 1974). *Raldh3* has also been detected in the mesonephros at 10.5 dpc (Zhao *et al.,* 2009). Using two transgenic assays in which LacZ expression is driven by the RARβ promoter to provide a read-out for RA production, F9 RARE reporter cells (Wagner *et al.,* 1992) and the *RARE-lacZ* mouse line (Rossant *et al.,* 1991), mesonephric cells triggered a strong β-galactosidase response in both models indicating high levels of RA were being produced from this tissue (Bowles *et al.,* 2006). Therefore it was hypothesized that the mesonephros is the source of RA, which flows into the adjacent gonad in both sexes to induce meiosis in XX germ cells but is degraded in the *Cyp26b1*-expressing testis (Bowles *et al.,* 2006)(Fig. 1). A recent study of 11.5 dpc ovaries cultured without the mesonephros observed some XX germ cells entering meiosis, although the presence of the mesonephros and/or RA sped up meiotic progression (Guerquin *et al.,* 2010). This is somewhat surprising in view of recent independent studies that confirmed the necessity of the mesonephros (Kumar *et al.,* 2011); perhaps differing culture conditions, including media/sera, and timing of these assays account for the discrepancies. Additionally, because the ovary itself expresses *Raldh1* (Bowles *et al.,* 2009), albeit at low levels, it is possible that there was sufficient RA present in this culture system to allow some germ cells to enter meiosis. RALDH1 is known to be much less efficient in generating all-*trans* RA and has been identified at sites where low levels of RA are required for morphogenesis (Haselbeck *et al.,* 1999). As depicted in Fig. 1, RALDH1 expression in the ovary could provide another source of RA under normal conditions, as appears to be the case in the human ovary (Childs *et al.,* 2011).

How much retinoic acid?

The local concentration of RA has not been measured for the gonad or mesonephros, but concentrations at other sites in 13.5 dpc mouse embryos are reported to be between 10-40nM (Horton and Maden 1995). While exogenous RA was initially implicated in germ cell meiosis in organ culture using much higher concentrations (0.7-1μM; (Bowles *et al.,* 2006; Koubova *et al.,* 2006), more recent studies have demonstrated that much lower levels (as low as 1nM) are sufficient for not only activation of *Stra8* but also functional meiosis (Bowles *et al.,* 2010; Chen *et al.,* 2012; Ohta *et al.,* 2010; Zhou *et al.,* 2008). Further, in vitro, high levels of RA are detrimental to germ cell survival in both testes and ovaries (Best and Adams 2009). Therefore, it is likely that a very low levels of endogenous RA are sufficient to induce meiosis in the *in vivo* situation.

Challenging the retinoic acid theory

Recently, a study by Kumar *et al.,* 2011 investigating meiotic induction in the absence *Raldh2* and *Raldh3* has challenged the theory that meiotic induction is dependent on RA. Three conclusions are drawn from that study: Firstly, in the absence of RA-producing enzymes RALDH2 and RALDH3, *Stra8* was found to be expressed in fetal ovaries at 13.5 dpc, leading the authors to conclude that RA is unnecessary for meiotic induction. Secondly, *Stra8* expression was observed in fetal testes when CYP26b1 was chemically inhibited with ketoconazole, but only in the presence of the mesonephros, leading the authors to propose the existence of a CYP26B1-sensitive factor produced by the mesonephros, that is not RA, is necessary for meiotic induction. Thirdly, using ChIP, the putative RARE elements of the *Stra8* promoter were found to be bound by RAR antibodies only weakly, leading to the conclusion that *Stra8* is unlikely to be regulated by RA *in vivo*. Given the persuasive evidence pointing to a finely tuned mechanism of RA signaling controlling germ cell meiosis, the findings of Kumar *et al.,* 2011 are surprising. However, alternative explanations for the conclusions of that study are presented below (also thoroughly reviewed by (Griswold *et al.,* 2012)).

Analyzing *Raldh2-/-;Raldh3-/-* double-mutant embryos, no RA activity was detectable in the gonad or mesonephros using the *RARE-LacZ* reporter line, which the authors show to be sensitive to 25nM RA and above (Kumar *et al.,* 2011). As mentioned already, germ cells respond by up-regulating *Stra8* in the presence of very low levels of RA *in vitro* (as little as 1nM; (Bowles *et al.,* 2010; Chen *et al.,* 2012; Ohta *et al.,* 2010; Zhou *et al.,* 2008)). Additionally, redundancy within the RALDH family has been reported in tissues such as the dorsal retina (Fan *et al.,* 2003) and it is possible that RALDH1 expressed in the ovary produces sufficient levels of RA in the absence of RALDH2 and RALDH3 to induce meiosis even if not sufficiently high to elicit *RARE-LacZ* reporter expression. Therefore, biochemical quantification of RA levels in the *Raldh2-/-;Raldh3-/-* mutants should be sought before excluding a role for RA in meiosis induction.

Using ChIP analysis in gonadal tissue, the two putative RARE sites in the *Stra8* promoter showed only weak binding to RAR antibodies (Kumar *et al.,* 2011), leading the authors to suggest that RA does not bind to and stimulate *Stra8* expression *in vivo*. The extremely low contribution of *Stra8*-expressing germ cells in this assay however, has undoubtedly biased this result to reflect the situation in somatic cells of the gonad. We suggest that the weak binding of RARβ to the most proximal RARE (Kumar *et al.,* 2011) may instead be a very significant positive result, given the low germ cell: somatic cell ratio at 13.5 dpc.

Finally, RA is the only known substrate of CYP26B1, making the suggestion of a mesonephros-derived, non-RA, CYP26B1-degraded factor responsible for inducing meiosis untenable at present.

Meiotic competence: what makes germ cells so special?

Although RA directs differentiation and patterning in many embryonic tissues, only the germ cell lineage responds to this morphogen by entering meiosis. What is it that makes germ cells respond to RA in this unique way? A major clue comes from the epigenetic state of germ cells residing in the genital ridges.

Unlike the rest of the somatic cells in the developing embryo, germ cells undergo genome-wide de-methylation (Hajkova *et al.,* 2002; Sato *et al.,* 2003; Szabo *et al.,* 2002). Methylated promoter regions are generally associated with gene silencing and hypomethylated regions with gene transcription; *Ddx4*, *Dazl, Gcna1* and *Scp3* are common germ cell genes that become hypomethylated, and therefore expressed, upon entry into the gonad (Maatouk *et al.,* 2006). Hypomethylation largely occurs by 12.5 dpc for most genes investigated (Guibert *et al.,* 2012; Hajkova *et al.,* 2002; Henckel *et al.,* 2012; Szabo *et al.,* 2002; Szabo and Mann 1995) and female

germ cells display less global methylation than male germ cells at 13.5 dpc (Popp *et al.,* 2010). Sex-specific re-methylation is acquired during late gestation and perinatally for male and female germ cells, respectively (Davis *et al.,* 2000; Li *et al.,* 2004; Ueda *et al.,* 2000). In addition to global de-methylation, germ cells also lose repressive histone modifications, including H3K27me3 and H3K9me3, and are positive for the active histone modification H3K9ac (Abe *et al.,* 2011; Hajkova *et al.,* 2008; Seki *et al.,* 2005). These marks indicate weak histone-DNA interaction and, together with global DNA hypomethylation and chromosome decondensation (Hajkova *et al.,* 2008), germ cell chromatin is considered to be in an 'open' state. Undoubtedly, this chromatin configuration would have a bearing on the suite of genes that respond to RA exposure; however there have been few studies that test this hypothesis directly.

One factor potentially linking chromatin organization with RA-responsiveness is chromobox homologue 2 (CBX2). CBX2 is a polycomb group protein involved in chromatin remodelling and defines the RA-sensitive window for *Hox* gene expression during early embryogenesis (Bel-Vialar *et al.,* 2000). XY germ cells devoid of *Cbx2* exhibit premature meiosis suggesting that the chromatin restructuring by CBX2 is necessary to avoid RA stimulation of meiosis in these cells (Baumann and De La Fuente 2011).

As mentioned, HDACs are among many co-factors with which RARs associate to negatively regulate transcription (Hong *et al.,* 1997). In pre-meiotic germ cells, HDACs have been shown by co-immunoprecipitation to directly interact with RARγ (Wang and Tilly 2010). When histone acetylation (repression) was artificially abolished using the HDAC inhibitors trichostatin-A (TSA) and suberoylanilide hydroxamic acid (SAHA), the meiotic markers STRA8 and SYCP3 were detected in a few XY germ cells, as was the case when they were treated with RA (Wang and Tilly 2010). In F9 pre-meiotic cells, HDAC inhibition with TSA amplified RA-induced *Stra8* promoter activation. Using mutation analysis, a region about 920bp upstream of the *Stra8* transcription start site was required for the HDAC-mediated repression *in vitro* (Wang and Tilly 2010). Whether such a mechanism is relevant to *in vivo* germ cell development remains to be determined: as mentioned previously, pre-meiotic germ cells already possess 'open' and accessible chromatin.

Conclusions

Although vitamin A deficiency was associated with infertility several decades ago (Dowling and Wald 1960), it is only recently that we have begun to unravel how RA metabolism directs meiosis during fetal ovarian development and postnatal testis development. A complex picture of RA metabolism and catabolism is emerging: RA is synthesised in the mesonephros of both sexes but is catabolised in the developing mouse testis to prevent germ cells from responding to the morphogen. In the fetal mouse ovary, RA induces *Stra8* expression in germ cells and they subsequently enter meiosis. This paradigm appears to hold true for multiple species, including human, chicken and amphibian.

But can we conclude that RA is necessary and sufficient for meiotic entry? Certainly, when RA levels are reduced through insufficient dietary intake of vitamin A, meiosis is compromised in a dose-dependent fashion. Similar effects are observed *in vitro* when RA synthesis is antagonised, indicating that RA is necessary for correct entry into meiosis for XX germ cells. Exogenous addition of RA, either chemically or genetically, induces XY germ cells to aberrantly enter meiosis, confirming that RA is sufficient for meiotic entry. Given the importance of correctly differentiating the germ cell lineage for fertility, it is unlikely that RA alone would regulate the process of meiotic entry. Indeed, we now know that, in addition to degrading RA with expression of CYP26B1, the fetal testis re-enforces the male differentiation pathway by expressing fibroblast growth factor 9 (FGF9), which is inhibitory to meiosis induction in XY germ cells (Barrios *et al.,* 2010; Bowles *et al.,* 2010). Future studies will likely uncover additional regulators of meiosis (both positive and negative) in this system, where germ cell fate cannot be left to chance.

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