The Role of Retinoic Acid in Germ Cell Development in Embryonic Mouse Gonads

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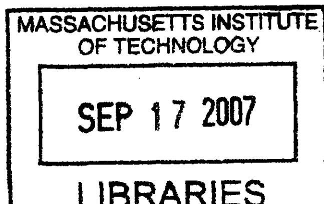
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Submitted to the Department of Biology on May 2, 2007
in Partial Fulfillment of the Requirements for the Degree of
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Abstract

Germ cells are the only cell type to undergo meiosis, a specialized cell division process necessary for the formation of haploid gametes. Timing of this process is sex-specific. Ovarian germ cells initiate meiosis during embryonic development, while testicular germ cells initiate meiosis after birth.

In a series of gonad explant culture experiments, I show that retinoic acid (RA) is required for meiotic initiation in embryonic ovaries, because it is necessary for *Stra8* (*Stimulated by retinoic acid gene 8)* expression. *Stra8* is required for pre-meiotic DNA replication in embryonic ovaries and it is only expressed in testes after birth. I also show that a cytochrome p450 enzyme CYP26B1, specifically expressed in embryonic testes but not ovaries, prevents *Stra8* expression in testes during embryonic development.

To confirm our results *in vivo,* and to examine if RA is sufficient to induce meiosis in embryonic testes, I generated *Cyp26b1-/-* and *Cyp26b1-/-Stra8-/-* mice. I show that germ cells in *Cyp26b1-/-* embryonic testes initiate a meiotic program but fail to complete meiotic prophase. Instead, germ cells proliferate until birth. RA also causes somatic cell defects. It inhibits Leydig cell differentiation and disturbs testis cord maintenance.

Thus, RA has distinct effects in embryonic ovaries and embryonic testes. In ovaries, it is required for meiotic entry with no known effects on somatic cell development. In embryonic testes, RA is not sufficient for functional meiotic prophase and it induces proliferation in germ cells. RA also disrupts embryonic testicular somatic cell development.

Thesis Supervisor: David C. Page
Title: Professor of Biology, Howard Hughes Medical Institute

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CHAPTER 1

Introduction to the development of germ cells in embryonic mouse gonads

Unique features of germ cell development have long fascinated scientists. First, germ cells are the only cell lineage which carries with it the DNA for the next generation. This presents a conundrum: Are germ cells the soma's way of making a new soma or is the soma the germ cells' way of making more germ cells? Second, to successfully dispatch the DNA to the next generation, germ cells are the only cell type in the body to undergo meiosis. This specialized cell division process results in haploid chromosome content and DNA recombination making gametes unique from the rest of the somatic cells. Third, germ cells, which initially look the same in both sexes, give rise to gametes with sex specific characteristics. The differences between the cellular structure of eggs (female gametes) and sperm (male gametes) are striking. How and why did they evolve to be so different?

This introduction will focus on one window of the mouse germ cell lifecycle – from their arrival to the gonads to the birth of the newborn mouse. During this critical ten-day period, germ cell sex is determined and sexual differentiation of the future gametes begins. This interval also provides a unique window of opportunity to study meiotic initiation in ovarian germ cells.

Homecoming

The somatic gonad and the germ cells originate in different locations in the embryo. The gonad (initially called the genital ridge) forms as a thickening of the coelomic epithelium on the ventral surface of the mesonephros (Byskov, 1994). Germ cells (also called primordial germ cells or PGCs) are derived from the epiblast (Falconer

and Avery, 1978; Gardner et al., 1985). Germ cells migrate to the gonad and proliferate during their migration. Approximately 1500 primordial germ cells enter each gonad between E10.5 and E11.5 (McLaren, 1984). The arrival of germ cells into the gonad is still shrouded in mystery. How do the germ cells know that they have reached their final destination and should not proceed any further? What molecular signals does the gonad use to communicate with the germ cells and vice versa? What are the first actions germ cells take once they arrive at the gonad?

During the last stretch of their migration (i.e. from the hindgut to the gonad), the characteristics of germ cells change substantially. Confocal microscopy revealed that germ cells leave the hindgut independently, but at the time of gonad colonization they start forming long processes (40 microns) to interface with each other and form networks. This clustering requires E-cadherin, which is upregulated after the germ cells leave the hindgut (Bendel-Stenzel et al., 2000). Once the germ cells reach the gonad, they lose the processes and their motility, and they aggregate. Interestingly, this sequence of movements can be reproduced in culture suggesting that this behavior may be intrinsic to germ cells at this stage of their development (Gomperts et al., 1994).

At the time of colonization, dramatic changes in gene expression can be observed in germ cells. They begin expressing highly conserved germ cell-specific genes like *mouse vasa homologue (Mvh)* and *deleted in azoospermia-like (Dazl)* (Toyooka et al., 2000; Hu, 2006). They also begin to express *germ cell-less (Gcl),* a mouse homologue of a component of *Drosphila* germ plasm (Toyooka et al., 2000).

Mvh, Dazl and *Gcl* are expressed in the germ cells of both sexes; however the knockout phenotypes differ between the sexes. *Mvh*-deficient germ cells in the testis exhibit a lower proliferation rate after colonization, while *Mvh*-deficient germ cells in ovaries proliferate normally (Tanaka, Toyooka et al. 2000). A knockout of *Gcl* exhibits a later spermatogenesis phenotype in testes while no phenotype has been reported in ovaries to date (Kimura et al., 2003a). Interestingly, *Dazl*-deficient germ cells in testes start dying several days after entering the gonad while *Dazl*-deficient germ cells in ovaries survive much longer (Lin and Page, 2005; Lin, 2005). These results suggest that while expression of these early germ cell genes is not sexually dimorphic, the process underlying proliferation in the arriving germ cells may already be different between the sexes (DiNapoli et al., 2006).

Postmigratory germ cells also start expressing *germ cell nuclear antigen 1 (Gcna1)* but the gene coding for this antigen has not yet been identified (Enders and May, 1994). Surprisingly, *Gcna1* expression does not require the germ cell's arrival at the gonad – in fact when germ cells are placed in the adrenal, they start expressing *Gcna1* at the time when they would normally enter the gonad (Wang et al., 1997; Richards et al., 1999). Unfortunately, similar experiments have not been done with *Mvh* or *Dazl.* However, *Dazl* is known to be expressed in some lines of embryonic stem cells suggesting that its expression can also be induced outside of the gonad (Lin, 2005). These results suggest that whatever factor induces the expression of these genes upon arrival to the gonad, it must also be present in other tissues. Alternatively, germ cells may be

preprogrammed to express these genes autonomously by a clock mechanism (e.g. after a certain number of cell divisions, regardless of their arrival to the gonad).

During their migration to the gonads, germ cells proliferate. After their arrival at the gonad, they undergo two or three more rounds of cell division (McLaren, 2003). During the proliferative phase inside the gonad, germ cells develop in clusters or nests. Some controversy surrounds formation of these clusters. *In vitro,* it has been shown that these clusters form by aggregation (Gomperts et al., 1994) but other studies have suggested that germ cell clusters arise by successive divisions of a single progenitor cell similar to cysts, which have been described in *Drosophila* (Pepling and Spradling, 1998). Whether the germ cells really form cysts by successive divisions of a progenitor or whether they simply aggregate into clusters is unknown but it has been conclusively shown that germ cells at this stage are connected by intercellular bridges and divide synchronously (Pepling and Spradling, 1998; Pepling, 2006).

Immediately after arriving into the gonad until about E13.5, germ cells themselves are not yet morphologically distinguishable between the sexes. However, during this time period, their somatic surroundings are changing dramatically. While it is not known how this changing environment influences the germ cells, it is hard to imagine that germ cells could be oblivious to these changes. Between E10 and E11.5, the gonads of both sexes are growing rapidly through cell proliferation and cell migration from the neighboring mesonephros (Schmahl et al., 2000). The somatic gonads are undergoing sex determination and their cell lineages are becoming sexually differentiated.

As the germ cells start arriving into the XY gonad around E10.5, *sex determining region Y (Sry)* expression starts in pre-Sertoli cells and continues through E12.5 (Koopman et al., 1990; Gubbay et al., 1990; Albrecht and Eicher, 2001). While not much is known about the molecular function of *Sry,* its expression is required for sex determination and sex differentiation of cell lineages in the developing testes. By E12.5, the testicular cords enclose the germ cells and the Sertoli cells, the somatic cells which support the germ cell development in the testes. Peritubular myoid cells shape the cords by forming a filamentous layer around the germ cells and the Sertoli cells. Fetal Leydig cells, the steroid producing cells, become separated from the germ cells and are located in the interstitial spaces between the cords (Fig.1).

Mesonephric cell migration into XY gonads continues until E16.5 but there is little or no mesonephric migration into the XX gonads after E11.5 (Martineau et al., 1997). In comparison to the developing testes, changes in the morphology of the ovary appear to be much less dramatic than in the testes. Germ cells and pregranulosa cells, the somatic cells which support germ cell development in the ovary, become organized into ovigerous (or ovarian) cords (Byskov, 1986; Guigon and Magre, 2006). The ovigerous cords are outlined by a basement membrane (Sawyer et al., 2002; Mazaud et al., 2005) instead of myoid cells, which are not present in the ovary. The function of these cords is

Figure 1. Illustration depicting sexual differentiation of the XX and XY mouse gonads (adapted from Menke, 2002).

XX Genital Ridge

E10.5

E11.5

E12.5

Ceolomic Epithelium of the Gonad

Mesonephros

Wolffian Duct

Mullerian Duct

Germ Cells Migrate Into Gonads

Ceolomic Epithelium of the Gonad

Mesonephros

Wolffian Duct

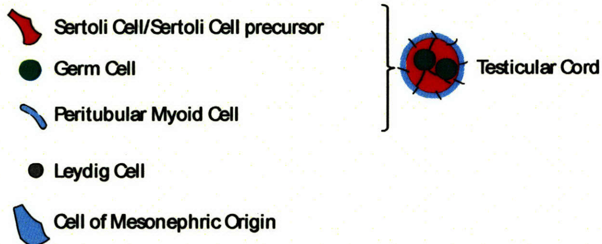
Mullerian Duct

E10.5

E11.5

E12.5

XY Genital Ridge



unknown. In mice, ovigerous cords are not very prominent; however, these cords are better defined in other mammalian species (Byskov, 1986).

While no equivalent of *Sry* has been identified in the ovary, recent microarray analyses show that somatic cells in ovaries undergo dramatic gene expression changes around the time germ cells arrive (Nef et al., 2005; Beverdam and Koopman, 2006). These results suggest that, similarly to the testis, the ovary is undergoing active sex determination and sex differentiation processes even though the somatic tissue appears much less organized than the testis at this time.

During migration, XX germ cells inactivate one of their X chromosomes similar to the X-inactivation of somatic cells. Soon upon their entry into the gonads, germ cells reactivate their inactive X chromosome (McLaren and Monk, 1981; Monk and McLaren, 1981; Nesterova et al., 2002). The exact time and mechanism of X chromosome reactivation is not known except that it begins shortly after arrival into the gonad and before germ cells initiate meiosis at E13.5 (McLaren, 2003). It is also not known if the reactivation is a cell autonomous process but it has been shown that XXY germ cells undergo X-chromosome reactivation upon arrival at the testes, suggesting that X-reactivation may not be sex-specific (Mroz et al., 1999).

During migration, germ cells carry methylation patterns. Once they enter the gonads, they undergo genome-wide demethylation and become demethylated by E13.5 (Monk et al., 1987). Both imprinted genes (*Peg3, Snrpn, H19* etc.) as well as non-

imprinted genes (*Actin*) become demethylated but certain sequences like repetitive elements and minor satellites appear to be only partially demethylated in germ cells (Hajkova et al., 2002). It is not known what regulates the rapid genome-wide demethylation in germ cells upon entry into the gonad and active demethylating processes have been suggested (Hajkova et al., 2002; Morgan et al., 2005). It is also not known if erasure of methylation patterns upon entry into the gonads is a conserved process in other species. The significance of this erasure is poorly understood – it has been suggested that genome-wide demethylation serves to prevent epimutations that may have occurred during prior germ cell development. However, several studies have shown that newly acquired epigenetic abnormalities are not effectively erased in germ cells and can persist for several generations (Morgan et al., 1999; Anway et al., 2005).

The effects of germ cells on the development of the somatic gonad have been studied. In gonads of both sexes, the absence of germ cells does not affect the early proliferation of somatic cells (Merchant, 1975). Furthermore, in the testes, germ cells appear dispensable because testes develop normally (though with a small delay) in the absence of germ cells (Merchant, 1975; Brennan and Capel, 2004). In ovaries, initial development also appears normal and the presence of germ cells is not required for the formation of the ovigerous cords (Merchant, 1975). However, the absence of germ cells has dramatic effects on the ovaries later in development. Germ cells are required for ovigerous cord breakdown and the somatic cell differentiation during follicle formation around birth. In germ cells' absence, follicles are not formed and the ovigerous cords stay intact (Merchant-Larios and Centeno, 1981; Guigon and Magre, 2006).

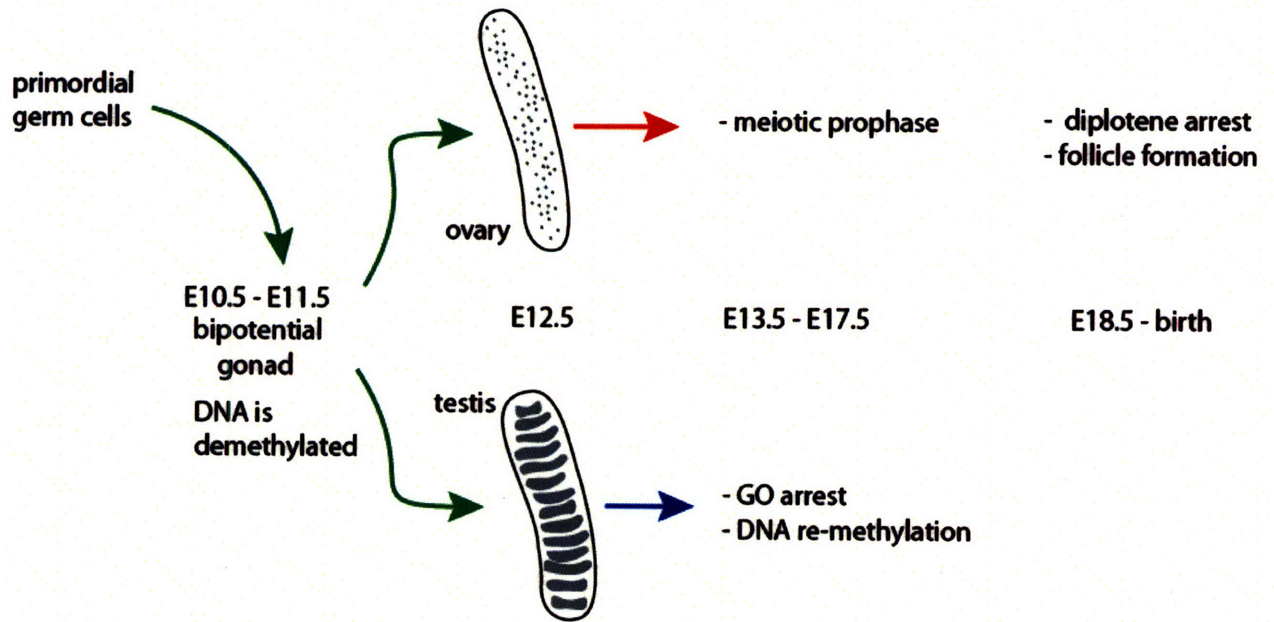
In conclusion, germ cells arrive at their home at a turbulent time when the somatic gonad has begun sex determination and sexual differentiation. Striking changes are also taking place in the germ cells upon their arrival. Within a short period, germ cells of both sexes undergo whole-genome demethylation and upregulate new genes. Germ cell morphology changes and their contact with each other increases. Germ cells form clusters and divide synchronously within them. XX germ cells reactivate their X chromosomes. The connections between these various processes and their functions are unknown but they are suggestive of a remarkable transition in germ cells.

To enter or not – the question of germ cell sex determination

Germ cell sex is thought to be determined by the somatic environment of the gonad into which the germ cells migrate (McLaren, 1984). Germ cells in ovaries are sex determined when they start entering meiotic prophase at E13.5. Sex determination of germ cells in the testes is much harder to define. Traditionally, it has been suggested that germ cells in testes are sex-determined when they enter G0 arrest at about the same time germ cells in ovaries enter meiosis (Fig. 2). However, reconstitution and culture experiments suggest that their sex must be determined earlier.

E12.5 and E13.5 XX germ cells placed into reconstituted testes develop as prospermatogonia and do not enter meiotic prophase as judged by chromosome condensation patterns (McLaren and Southee, 1997; Adams and McLaren, 2002). In contrast, E11.5 but not E12.5 or E13.5 XY germ cells cultured outside of the testes (e.g. in reconstituted ovary) will enter meiotic prophase

Figure 2. XX and XY germ cell development from E10.5 until birth.


primordial germ cells
ovary
- meiotic prophase
- diplotene arrest
- follicle formation
E10.5 - E11.5
bipotential gonad
DNA is demethylated
E12.5
E13.5 - E17.5
E18.5 - birth
testis
- G0 arrest
- DNA re-methylation

(Zamboni and Upadhyay, 1983; McLaren and Southee, 1997). These results show that germ cell sex determination in the testes must take place approximately between E11.5 and E12.5 (i.e. before germ cells start entering the G0/G1 arrest, which occurs between E13.5 and E16.5). What this process entails and how the testicular germ cells lose their sexual plasticity is unknown.

The chromosome dance

In females, meiotic prophase begins during embryonic development. In males, meiosis is initiated after birth. Why mammals have sexually dimorphic timing of meiotic entry is unknown but the consequences are clear. At birth, a pool of primordial follicles is established in ovaries representing the total number of germ cells available to females during their reproductive lifespan. In testes, foundations are laid out for generating an adult spermatogonial stem cell population replenishing male gametes throughout life.

Meiosis consists of two consecutive cell divisions without an intervening S phase and results in haploid chromosome content in gametes. In addition, during prophase of the first meiosis, recombination takes place. During embryonic development, XX germ cells progress only through leptotene, zygotene and pachytene stages and arrest in diplotene of the first meiotic prophase. This arrest is sometimes called dictyate arrest.

The decision to enter meiotic cell cycle is triggered by different cues in different organisms but its core molecular machinery appears to be highly conserved across species. Work in yeast suggests that the decision to enter meiosis occurs during the G1

phase of the cell cycle (Marston and Amon, 2004). Recent evidence in mice shows that a germ cell's commitment to meiosis must occur prior to or during DNA replication (Baltus et al., 2006), consistent with the decision taking place in G1.

Although pre-meiotic DNA replication during S phase likely uses much of the same machinery as pre-mitotic replication, differences exist. First, pre-meiotic DNA replication takes longer than pre-mitotic replication in all organisms studied so far (Crone et al., 1965; Cha et al., 2000; Marston and Amon, 2004). Second, pre-meiotic DNA replication produces sister chromatids held together by meiosis-specific cohesins such as *rec8-like 1 (Rec8L1)* (Klein et al., 1999; Eijpe et al., 2003). Third, in both yeast and in mice, factors required for pre-meiotic but not pre-mitotic DNA replication exist (Davis et al., 2001; Baltus et al., 2006). Unfortunately, no specific marker of pre-meiotic S phase in mice is currently known.

Leptotene is the first stage of the first meiotic prophase. The pairing of homologous chromosomes – the synapse - has not occurred yet. However, protein structures called axial elements start forming in short patches along the chromosomes. These axial elements contain *synaptonemal complex protein 3 (Sycp3).* Interestingly, double-stranded breaks associated with recombination and generated by the meiotic enzyme Sporulation protein 11 (SPO11) are detectable at this stage. This suggests that recombination is initiated prior to synapsis during leptotene (Mahadevaiah et al., 2001).. The H2A histone family protein, member x (H2AX) is phosphorylated near the sites of double-stranded DNA breaks. An antibody against phosphorylated H2AX labels cells during leptotene (Mahadevaiah et al., 2001). At this stage, chromosome ends are

attached to the inner surface of the nuclear envelope over most of the nuclear periphery and appear like thin bands or pearls on a necklace (Speed, 1982; Zickler and Kleckner, 1998).

At the leptotene-zygotene transition, chromosomes attached to the nuclear envelope become rapidly redistributed to one region, resulting in telomere clustering called the "bouquet" conformation. The "bouquet" conformation persists through zygotene (Zickler and Kleckner, 1999; Scherthan, 2003).

Zygotene, the second stage of the first meiotic prophase, is characterized by partial synapsis. Homologous chromosomes are now partly held together by the synaptonemal complex. This zipper-like structure is composed of two lateral elements (whose core is formed by the above-mentioned SCP3-containing axial elements) joined together by a transverse filament. An antibody against the phosphorylated H2AX labels cells also during zygotene but the staining is less intense than during leptotene (Mahadevaiah et al., 2001)

At pachytene, full chromosome synapsis is completed (Zickler and Kleckner, 1999). Chromosomes contract and appear short and thick (Speed, 1982). In mid pachytene, they are released from the "bouquet" organization and re-organize themselves evenly on the nuclear periphery. Shortly after, they are released from their nuclear envelope attachments (Zickler and Kleckner, 1999).

Diplotene is characterized by the desynapsis of homologous chromosomes (Speed, 1982). The dictyate nuclei are larger and the chromosomes appear decondensed (Speed, 1982). In oocytes, *y box protein 2 (Ybx2* or *Msy2)* is a convenient cytoplasmic marker for the diplotene or dictyate stages (Gu et al., 1998; Yu et al., 2001; Pangas et al., 2006). Little is known about the causes of diplotene arrest. However, granulosa cells may be responsible for its maintenance. If they are removed from the follicle, oocytes will resume the meiotic progression through the first meiotic division and arrest at metaphase 2 (Libby et al., 2002).

The length of each phase of the first meiotic prophase has been estimated and measured. It has also been noted that the duration of each phase appears to be similar between male and female germ cells. Pre-meiotic S phase is thought to last approximately 12 hours in contrast to the pre-mitotic S phase, which lasts approximately 6-8 hours. Leptotene duration is roughly 3-6 hours, zygotene 12-40 hours, and pachytene more than 60 hours. G2 phase is thought to be very short in germ cells – only about half an hour (Crone et al., 1965).

Unlike the postnatal testis, the embryonic ovary provides a convenient model for studying meiotic prophase. In the postnatal testis, except for the first round, not all germ cells initiate meiotic prophase at the same time. In an adult, each tubule contains cells at different stages of the cell cycle - a heterogeneous population that is difficult to study. In the embryonic ovary, all germ cells enter meiotic prophase. This makes it a particularly useful model for the study of meiotic prophase and its progression in mammals.

Meiotic prophase at the molecular level

Immediately after their arrival in the gonads, germ cells inside the XY or XX gonads appear indistinguishable and behave similarly. At E13.5, the cellular morphology of germ cells in the testes begins to differ from their counterparts in the ovaries. A subset of germ cells in ovaries show signs of chromosome condensation associated with meiotic prophase, while a subset of germ cells in testes starts entering G0 (also called G0/G1 or G1) arrest.

In ovarian germ cells, several genes required for meiotic cell cycle begin their expression around E12.5 in an anterior to posterior wave. This wave of expression includes all meiotic markers tested to date. Their expression sweeps through the ovary over a span of approximately four days (Menke et al., 2003; Yao et al., 2003; Bullejos and Koopman, 2004).

The earliest female specific germ cell gene expressed in a wave is *stimulated by retinoic acid gene 8 (Stra8)* (Baltus et al., 2006). *Stra8* was first discovered in a screen for retinoic acid inducible genes in embryonic carcinoma cells and subsequently characterized as a gene expressed exclusively in male but not female germ cells (Oulad-Abdelghani et al., 1996). A more recent study has corrected this finding and shown that *Stra8* is expressed in ovarian germ cells during embryonic development and testicular germ cells after birth (Menke et al., 2003). *Stra8* is expressed in an anterior to posterior pattern starting at E12.5 and disappears around E16.5 (Menke et al., 2003). It is unknown what induces *Stra8* upregulation in this characteristic wave-like pattern but it has been

shown that a transcriptional regulator *Sohlh1* is required for its downregulation (Pangas et al., 2006). Characterization of *Stra8-/-* gonads revealed that *Stra8* is required for pre-meiotic but not pre-mitotic DNA replication in embryonic mouse ovaries (Baltus et al., 2006).

Stra8 expression is closely followed by *Dmc1*, a meiotic recombinase, also expressed in an anterior to posterior wave (Menke et al., 2003). Interestingly, *Stra8-/-* ovaries fail to express *Dmc1*, suggesting that *Stra8* regulates this gene's expression. However, it remains unknown if *Stra8* is directly responsible for the *Dmc1* wave of expression.

Additional genes observed in wave-like expression patterns include *Scp3,* and gH2AX (Yao et al., 2003; Bullejos and Koopman, 2004). Many meiotic markers have not yet been tested for anterior-to-posterior expression – e.g. it remains unknown if *Spo11,* which generates double-stranded DNA breaks, or *Rec8,* a meiotic cohesin, are expressed in waves as well.

Interestingly, one of these genes, *Scp3,* is also expressed in the germ cells of embryonic testes at E12.5 but its expression is subsequently downregulated (Di Carlo et al., 2000; Chuma and Nakatsuji, 2001). Based on its early expression pattern and on the morphological similarities between ovarian and testicular germ cells just prior to the meiotic entry in ovaries, it has been argued that a pre-meiotic or "preleptotene" stage exists in both testicular and ovarian germ cells at E12.5 (McLaren, 1984; Di Carlo et al.,

2000). At this time, germ cells of both sexes exhibit large nuclei and condensed chromatin and it has been proposed that this stage represents "the last mitotic prophase before the female germ cells enter meiosis and the male germ cells enter mitotic arrest" (Hilscher et al., 1974; McLaren, 1984). However, staining with Ki-67 antibody which labels cells at all stages of the cell cycle except for the resting (G0) phase (Scholzen and Gerdes, 2000), shows that germ cells in embryonic testes do not enter G0 arrest simultaneously but over a span of several days from E13.5 through E16.5 (Hu, 2006). *Scp3* expression in all testicular germ cells is long diminished before all testicular germ cells enter G0. Thus "premeiotic" stage as marked by *Scp3* expression would have to be followed by mitotic cell cycle in at least a subset of germ cells in the testes before they enter G0 arrest.

Why do ovarian germ cells initiate meiosis and testicular germ cells do not? Ectopic germ cells in the adrenal gland enter meiotic prophase at the same time that they would normally enter if they were in the ovaries (Zamboni and Upadhyay, 1983). *In vitro* co-culture experiments with embryonic lung tissue have also shown that germ cells can enter meiosis inside the lung (McLaren and Southee, 1997). These and similar results demonstrate that germ cells of both sexes enter meiosis in every tissue tested except for the undisrupted embryonic testes. Therefore, testes must produce a meiosis preventing substance, since it is the only tissue in which germ cells do not initiate meiosis (McLaren and Southee, 1997). It has been a matter of some controversy whether meiotic entry is a cell autonomous event regulated by an internal germ cell clock or induced by a meiosis

inducing substance. This substance would have to exist in many other tissues since germ cells appear to enter meiosis in many different places (McLaren and Southee, 1997).

The identity of either one of these substances was not known but several studies have suggested their properties. Biochemical experiments showed that meiosis inducing substance was not a protein but a small steroid-like molecule, while meiosis preventing substance was likely a diffusible protein (Grinsted and Byskov, 1981; Andersen, 1981). Interestingly, disruption of the testes cords lead to germ cell meiotic entry suggesting that meiosis-preventing substance required the germ cells to be protected inside the cords (McLaren and Southee, 1997). The anterior-to-posterior expression pattern of meiotic markers is consistent with diffusion of a meiosis inducing substance. This would provide a mechanistic explanation for the spatio-temporal expression pattern of these genes (Menke et al., 2003).

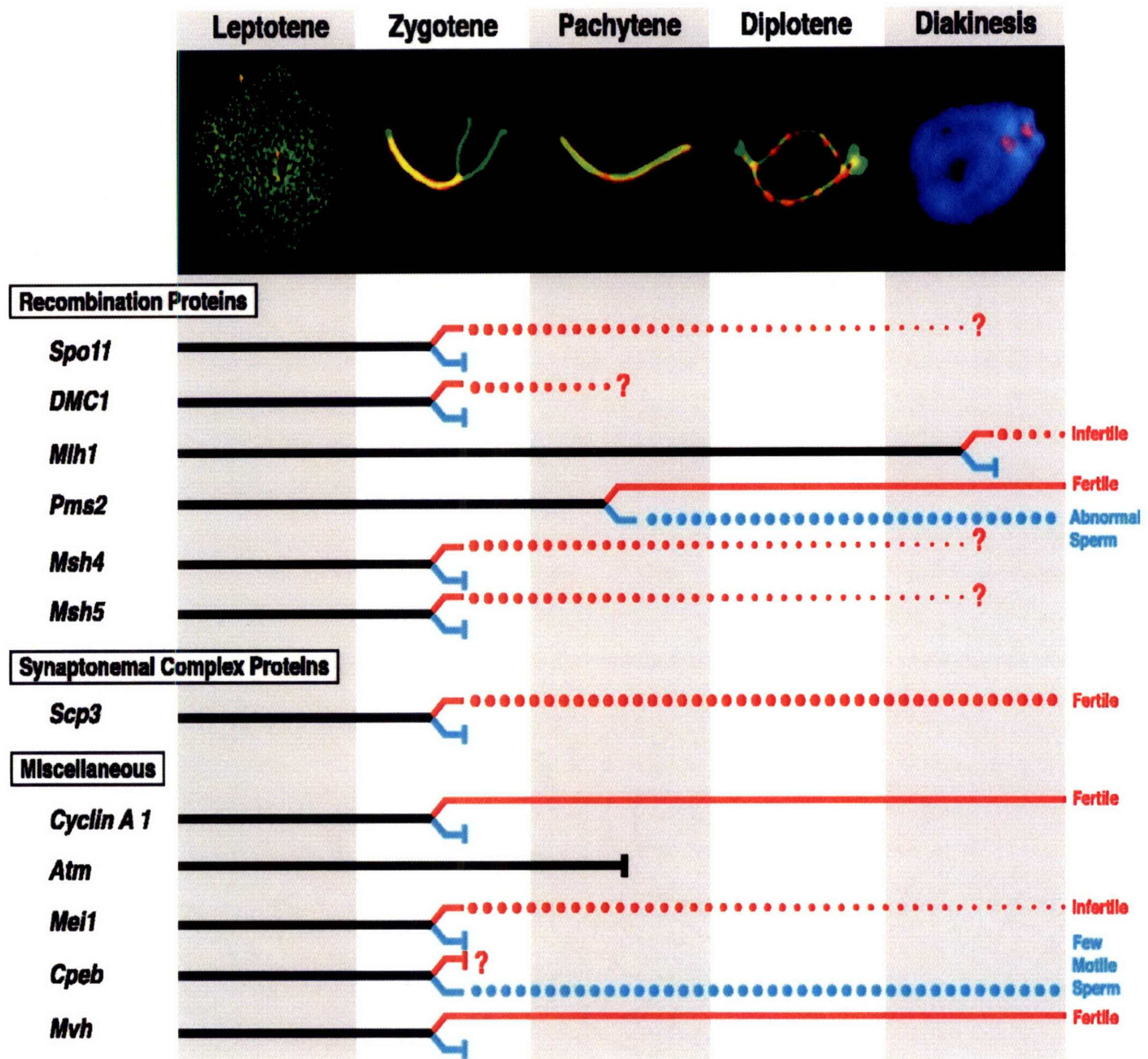
Search for the meiosis inducing substance has yielded multiple candidates. Cultures of embryonic explant ovaries demonstrated that certain growth factors such as insulin-like growth factor 1 or leukemia inhibitory factor can significantly increase the number of meiotic germ cells in the gonads (Lyrakou et al., 2002). Interestingly, analogous cultures with retinoic acid or retinoic acid receptor alpha agonists also promote meiotic entry and progression through zygotene but delay the zygotene-pachytene transition (Livera et al., 2000). While these studies provide interesting candidates, convincing evidence of their action *in vivo* and the mechanism by which they promote meiosis in ovary but not the testes is missing.

Sex and meiosis

How similar is meiosis in embryonic female germ cells to that in postnatal male germ cells? The timing and the continuity of the process differ between the sexes. Meiosis in female germ cells makes several starts and stops and can span decades. Male germ cell meiosis only lasts a little over a week and is a continuous process. Emerging evidence from meiotic knockouts provides an additional insight – female meiosis appears to be more resilient to mutations than male meiosis (Hunt and Hassold, 2002). Most meiotic mouse mutants analyzed so far exhibit different phenotypes and arrest points in males and females (Fig. 3). For example, males deficient for *Scp3* are infertile and exhibit a zygotene arrest (Yuan et al., 2000), while *Scp3* deficient females are only subfertile (Yuan et al., 2002). Unfortunately, most of these mutants have not been analyzed in pure strain mice and variable genetic background may influence the phenotype. Also, several meiotic mutants also remain characterized only in one sex and not the other, and it remains to be seen if this trend holds up in most meiotic mutants.

Why do most male meiotic mutants arrest earlier than female meiotic mutants? In yeast, it has been shown that an early control mechanism operates in meiotic prophase – a pachytene checkpoint (Roeder and Bailis, 2000). It is thought that this checkpoint may be similar to the DNA damage checkpoint and respond to unresolved double-stranded breaks (Roeder and Bailis, 2000). Sex-specific regulation of this checkpoint in mice may explain a more stringent response to mutations in males (Hunt and Hassold, 2002).

Figure 3: Arrest points in mutants with meiotic phenotypes. Black line implies similar development in both sexes while blue (male) and red (female) lines denote sex-specific phenotypes, vertical line shows meiotic arrest, dotted line shows that only a subset of cells is surviving (adapted from Hunt and Hassold, 2002).



Leptotene
Zygotene
Pachytene
Diplotene
Diakinesis
Recombination Proteins
Spo11
DMC1
Mlh1
Pms2
Msh4
Msh5
Synaptonemal Complex Proteins
Scp3
Miscellaneous
Cyclin A 1
Atm
Mei1
Cpeb
Mvh
Infertile
Fertile
Abnormal Sperm
Fertile
Fertile
Infertile
Few Motile Sperm
Fertile

Germ cell reduction – a biological clock ticking before birth?

Germ cell death is a prominent feature of ovarian germ cell embryonic development. After ovarian germ cells cease to proliferate mitotically at about E13.5, their population is at its peak. In mice, this number is approximately 25,000 – 30,000. From the onset of meiosis until two days after birth, this pool is depleted continuously resulting in a two-thirds reduction (Borum, 1961; Bakken and McClanahan, 1978; Morita and Tilly, 1999).

The continuous germ cell reduction model has been challenged recently by using the MVH antibody to label germ cells. It has been proposed that the decrease of oocyte number is limited to two days after birth when germ cell cysts are breaking down (Pepling and Spradling, 2001). However, a more recent study using GCNA and MVH antibodies to label germ cells contradicted these results and confirmed a continuous decline in the number of germ cells through the progression of meiotic prophase during embryonic development (McClellan et al., 2003).

Little is understood about the cause of this massive culling of germ cells inside ovaries before and shortly after birth. It has been suggested that much of the cell death could be accounted for by aberrant progression through meiotic prophase (Borum, 1961). In classical studies, a major reduction of germ cells was documented specifically in pachytene (Borum, 1961; Bakken and McClanahan, 1978). An alternative theory suggests that germ cell death does not necessarily target cells which are defective but rather kills a proportion of the germ cell population so that they can serve as nurse cells

and provide nutrients and mitochondria to the surviving germ cells via the cytoplasmic bridges (Pepling and Spradling, 2001). Alternatively, massive germ cell loss would increase the number of granulosa support cells per surviving germ cell (Ohno and Smith, 1964).

The mechanism of germ cell death is no better understood. From the onset of meiosis until two days after birth, germ cells are progressing through many different stages of the cell cycle. It is hard to imagine that a single mechanism could kill germ cells at such different times. Recent studies have also suggested that multiple mechanisms might be at play. Mice lacking *Bcl-2 associated X protein (Bax),* a key apoptotic component, end up with a larger number of oocytes than their wild type siblings – however – they still experience oocyte loss during the progression of meiotic prophase similarly to their wild type siblings. These results demonstrate that germ cells in meiotic prophase are lost by a *Bax* independent mechanism (Alton and Taketo, 2007).

Pluripotency wave

In addition to meiotic markers, another set of genes exhibits an intriguing expression pattern in ovarian germ cells. Several markers of pluripotency - namely *POU domain, class 5, transcription factor 1 (Oct4/Pou5f1), sry-box containing gene 2 (Sox2), developmental pluripotency associated 5 (Dppa5),* and *developmental pluripotency associated 3 (Dppa3)* - have been shown to be downregulated in an anterior to posterior expression pattern in ovarian germ cells but are maintained longer in testicular germ cells (Menke et al., 2003; Bullejos and Koopman, 2004; Western et al., 2005; Lin, 2005).

Little is understood about pluripotency markers and their regulation in the germ cells of both sexes. During migration and upon arrival at the gonad, germ cells express pluripotency markers (Western et al., 2005). Subsequently, ovarian germ cells downregulate pluripotency markers in an anterior to posterior wave by E16.5 (Menke et al., 2003; Bullejos and Koopman, 2004; Western et al., 2005). Interestingly, at least one of these markers, *Oct4,* is re-expressed again in the postnatal ovary. The function of this *Oct4* expression pattern in postnatal ovary is unknown.

Male germ cells have also been shown to downregulate mRNAs of pluripotency markers *Sox2* and *Dppa5* before birth (Western et al., 2005). However, this down regulation is much faster than in ovaries and does not appear directional. Furthermore, pluripotency markers are detectable in testicular germ cells in the postnatal testes. The significance and regulation of this process is currently unknown.

It is not known if there is a link between the meiotic and the pluripotency waves. In mice of C57BL/6 background, the *Oct4* anterior-to-posterior disappearance starts 12-24 hours after the first appearance of *Stra8* (Menke et al., 2003). This observation suggests that the meiotic wave precedes the pluripotency downregulation. *Stra8* expression may cause the downregulation of *Oct4* (and/or its downstream targets) or both of them may be regulated independently by a third factor.

What causes the ovarian anterior-to-posterior waves in general? It has been suggested that the order in which the germ cells arrive into the gonad may establish the

anterior-to-posterior distribution. However, time lapse movies suggest that germ cells do not colonize the gonad in any particular order (e.g. anterior-to-posterior) and quickly become immobilized within the gonad. While some germ cells arrive earlier and others later, their location within the gonad appears to be random (Molyneaux et al., 2001; Molyneaux, 2006). The somatic gonad itself differs between the anterior and posterior ends suggesting that perhaps some intrinsic differences between the poles of the gonad might lead to the waves. Another option is that diffusion of factor(s) in an anterior-to posterior direction could induce gene expression in a similar direction. However, evidence to support any of these models is lacking.

Follicle formation

Primordial follicles are formed in ovaries perinatally. Oocytes arrested in the diplotene stage become surrounded by a single layer of somatic cells to form primordial follicles (Matzuk et al., 2002). This surrounding process and subsequent transition to primary follicles requires the breakdown of ovigerous cords and the remodeling of the basal membrane. Each primordial follicle is composed of a single germ cell surrounded by flattened granulosa cells.

In mice, there is sufficient evidence that germ cells are the directors of primordial follicle formation as well as the subsequent progression into primary follicles and later stages of folliculogenesis. Without germ cells, primordial follicle formation never occurs. *Folliculogenesis specific basic helix loop helix (Figla),* a germ cell-specific gene expressed from E13.5 onwards, is required for primordial follicle formation (Soyal et al.,

2000). In the *Figla-/-* ovaries, germ cells eventually die a few days after birth when they fail to recruit granulosa cells around them. It is not known by what mechanism *Figla* regulates primordial follicle formation and how the timing is regulated.

Growth differentiation factor 9 (Gdf9), another germ cell-specific gene, is required for further maturation of primordial follicles into primary follicles (Dong et al., 1996). *NOBOX oogenesis homeobox (Nobox)* also plays a role in follicle formation, because *Nobox* deficient ovaries exhibit a delay in primordial follicle formation and do not transition into primary follicles (Rajkovic et al., 2004). Furthermore, they express decreased levels of *Gdf9* suggesting that *Nobox* regulates its expression (Rajkovic et al., 2004).

What are the XY germ cells up to?

What happens to XY germ cells in the testes after E13.5, when their ovarian counterparts start entering meiotic prophase? In comparison to the many processes going on in XX germ cells during this time period until about birth, germ cells in the testes appear remarkably passive. However, this observation may reflect gaps in our understanding of the processes occurring in testicular germ cells at this time.

Germ cells in the testes start entering mitotic arrest at E13.5. Whether this arrest represents the G0 or the G1 phase of the cell cycle is unknown. Testicular germ cells do not enter mitotic arrest synchronously but over a span of three days. Unfortunately, it is not known if any sort of directionality exists (e.g. anterior to posterior or center to pole)

or if the arrest is a random process. It is also not known what mechanism underlies the arrest. XX germ cells placed in testes also arrest, suggesting that the testis itself is somehow involved in its regulation. Testicular germ cells remain arrested until after birth when mitosis is re-initiated prior to meiotic prophase.

Currently, only a few mouse knockouts have shown defects in achieving G0 arrest in male germ cells. In each case, failure to arrest coincides with ongoing germ cell death and postnatal testicular tumor formation. Conditional loss of *phosphatase and tensin homolog (PTEN)* in germ cells leads to defective entry into G0 arrest, increased apoptosis of testicular germ cells and tumor formation (Kimura et al., 2003b). Authors note that their phenotype is very similar to that of 129/Sv-ter mice (Noguchi and Stevens, 1982; Noguchi, 1996). The ter mutation in the *dead-end 1(Dnd1)* gene has been shown to cause germ cell loss and testicular germ cell tumors (Youngren et al., 2005).

Between E15.5 and E18.5, XY germ cells show marked increase in methylcytosine antibody staining, suggesting that *de novo* DNA methylation has been initiated in testicular germ cells (Coffigny et al., 1999; Hajkova et al., 2002; Kimura et al., 2003b; Allegrucci et al., 2005). In contrast, ovarian germ cells delay re-methylation until after birth (Obata and Kono, 2002; Hiura et al., 2006). The methyltransferase *Dnmt3a* and *Dnmt3L* are the most likely players in *de novo* DNA methylation of male germ cells (Bourc'his and Bestor, 2004; Kaneda et al., 2004). It is unknown how sex-specific timing of *de novo* DNA methylation is regulated or why it exists.

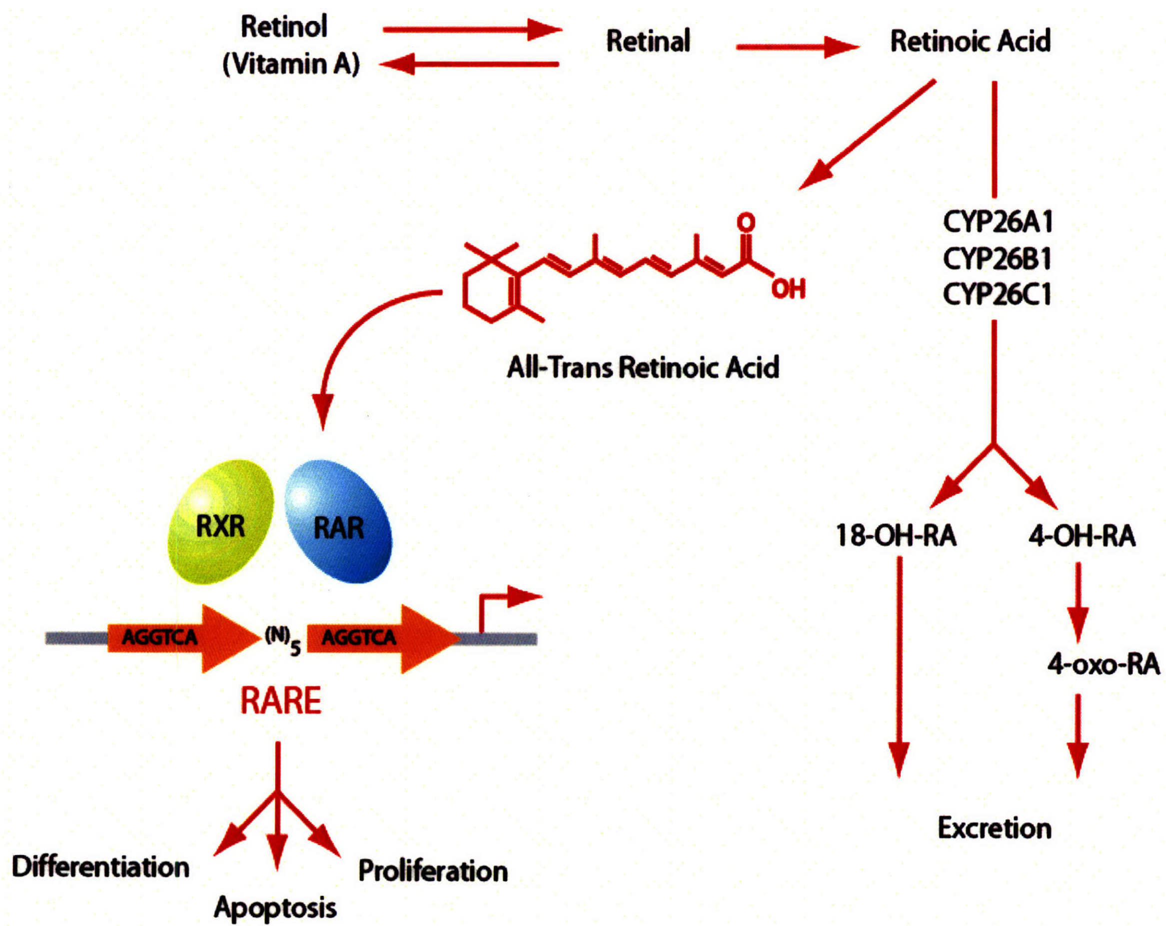
Aims of my thesis

Germ cell behavior inside the embryonic gonads has been studied during much of the last century, primarily through physical manipulation of cells, microscopy and morphological observations. Advances in molecular biology have facilitated generation of new insights and reexamination of established theories. Importantly, a number of markers for different processes have been identified in germ cells. These markers allow a closer look at the regulation of germ cell embryonic development at the molecular level.

I have become interested in germ cell sex determination – specifically, why embryonic XX, but not XY germ cells express *Stra8*. Since *Stra8* is one of the earliest known molecular markers specific to XX germ cells and the only known gene required for pre-meiotic DNA replication, development of new insights into its transcriptional regulation will improve our understanding of germ cell sex determination.

My work examines the role of retinoic acid in embryonic germ cell development and regulation of *Stra8* expression. Retinoic acid is a derivative of vitamin A (retinol). The biosynthetic pathway for generating retinoic acid from retinol involves two oxidation steps, the first of which generates retinaldehyde (retinal). However, the rate limiting step in the retinoic acid biosynthesis pathway is the second and final step, which is catalyzed by retinaldehyde dehydrogenases. These enzymes are expressed selectively in some tissues

Figure 4: The biosynthetic pathway for the synthesis and metabolism of all-*trans* retinoic acid and its signaling mechanism in the cell (modified from Petkovich, 2007).


Retinoic acid synthesis, metabolism and signaling
Retinol
(Vitamin A)
Retinal
Retinoic Acid
O
OH
All-Trans Retinoic Acid
CYP26A1
CYP26B1
CYP26C1
RXR
RAR
AGGTCA
(N)5
AGGTCA
RARE
18-OH-RA
4-OH-RA
4-oxo-RA
Excretion
Differentiation
Apoptosis
Proliferation

during embryonic development to ensure site specific exposure to retinoic acid (Niederreither et al., 2002b). Local concentration of retinoic acid is negatively regulated by *cytochrome p450 enzyme, family 26 (Cyp26)* enzymes, which hydroxylate retinoic acid and render it inactive. It has been shown that the main function of CYP26 enzymes is to protect tissues from retinoic acid exposure (Niederreither et al., 2002a) (Fig. 4).

Retinoic acid is an isoprenoid that binds to retinoic acid receptors (RARs). These receptors are ligand-regulated transcription factors which belong to the nuclear hormone receptor superfamily (Aranda and Pascual, 2001). In both deficiency and excess, retinoic acid causes many developmental defects in mouse embryos. Knockouts of genes involved in its signaling and chemical manipulations *in vitro* and *in vivo* have demonstrated that retinoic acid is required for the development of many tissues in the embryo including lungs, limbs, and the central nervous system.

Prior to this work, there was little evidence for retinoic acid signaling in embryonic gonads with the exception of one study that suggested retinoic acid hastened meiotic entry in cultured embryonic ovaries (Livera et al., 2000). The most suggestive piece of evidence for the *in vivo* role of retinoic acid in gonads came from our laboratory. A cDNA subtraction experiment revealed that E12.5 embryonic testes but not embryonic ovaries, expressed *cytochrome p450, family 26, subfamily b, polypeptide 1* (*Cyp26b1),* a member of the retinoic acid-metabolizing enzyme family (Menke and Page, 2002). This discovery, together with our improved understanding of *Stra8* function, led to the present work exploring the role of retinoic acid in embryonic germ cell development.

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CHAPTER 2

Retinoic acid regulates sex-specific timing of meiotic initiation in mice

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Author contributions:

Jana Koubova designed, performed and analyzed all experiments except for the experiment involving vitamin A deficient (VAD) mice. Doug Menke provided *Stra8-/-* ovaries, and participated in the design of several experiments. Qing Zhou and Mike Griswold designed, performed and analyzed the VAD mouse experiment (mentioned above). Blanche Capel taught Jana Koubova the organ culture technique. David Page provided helpful discussions throughout the project and assisted during the writing of the manuscript.

Abstract

In mammals, meiosis is initiated at different time points in males and females, but the mechanism underlying this difference is unknown. Female germ cells begin meiosis during embryogenesis. In males, embryonic germ cells undergo G0/G1 mitotic cell cycle arrest, and meiosis begins after birth. In mice, the *Stra8* gene has been found to be required for the transition into meiosis in both female and male germ cells. *Stra8* is expressed in embryonic ovaries just prior to meiotic initiation, while its expression in testes is first detected after birth. Here we examine the mechanism underlying the sex-specific timing of *Stra8* expression and meiotic initiation in mice. Our work shows that signaling by retinoic acid (RA), an active derivative of vitamin A, is required for *Stra8* expression and thereby meiotic initiation in embryonic ovaries. We also discovered that RA is sufficient to induce *Stra8* expression in embryonic testes and in vitamin A-deficient adult testes *in vivo*. Finally, our results show that cytochrome p450 (CYP)-mediated RA metabolism prevents premature *Stra8* expression in embryonic testes. Treatment with an inhibitor specific to RA-metabolizing enzymes indicates that a CYP26 enzyme is responsible for delaying *Stra8* expression in embryonic testes. Sex-specific regulation of RA signaling thus plays an essential role in meiotic initiation in embryonic ovaries and precludes its occurrence in embryonic testes. Since RA signaling regulates *Stra8* expression in both embryonic ovaries and adult testes, this portion of the meiotic initiation pathway may be identical in both sexes.

Embryonic development of the mammalian gonad is a dynamic process during which both germ and somatic cells acquire sex-specific characteristics. In mice, differences between the somatic components of ovaries and testes are microscopically evident by embryonic day 12.5 (E12.5). Germ cells, however, remain indistinguishable between the sexes until E13.5, when ovarian germ cells initiate prophase of meiosis and testicular germ cells arrest in the G0/G1 stage of the mitotic cell cycle (McLaren, 1984). In this study, we address the question of why ovarian germ cells initiate meiosis embryonically, while testicular germ cells postpone meiosis until after birth. In this context, we examine the role of retinoic acid (RA) signaling in meiotic initiation.

Our studies are based on the recent finding that *Stimulated by Retinoic Acid Gene 8* (*Stra8*) is required for meiotic initiation in both sexes (Baltus et al., 2006). *Stra8* expression in ovarian germ cells occurs over a period of four days starting at E12.5, just a day before meiotic germ cells with characteristically condensed chromatin can be observed (Menke et al., 2003). *Stra8* expression in the ovary occurs in an anterior-to-posterior wave, which is followed by anterior-to-posterior waves of expression of meiotic markers such as *Dmc1* (a meiotic recombinase) and *Scp3* (a synaptonemal complex protein) (Menke et al., 2003; Yao et al., 2003; Bullejos and Koopman, 2004). In contrast, embryonic testes do not express *Stra8* (Menke et al., 2003). Instead, *Stra8* is first expressed in the mitotic germ cells of postnatal testes, and subsequently in the premeiotic germ cells (spermatogonia) of adult testes (Oulad-Abdelghani et al., 1996; Menke et al., 2003).

Stra8 expression is stimulated by RA in embryonal carcinoma cells and embryonic stem cells in culture (Oulad-Abdelghani et al., 1996). We wondered whether RA also regulates endogenous *Stra8* expression in the germ cells of embryonic ovaries and postnatal testes. RA is an important regulator of embryonic patterning and development (Ross et al., 2000). It is generated by a series of oxidative reactions from dietary vitamin A (retinol) (Ross et al., 2000). Local levels of RA are regulated by retinaldehyde dehydrogenases, which catalyze the last step of RA synthesis, and by the CYP26 family of cytochrome p450 enzymes, which degrade RA (Abu-Abed et al., 2001; Niederreither et al., 2002; Duester et al., 2003). RA serves as a ligand to a family of nuclear hormone receptors known as RARs (retinoic acid receptors), which bind to RA response elements (RAREs) in the regulatory regions of target genes (Chambon, 1996). Three major RAR isotypes have been identified (RAR-alpha, RAR-beta and RAR-gamma), and these exhibit overlapping expression patterns and functional redundancy in many tissues (Kastner et al., 1995; Chambon, 1996).

Retinoic acid receptors (RARs) are expressed in the gonads of both sexes (Dolle et al., 1990; Morita and Tilly, 1999; Vernet et al., 2005) and several lines of evidence suggest that RA may play a role in the regulation of meiosis in both postnatal testes and embryonic ovaries. First, spermatogenesis is blocked in males fed a vitamin A-deficient (VAD) diet (Wolbach and Howe, 1925). Their testicular tubules are depleted of all meiotic cells, and the only remaining germ cells are undifferentiated spermatogonia and preleptotene spermatocytes (Thompson et al., 1964; Morales and Griswold, 1987). Since administration of RA to VAD animals is sufficient to restore and synchronize

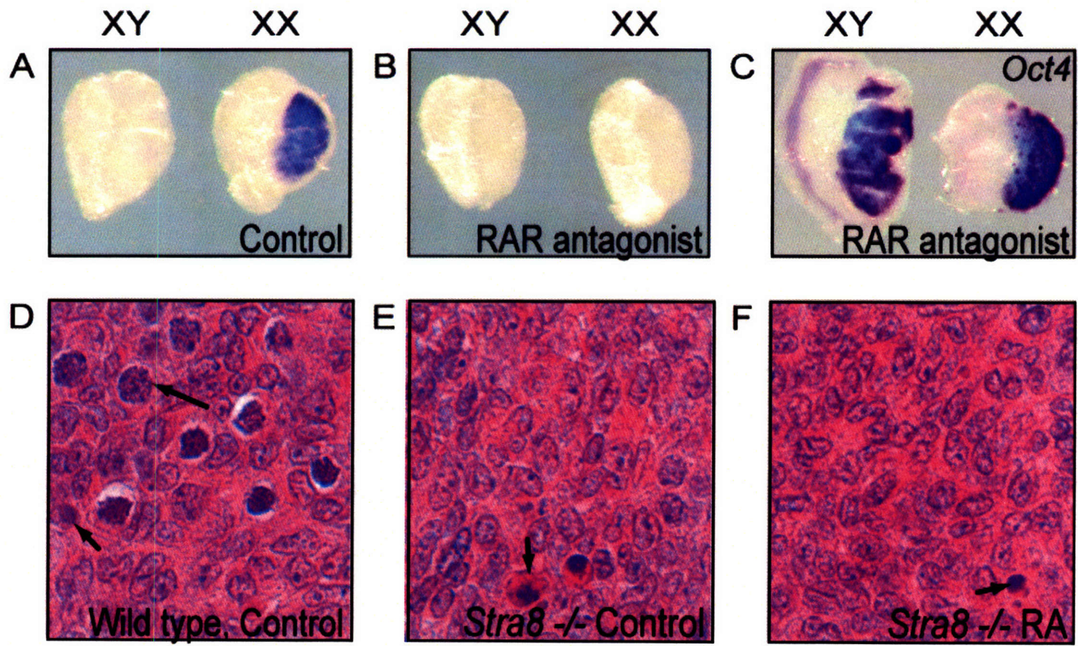
spermatogenesis in all seminiferous tubules, it has been suggested that RA is necessary for proper meiotic prophase (Morales and Griswold, 1987; van Pelt and de Rooij, 1991; de Rooij D.G., 1994). Second, RA treatment of embryonic rat ovaries in culture hastens meiotic prophase (Livera et al., 2000). Here, we examine the role of RA in the regulation of *Stra8,* a gene necessary for meiotic initiation, and in this way explore the mechanism behind the sex-specific timing of meiotic initiation.

Results

***Stra8* Expression in Embryonic Ovaries Requires RAR Signaling.** To determine if RA is required for *Stra8* expression in embryonic ovaries, we assayed the gene's expression in gonads cultured in the presence of RAR pan-antagonist BMS-204493. This compound, which antagonizes all three RAR isotypes, prevents RA signaling by stabilizing interactions between RARs and co-repressor proteins, thereby silencing expression of genes normally up-regulated by RA (Germain et al., 2002). Ovaries dissected at E11.5 and cultured for two days in control medium expressed *Stra8*, while testes did not (Fig. 1A), recapitulating the endogenous expression pattern. In contrast, ovaries cultured in the presence of RAR antagonist BMS-204493 did not express *Stra8* (Fig. 1B), though expression of a control germ cell marker, *Oct4*, was maintained (Fig. 1C). We conclude that RAR signaling is required for *Stra8* expression in embryonic ovaries.

These results, taken together with the observation that *Stra8* is required for meiotic initiation in embryonic ovaries *in vivo* (Baltus et al., 2006), imply that meiotic

Fig. 1. RAR signaling is required for *Stra8* expression in embryonic ovaries. (A, B) Whole-mount *in situ* hybridization with *Stra8* probe in gonads dissected at E11.5 and cultured for two days in (A) control medium or (B) with RAR pan-antagonist BMS-204493. (C) Whole-mount *in situ* hybridization with *Oct4* probe in gonads dissected at E11.5 and cultured for two days with BMS-204493. (D-F) Photomicrographs of hematoxylin and eosin-stained sections of (D) wild-type ovary dissected at E11.5 and cultured for four days in control medium or (E, F) *Stra8 -/-* ovaries cultured (E) in control medium or (F) with RA. Long arrow indicates representative cell with meiotic prophase condensation (D). We observed a few pyknotic nuclei in all samples (D-F, short arrows).


XY
XX
A
Control
B
RAR antagonist
C
Oct4
RAR antagonist
D
Wild type, Control
E
Stra8 -/- Control
F
Stra8 -/- RA

initiation there requires RAR signaling. To confirm that RA acts through *Stra8* to induce meiotic prophase condensation in embryonic ovaries, we cultured *Stra8 -/-* ovaries with and without RA. As expected, germ cells in wild-type ovaries dissected at E11.5 and cultured for four days in control medium displayed chromatin condensation (Fig. 1D) indicative of early meiotic prophase (McLaren and Southee, 1997; Livera et al., 2000). No such condensation was observed *Stra8 -/-* ovaries cultured in control medium (Fig. 1E) or with RA (Fig. 1F). Thus, RA failed to rescue the loss of meiotic prophase condensation in *Stra8 -/-* ovaries. We conclude that RA induction of *Stra8* expression in ovarian germ cells is essential to achieve the chromatin condensation of meiotic prophase.

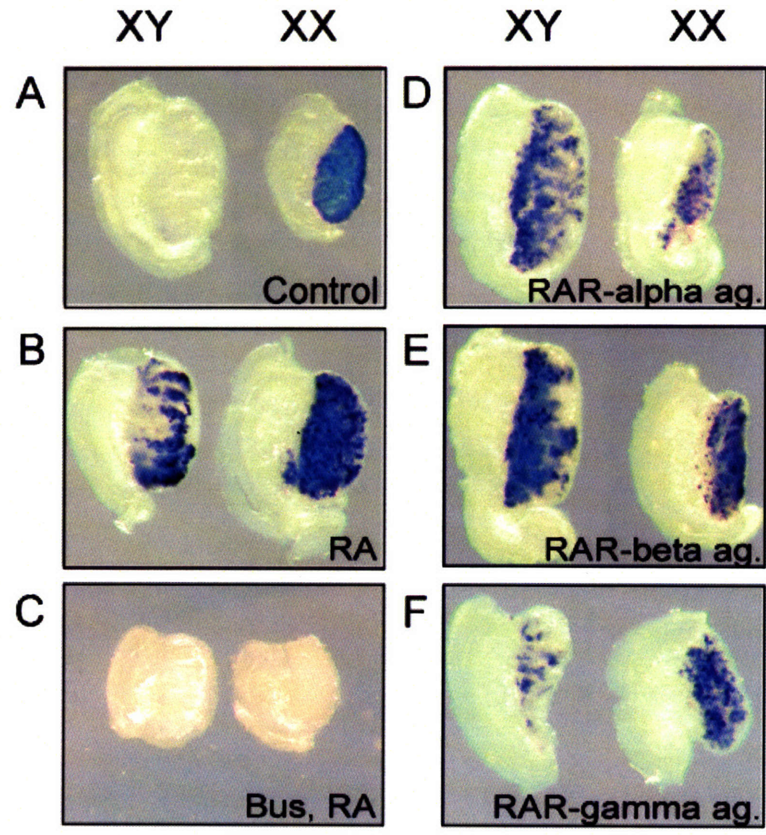
Exogenous RA Induces *Stra8* Expression in Embryonic Testes. Testicular germ cells do not express *Stra8* until after birth (Menke et al., 2003) despite the fact that testicular cells express RARs during embryonic development (Dolle et al., 1990). Perhaps the germ cells of embryonic testes are not exposed to RA *in vivo*. To determine if exogenous RA can induce *Stra8* expression in embryonic testes, we assayed the gene's expression in gonads cultured with all-*trans*-RA added to the medium. As expected, testes dissected at E12.5 and cultured for two days in control medium expressed no *Stra8* (Fig. 2A). By contrast, testes cultured with all-*trans*-RA displayed abundant expression of *Stra8* (Fig. 2B).

Although this RA-induced expression of *Stra8* appeared to be limited to embryonic testis cords, we wanted to determine whether it was restricted to germ cells,

where *Stra8* is normally expressed in adult testes and embryonic ovaries. Embryonic gonads were depleted of germ cells by busulfan treatment (Merchant, 1975), and then cultured with all-*trans*-RA added to the medium. *Stra8* expression was lost in both embryonic testes and ovaries treated in this manner (Fig. 2C). The simplest interpretation of these findings is that RA signaling induces *Stra8* expression only in the germ cell lineage. (Though less likely, we cannot exclude the possibility that, in this experimental context, *Stra8* is expressed in somatic cells, but only in the presence of germ cells.) Similar experiments were performed with testes from W^v/W^v embryos, which are severely depleted of germ cells due to a point mutation in the c-kit tyrosine kinase receptor (Nocka et al., 1990). Like busulfan-treated testes, testes from W^v/W^v embryos failed to express *Stra8* when cultured with all-*trans*-RA (data not shown). Again, the simplest interpretation of these findings is that exogenous RA induces *Stra8* expression in the germ cells but not in the somatic cells of embryonic testes.

In rats, all three *RAR* isotypes are known to be expressed in embryonic testes (Dolle et al., 1990; Cupp et al., 1999), and, in theory, any one might mediate the premature induction of *Stra8* observed in mouse embryonic testes treated with exogenous RA. To address this question empirically, we cultured embryonic gonads in the presence of RAR agonists selective for each of the three RAR isotypes: BMS-194753 (RAR-alpha), BMS-213309 (RAR-beta), and BMS-270394 (RAR-gamma). Treatment with any of these selective RAR agonists induced *Stra8* expression in embryonic testes (Fig. 2D, E, F). These results suggest that, in embryonic testes, all three RAR isotypes are capable of activating *Stra8* transcription.

Fig. 2. RA and agonists to all three RARs induce *Stra8* expression in testes. Whole-mount *in situ* hybridization with *Stra8* probe in gonads dissected at E12.5 and cultured for two days (A) in control medium, (B) with RA, (C) with RA after busulfan treatment, (D) with RAR-alpha agonist BMS-194753, (E) with RAR-beta agonist BMS-213309, or (F) with RAR-gamma agonist BMS-270394.

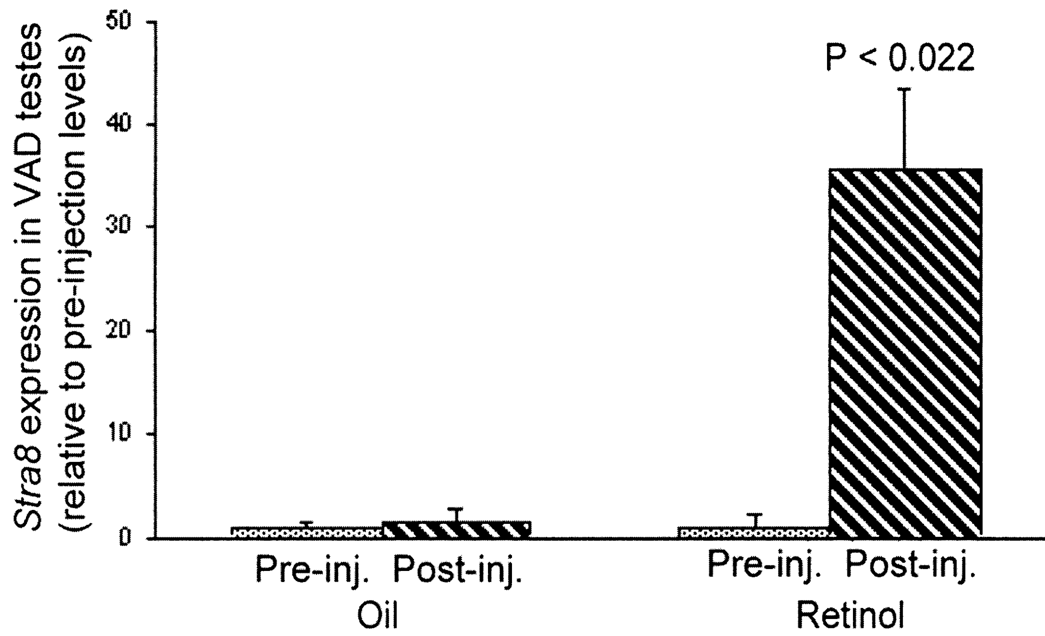


XY
XX
XY
XX
A
D
Control
RAR-alpha ag.
B
E
RA
RAR-beta ag.
C
F
Bus, RA
RAR-gamma ag.

RA Induces *Stra8* Expression in VAD Adult Testes. Does RA also regulate *Stra8* expression in the premeiotic cells of adult testes? If so, we might expect *Stra8* expression to be low in the testes of VAD (vitamin A-deficient) male mice, but to increase following injection of such mice with retinol (vitamin A), the precursor of RA. To test this prediction, we removed a single testis from each of several adult VAD males, after which each of the mice was injected with all-*trans*-retinol (ROL) in oil, or with oil alone. The second testis from each animal was harvested 24 hours later, and *Stra8* transcript levels – pre- and post-injection – were compared by quantitative RT-PCR. *Stra8* expression increased dramatically in the testes of VAD males following injection of ROL, but not following injection of oil alone (Fig. 3). This result demonstrates that RA signaling can induce *Stra8* expression in adult testes *in vivo* and thus represents a shared element of the regulatory pathway required for meiotic initiation in adult testes and embryonic ovaries.

CYP-mediated Metabolism of RA Prevents *Stra8* Expression in Embryonic Testes. The absence of *Stra8* expression in embryonic testes *in vivo* implies that RA signaling there differs from that in embryonic ovaries. In theory, embryonic testes might synthesize less RA, or metabolize RA more efficiently, or both. Lower levels of RA synthesis in embryonic testes have not been reported. However, several cytochrome p450 (CYP) enzymes, including some that may degrade RA, are known to be expressed in embryonic testes but not embryonic ovaries (Abu-Abed et al., 2002; Menke and Page, 2002; Nef et al., 2005). One of these CYP enzymes could metabolize RA and thereby shield testicular germ cells from RA signaling.

Fig. 3. Quantitative RT-PCR analysis of *Stra8* expression in retinol-injected or control (oil-injected) adult VAD testes compared to pre-injection, contralateral testes. (Error bars indicate standard deviation; $P < 0.022$, paired *t* test, two-sided.)

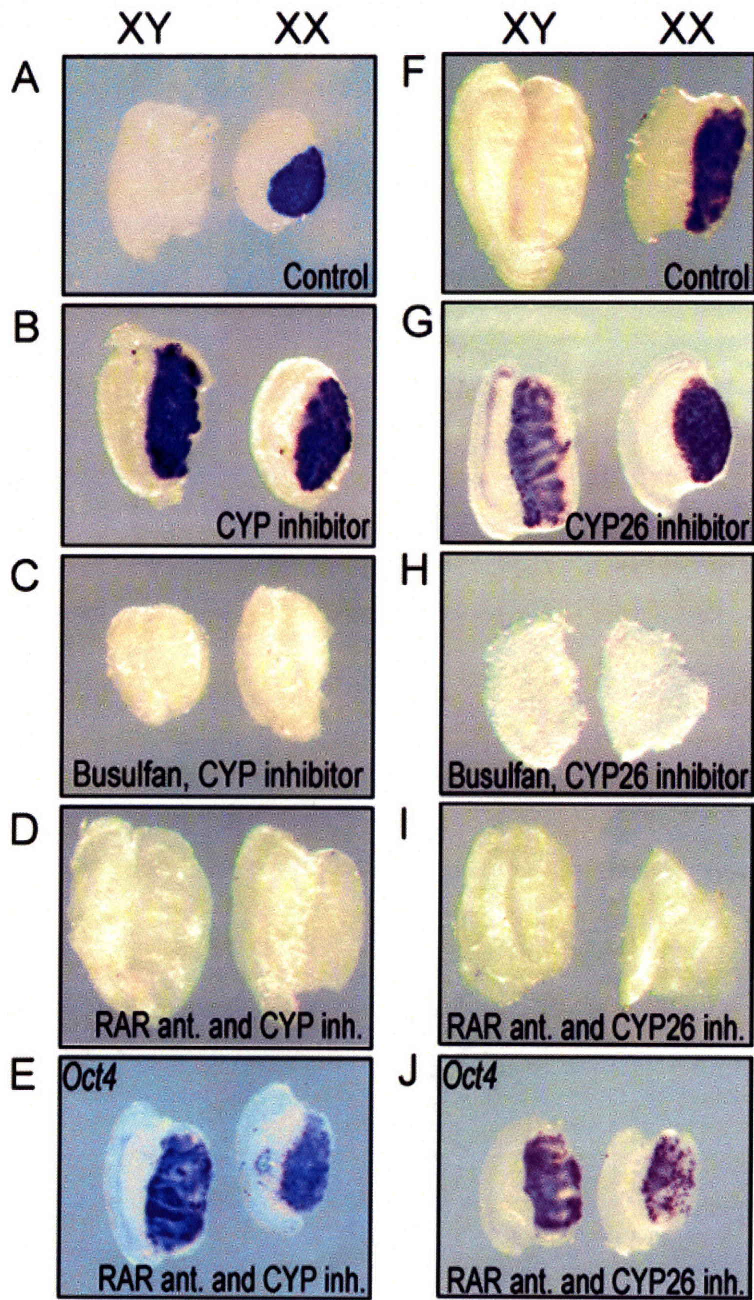

Stra8 expression in VAD testes
(relative to pre-injection levels)
50
40
30
20
10
0
P < 0.022
Pre-inj.
Post-inj.
Oil
Pre-inj.
Post-inj.
Retinol

To determine whether a testis-specific CYP enzyme could be responsible for the lack of *Stra8* expression in embryonic testes, we assayed *Stra8* expression in gonads cultured in the presence of ketoconazole, a potent but non-specific CYP inhibitor (Higashi et al., 1987; Van Wauwe et al., 1988). As expected, testes dissected at E12.5 and cultured for two days in control medium did not express *Stra8* (Fig. 4A). However, embryonic testes cultured in the presence of ketoconazole displayed robust expression of *Stra8* (Fig. 4B). Ketoconazole had no detectable effect on embryonic ovaries. Busulfan-treated testes failed to express *Stra8* after treatment with ketoconazole (Fig. 4C), suggesting that ketoconazole induced *Stra8* expression only in germ cells.
Given the diversity of CYP enzymes inhibited by ketoconazole, it was important to confirm that the compound's effects on *Stra8* expression required RAR activation.

Accordingly, we dissected testes at E12.5 and cultured them for two days in the presence of both ketoconazole and RAR pan-antagonist BMS-204493. As expected, the RAR antagonist blocked the effect of ketoconazole in embryonic testes (Fig. 4D). This dual treatment was not toxic to germ cells, as judged by expression of the germ cell marker *Oct4* (Fig. 4E). Taken all together, the experiments involving ketoconazole strongly suggest that, in embryonic testes, germ-cell transcription of *Stra8* is prevented by a CYP enzyme that metabolizes RA.

Implicating the CYP26 Family in RA Degradation in Embryonic Testes. Several CYP enzymes are capable of metabolizing RA, but enzymes of the CYP26 family are

Fig. 4. CYP26 activity prevents *Stra8* expression in embryonic testes. (A-D, F-I) Whole-mount *in situ* hybridization with *Stra8* probe in gonads dissected at E12.5 and cultured for two days (A, F) in control medium, (B) with CYP inhibitor ketoconazole, (C) with ketoconazole after busulfan treatment, (D) with RAR pan-antagonist BMS-204493 and ketoconazole, (G) with CYP26 inhibitor R115866, (H) with R115866 after busulfan treatment, or (I) with BMS-204493 and R115866. (E, J) *In situ* hybridization with *Oct4* probe in gonads dissected at E12.5 and cultured for two days (E) with BMS-204493 and ketoconazole or (J) with BMS-204493 and R115866.



XY
XX
XY
XX
A
F
Control
Control
B
G
CYP inhibitor
CYP26 inhibitor
C
H
Busulfan, CYP inhibitor
Busulfan, CYP26 inhibitor
D
I
RAR ant. and CYP inh.
RAR ant. and CYP26 inh.
E
J
Oct4
Oct4
RAR ant. and CYP inh.
RAR ant. and CYP26 inh.

particularly specific and efficient (White et al., 1997). While ketoconazole is a nonspecific inhibitor of CYP enzymes, the compound R115866 is a highly selective inhibitor of CYP26-mediated metabolism of RA *in vivo* (Stoppie et al., 2000). To test if CYP26-mediated RA metabolism in embryonic testes prevents *Stra8* expression, we dissected embryonic gonads at E12.5 and cultured them for two days in the presence of R115866. This CYP26 inhibitor induced *Stra8* expression in embryonic testes, but had no detectable effect on embryonic ovaries (Fig. 4G) when compared with controls (Fig. 4F). As expected, R115866 failed to induce *Stra8* expression in testes from busulfan-treated and W^v/W^v embryos (Fig. 4H; data not shown), suggesting that only germ cells express *Stra8* in response to inhibition of CYP26. Embryonic testes cultured in the presence of both the CYP26 inhibitor and the RAR pan-antagonist failed to express *Stra8* (Fig. 4I), confirming that RARs mediate the effects of the CYP26 inhibitor. This dual treatment did not affect the control germ cell marker *Oct4* (Fig. 4J). Based on all these findings, we conclude that a CYP26 enzyme degrades RA in embryonic testes and thereby precludes germ-cell transcription of *Stra8*.

***Cyp26b1* Is Expressed in Embryonic Testes but Not in Embryonic Ovaries.** In mice, the *Cyp26* gene family has three members, only one of which, *Cyp26b1*, is expressed in embryonic gonads (MacLean et al., 2001; Abu-Abed et al., 2002; Tahayato et al., 2003). The *Cyp26b1* gene was shown previously to be expressed in somatic cells of embryonic testes (Abu-Abed et al., 2002). We confirmed and extended these findings by whole-mount *in situ* hybridization of *Cyp26b1* transcripts in embryonic gonads. *Cyp26b1* expression in testes begins by E11.5 and is maintained until at least E15.5 (Fig. 5). No

Fig. 5. Developmental time course of *Cyp26b1* expression in embryonic gonads as revealed by whole-mount *in situ* hybridization.

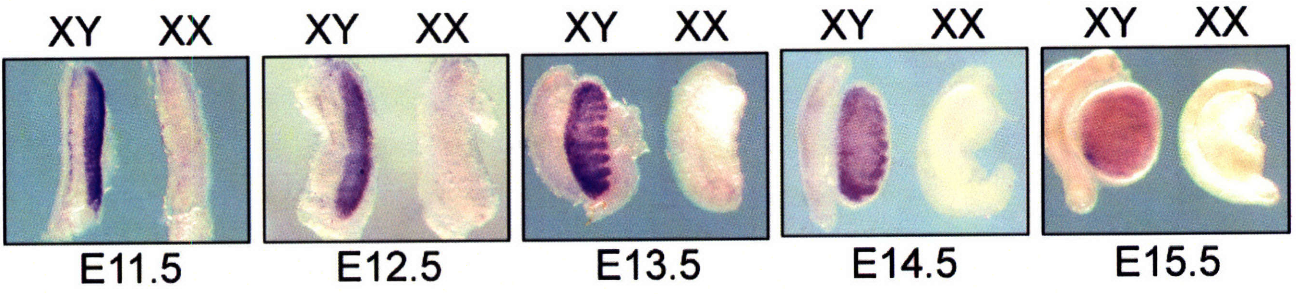
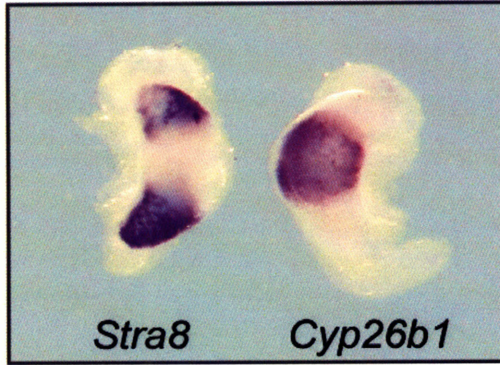

XY
XX
XY
XX
XY
XX
XY
XX
XY
XX
E11.5
E12.5
E13.5
E14.5
E15.5

Fig. 6. *Stra8* and *Cyp26b1* expressed in mutually exclusive domains in E14.5 XY^{ZAL} ovotestes. *Stra8* is expressed in the polar, ovarian regions. *Cyp26b1* is expressed in the central, testicular region.


Stra8
Cyp26b1

expression of *Cyp26b1* in ovaries was seen during the same period (Fig. 5). Based on *Cyp26b1*'s unique expression pattern, and our finding that inhibiting CYP26 activity induces *Stra8* expression in embryonic testes, we hypothesize that CYP26B1 is the RA-metabolizing enzyme responsible for delaying *Stra8* expression in embryonic testicular germ cells.

When a Y chromosome derived from Zalende mice is crossed onto a C57BL/6 genetic background, many of the resulting XY^{ZAL} embryos are partially sex-reversed, with ovotestes (Eicher et al., 1982; Eicher and Washburn, 1983; Menke et al., 2003). An XY^{ZAL} ovotestis is typically comprised of a central region exhibiting testicular histology and two polar regions displaying ovarian histology (Eicher et al., 1982; Eicher and Washburn, 1983). Whole-mount *in situ* hybridization to XY^{ZAL} ovotestes revealed mutually exclusive realms of expression for *Stra8* and *Cyp26b1* (Fig. 6). *Stra8* expression is confined to the ovarian (polar) portions of the ovotestis, while *Cyp26b1* expression is limited to the testicular (central) region. Thus, *Stra8* and *Cyp26b1* appear to be expressed in non-overlapping domains, consistent with the hypothesis that *Cyp26b1* activity prevents transcription of *Stra8*.

Discussion

Based on the findings reported here, we propose a model in which RA signaling and metabolism regulate whether female and male germ cells initiate meiosis during embryogenesis (Fig. 7). The model posits that, in embryonic ovaries, RA induces germ cells to express *Stra8*, which in turn leads to initiation of meiosis. In embryonic testes, an

enzyme of the CYP26 family – likely CYP26B1 – degrades RA and thereby prevents expression of *Stra8* and precludes initiation of meiosis. According to our model, and as discussed below, both production and degradation of RA occur outside germ cells, in the somatic cells and tissues of the embryo. Thus, we propose that germ cells respond to sex differences in the RA environments offered by embryonic ovaries and testes.

Further, our findings in VAD mice suggest that meiotic initiation in adult testes may be regulated by a pathway similar to that operating in embryonic ovaries. In testes of VAD adult males, *Stra8* is significantly upregulated in response to retinol (ROL), a precursor to RA (Fig. 3). Thus, at least a portion of the meiotic initiation pathway appears to be identical in the adult testis and embryonic ovary. The spermatogenic arrest observed in VAD adult males may be due in part to the absence of *Stra8* expression. Conversely, testis-wide induction of *Stra8* by RA may contribute to the spermatogenic synchronization observed in VAD animals after administration of ROL (Morales and Griswold, 1987).

In the 1970's, Byskov and Saxen suggested that a somatically generated "meiosis inducing substance" is present in the embryonic ovary, and that it is required for germ cells there to initiate meiotic prophase (Byskov and Saxen, 1976). However, other investigators noted that ectopic germ cells localized not in ovaries but in the adrenal glands, or in the mesonephros, or even in embryonic lung aggregates, also initiated meiosis (Zamboni and Upadhyay, 1983; McLaren, 1984; McLaren and Southee, 1997). These observations led investigators to argue that, if a meiosis inducing substance

Fig. 7. A proposed model for regulation of meiotic initiation in embryonic gonads.

Embryonic ovary: CYP26(B1)^{OFF} ⟶ high [RA] ⟶ *Stra8*^{ON} ⟶ Meiotic initiation

Embryonic testis: CYP26(B1)^{ON} ⟶ low [RA] ⟶ *Stra8*^{OFF} ⟶ No meiotic initiation

existed, it must be present in all of these somatic contexts (Zamboni and Upadhyay, 1983; McLaren and Southee, 1997). Alternatively, they suggested, germ cells in embryonic ovaries may initiate meiosis autonomously, without reference to somatic cues. Our findings suggest that RA is the somatically generated meiosis inducing substance. RA is known to be present in many embryonic tissues, including adrenal gland (Haselbeck et al., 1997; Haselbeck and Duester, 1998), mesonephros (Shen et al., 1992), and lung (Malpel et al., 2000). Thus, our proposal that RA is the meiosis inducing substance readily accounts for the ability of embryonic germ cells to initiate meiosis in diverse extragonadal sites.

Several findings lead us to suggest that RA, though somatically produced, acts directly on embryonic germ cells to induce expression of *Stra8*. As shown by other investigators, ectopic germ cells in diverse extragonadal sites initiate meiosis in the absence, or near absence, of gonadal somatic cells (Zamboni and Upadhyay, 1983; McLaren and Southee, 1997). The simplest interpretation of these observations, together with our present findings, is that RA acts via RARs located in germ cells, rather than signaling indirectly via gonadal somatic cells. In embryonic ovaries, RA receptors are readily detected in germ cells but are expressed at very low levels, if at all, in somatic cells (Morita and Tilly, 1999). Indeed, the promoter of the *Stra8* gene contains two putative RA response elements (RAREs), raising the possibility that RA up-regulates *Stra8* transcription by binding to RARs directly engaged at the *Stra8* promoter.

We do not yet know the tissue source of the RA that 1) induces *Stra8* expression in embryonic ovaries and 2) is eliminated by CYP26 activity in embryonic testes. While the gonads themselves could synthesize RA, at least two possible extragonadal sources merit consideration. First, the embryonic adrenal gland, located just anterior to the gonad, is a site of robust RA synthesis during embryogenesis (Haselbeck et al., 1997; Haselbeck and Duester, 1998). Could diffusion of RA from the embryonic adrenal gland account for the anterior-to-posterior wave of *Stra8* expression observed in the developing ovary (Menke et al., 2003)? Second, by studying mice carrying a *lacZ* transgene controlled by RA response elements (RAREs) (Rossant et al., 1991), we detected RA in the mesonephroi of both sexes at E11.5 and E14.5 (Suppl. Fig. 1), corroborating other investigators' findings (41). (Our assay did not reveal RA in the gonads of either sex at these time points, suggesting that RA concentrations are low in embryonic gonads or, perhaps more likely given our findings, that *RARE-lacZ* detection of RA is ineffective there.)

We discovered that germ cells in cultured embryonic testes will express *Stra8* if exposed to RA, to RAR agonists, or to inhibitors of CYP26 activity (Figs. 2 and 4). An important but unresolved question is whether such treatments of embryonic testes will induce premature meiotic prophase. For example, in embryonic testes cultured in the presence of CYP26 inhibitor R115866, we observed some degree of chromatin condensation in a few cells (data not shown), but this was typically accompanied by and might potentially be confused with apoptosis. Further experiments will be required to determine whether inhibition of CYP26 activity induced *bona fide* meiotic prophase.

McLaren and Southee's results suggested that the somatic cells of the embryonic testis inhibit germ cells from entering meiotic prophase (McLaren and Southee, 1997). Our findings substantiate this hypothesis and identify CYP26-catalyzed degradation of RA as a central mechanism of meiotic inhibition. McLaren and Southee have noted that the embryonic testes' ability to inhibit meiosis is lost if the architectural integrity of the testis cords – rings of somatic cells that enclose the germ cells – is mechanically disrupted (McLaren and Southee, 1997). This observation accords with published data as to the site of *Cyp26b1* expression in testes. In early postnatal and adult testes, *Cyp26b1* expression is confined to the myoid cells that encircle the seminiferous tubules (Vernet et al., 2005). Similarly, in embryonic testes, *Cyp26b1* is expressed in cells outside the developing testis cords (Abu-Abed et al., 2002). Taken together, these observations suggest the possibility that CYP26B1-expressing cells surrounding the testis cords form a catabolic barrier that prevents RA generated outside the cords from reaching the germ cells within. A similar barrier has been described in the adult testis (Vernet et al., 2005).

How does our model (Fig. 7) relate to prevailing understandings of mammalian sex determination, specifically with respect to the role of *Sry*, the sex-determining gene on the Y chromosome? Viewed from the perspective of our present studies and model, *Sry* must function to ensure that cells surrounding the testis cords express *Cyp26b1*. *Sry* has been shown to function in the supporting cell lineage, which gives rise to the intratubular Sertoli cells (Palmer and Burgoyne, 1991; Albrecht and Eicher, 2001). Thus, we surmise that *Sry*'s role in regulating *Cyp26b1* expression is indirect, executed via signaling between somatic cells of different types.

Materials and Methods

Mice. CD1 random-bred mice (Charles River) were employed in all embryonic gonad culture experiments except where mutant strains are specified. *Stra8 -/-* embryos were generated by mating heterozygotes (Baltus et al., 2006). XY^{ZAL} embryos with ovotestes were obtained by crossing Zalende/Ei males (Jackson Laboratory) with C57BL/6 females to generate F_1 hybrids. F_1 males were then backcrossed to C57BL/6 females to create N2 XY^{ZAL} embryos with feminized gonads, as previously reported (Eicher and Washburn, 1983; Menke et al., 2003). Embryos carrying a *RARE-lacZ* transgene were obtained from matings of *RARE-lacZ*$^{/+}$ males (Rossant et al., 1991) with CD-1 females. W^v/W^v (Jackson Laboratory) embryos were obtained by mating heterozygotes.

Embryo Collection and Sexing. To establish timed matings, female mice were housed with male mice overnight. Noon of the day when a vaginal plug was evident was considered E0.5. E11.5 embryos were sexed by isolating DNA and using a multiplex PCR reaction involving two primer pairs. One pair of primers amplifies a 245-bp fragment of myogenin as an internal positive control (primer sequences: 5'-TTACGTCCATCGTGGACAGCAT-3' and 5'-TGGGCTGGGTGTTAGTCTTAT-3'). The second pair of primers amplifies a 342-bp segment of a Y-chromosome repeat sequence (5'-CAGTTACCAATCAACACATCAC-3' and 5'-CTGGAGCTCTACAGTGATGA-3').

Germ Cell Depletion. At 9.5 days *post coitum*, pregnant females were injected intraperitoneally with 200µL of 8mg/mL busulfan (Sigma) in 50% dimethyl sulfoxide

(Merchant, 1975). Embryos from these busulfan-treated mothers were dissected at E11.5 or E12.5.

Embryonic Gonad Cultures and Treatments. Pregnant mice were sacrificed by cervical dislocation, and embryonic gonads were dissected out in phosphate-buffered saline solution. Gonads were cultured atop 1.5-1.7ml agar blocks as described previously (Martineau et al., 1997). The following compounds were dissolved in ethanol and stored in aliquots at -80 °C before use: all-*trans*-retinoic acid (Sigma), RAR pan-antagonist BMS-204493, and selective RAR agonists BMS-194753, BMS-213309, and BMS-270394 (all gifts from Bristol-Myers Squibb). Ketoconazole (Sigma) and CYP26 inhibitor R115866 (a gift from Johnson and Johnson) were dissolved in dimethyl sulfoxide and stored at room temperature prior to use. All compounds were added to the culture media surrounding the agar blocks at least one hour before freshly dissected gonads were deposited on the blocks. The concentrations of compounds in culture media were as follows: BMS-204493, 5μM; all-*trans* RA, 0.7μM; BMS-194753, 0.5μM; BMS-213309, 0.5μM; BMS-270394, 0.5μM; ketoconazole, 0.7μM; and R115866, 0.7μM. Control cultures were treated with ethanol and/or dimethyl sulfoxide as appropriate.

Histology and *In Situ* Hybridization. Embryonic gonads for histological examination were dissected in phosphate-buffered saline solution and cultured as described above. Gonads were then fixed in Bouin's solution at 4°C overnight, sectioned, and stained with hematoxylin and eosin.

For *in situ* hybridizations, embryonic gonads were dissected in phosphate-buffered saline solution and fixed in 4% paraformaldehyde at 4°C overnight. Whole-mount *in situ* hybridizations were performed as previously reported (Wilkinson and Nieto, 1993). Digoxigenin-labeled RNA probes for *Stra8* and *Oct4* (Menke et al., 2003) were detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody and staining with BM Purple alkaline phosphatase substrate (Roche).

Analysis of *Stra8* Expression in VAD Testes. Adult female mice (129/SvJ, Jackson Laboratory) were maintained on a VAD diet (Harlan Teklad) for at least two weeks before mating and throughout pregnancy. Their male offspring were fed a VAD diet for 13-14 weeks. One testis was removed from each animal and cut into two pieces. One half was fixed in Bouin's solution for histological assessment of spermatogenesis. The other half was placed in Trizol (Invitrogen) for RNA extraction and served as a pre-injection control in RT-PCR analysis. Incisions were sealed and the animals allowed to recover for 24 hours. Five animals with similarly deficient spermatogenesis (as judged by pre-injection testicular histology) were selected for subsequent analysis. Three (experimental) animals were injected subcutaneously with 100µL of 15mg/mL all-*trans*-retinol (ROL) acetate (Sigma) in 10% ethanol and sesame oil. Two (control) animals were injected with 100µL of 10% ethanol and sesame oil. The experimental and control animals' remaining testes were harvested 24 hours after injection and placed in Trizol for RNA extraction. Quantitative RT-PCR analysis was performed on a Prism 7000 System (Applied Biosystems) using SYBR-Green labeling. Each RNA sample was analyzed, in triplicate, with *Stra8* primers that amplify a 151-bp product (primers: 5'-

GTTTCCTGCGTGTTCCACAAG-3’ and 5’-CACCCGAGGCTCAAGCTTC-3’) and control *Rps2* primers that amplify a 112-bp product (primers: 5'-CTGACTCCCGACCTCTGGAAA-3' and 5'-GAGCCTGGGTCCTCTGAACA-3’). *Stra8* expression was normalized to *Rps2* expression and compared in pre- and post-injection testes from each mouse.

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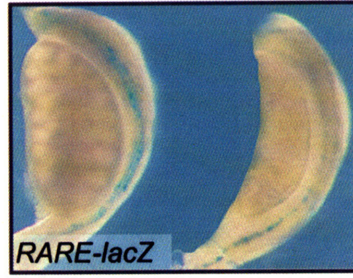
Suppl. Fig. 1. LacZ expression in the mesonephroi of (A) E11.5 or (B) E14.5 *RARE-lacZ* embryos.

A
XY
XX
RARE-lacZ


B
XY
XX
RARE-lacZ

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CHAPTER 3

In vivo, retinoic acid perturbs development of the embryonic testes without inducing functional meiotic prophase

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Author contribution: Targeted disruption of *Cyp26b1* in mice was performed by Jana Koubova except for blastocyst injections, which were performed by Mary Goodheart. All of the experiments were designed, performed and analyzed by Jana Koubova with the following two exceptions: Yueh-Chiang Hu designed, performed and analyzed the experiment with methylation markers. Jana Koubova designed and Yueh Chiang Hu performed and analyzed the SCP3 and REC8 loading experiments. David Page provided helpful discussions throughout the project and assisted in the writing of the manuscript.

Abstract

Retinoic acid (RA) is necessary for meiotic entry in mouse embryonic ovaries. In embryonic testes, RA is metabolized by a testis-specific enzyme CYP26B1 (Koubova et al., 2006; Bowles et al., 2006). It has been declared that RA is sufficient for "full-scale meiosis" in testes and that "CYP26B1 holds the key to preventing oogenesis in males by retarding meiosis"(Bowles et al., 2006). We examined the effects of RA on testes development and its ability to induce meiosis *in vivo,* using *Cyp26b1-/-* mice. Contrary to expectations, RA was not sufficient for meiotic prophase in embryonic testes. *Cyp26b1-/-* testicular germ cells failed to enter G0/G1 arrest and proliferated. They also failed to initiate male-specific DNA re-methylation and gradually died. Testis cords formed but Leydig cells did not differentiate and the cords became disorganized later in development. Thus, RA is toxic to embryonic testes and its metabolism by CYP26B1 is essential for development of both germ cells and somatic cells.

Germ cells are the only cells in our body that undergo meiosis – a specialized cell division process that results in recombination and haploid chromosome content. This process is the core of sexual reproduction, because it ensures haploid and unique DNA content in male gametes (sperm) and female gametes (eggs). Interestingly in mammals, the timing of meiotic entry is different between males and females. In ovaries, germ cells enter meiosis during embryonic development. In embryonic testes, germ cells enter G0/G1 arrest instead, and only enter meiosis after birth.

Recently, it has been shown that retinoic acid (RA) is necessary for germ cell meiotic entry in embryonic ovaries (Koubova et al., 2006; Bowles et al., 2006), because it up-regulates *Stimulated by retinoic acid gene 8 (Stra8),* which is required for pre-meiotic DNA replication (Baltus et al., 2006). In embryonic testes, RA is prevented from inducing *Stra8* expression because of the retinoic acid-degrading cytochrome p450 enzyme, CYP26B1, which is expressed in the testes at that time (Menke et al., 2003; Koubova et al., 2006; Bowles et al., 2006). It has been concluded that RA is not just necessary for meiotic entry, but also sufficient for "full-scale" meiosis in embryonic testes and that CYP26B1 prevents "oogenesis in males by retarding meiosis *in vivo*" (Bowles et al., 2006). This conclusion relied on cultures of embryonic testes with a non-specific cytochrome p450 inhibitor and two experiments with *Cyp26b1-/-* testes, which upregulated mRNAs of *Stra8* and the synaptonemal complex component, *Scp3.* It is unknown if expression of these genes is sufficient to induce functional meiosis, and if *Stra8* expression can induce other meiotic markers in the testes *in vivo.*

Cultures of rat testes with RA showed that retinoic acid negatively affects early testicular somatic development. *In vitro,* RA inhibited testicular cord formation by preventing mesonephric cell migration. RA also inhibited differentiation of Sertoli cells, and caused germ cell death (Li and Kim, 2004). In a different *in vitro* culture system, RA had the opposite effect on XY primordial germ cells and promoted germ cell survival and proliferation (Koshimizu et al., 1995). *In vivo,* the effects of RA on germ cell and somatic cell development in embryonic testes are unknown and it is not clear if physiological levels of RA in embryonic gonads could produce any of the effects observed in culture. To determine the *in vivo* effects of RA on testicular development and to establish if it is sufficient to induce meiotic prophase in XY germ cells, we generated a targeted deletion of *Cyp26b1* in mice (Suppl. Fig. 1).

Contrary to expectations, RA was insufficient for functional meiotic prophase and oogenesis in embryonic testicular germ cells. *Cyp26b1-/-* testicular germ cells upregulated markers of meiotic prophase including *Stra8, Rec8, Scp3,* and *Dmc1* (Fig. 1A). However, the protein or mRNA localization of these markers was abnormal and at least one early essential meiotic marker was not upregulated. *Stra8* expression in embryonic ovaries occurs in an anterior-to-posterior wave beginning at E12.5 and disappearing by E15.5 (Menke et al., 2003). *Stra8* expression is completely absent in wild type embryonic testes. In *Cyp26b1-/-* testes, *Stra8* expression was induced, but not downregulated by E15.5 as it would normally occur in embryonic ovaries (Fig. 1B). REC8, a meiosis-specific cohesin, is normally loaded onto DNA during pre-meiotic DNA replication (Klein et al., 1999; Eijpe et al., 2003). Cell spread experiments revealed that

REC8 is present but fails to load onto DNA in over 95% of all germ cells inside *Cyp26b1-/-* testes. In contrast, over 90% of XX germ cells have loaded REC8 on their chromosomes by the same time (Fig. 1C, Suppl. Table 1). Similarly, SCP3, a component of the synaptonemal complex (Yuan et al., 2000), also fails to load onto DNA in 95% of all *Cyp26b1-/-* testicular germ cells while it is loaded in nearly all ovarian germ cells (Fig. 1D, Suppl. Table 1). *Spo11* is one of the most highly conserved factors in meiosis. It is required to generate double-stranded breaks for recombination that are observed abundantly during the leptotene stage of meiotic prophase (Romanienko and Camerini-Otero, 2000; Mahadevaiah et al., 2001). *Spo11* is present in negligible amounts in *Cyp26b1-/-* testes in comparison to control ovaries (Fig. 1A). *Figla*, an oocyte-specific gene first expressed at E13.5 and required for the formation of primordial follicles (Soyal et al., 2000), is not expressed in *Cyp26b1-/-* testes (Fig. 1E). Finally, MSY2, a marker of diplotene germ cells (Gu et al., 1998; Yu et al., 2001), did not stain E16.5 (Fig. 1F) *Cyp26b1-/-* testes, while it is abundantly present in control ovaries. Thus, RA is not sufficient for functional meiotic prophase or oogenesis in testicular germ cells.

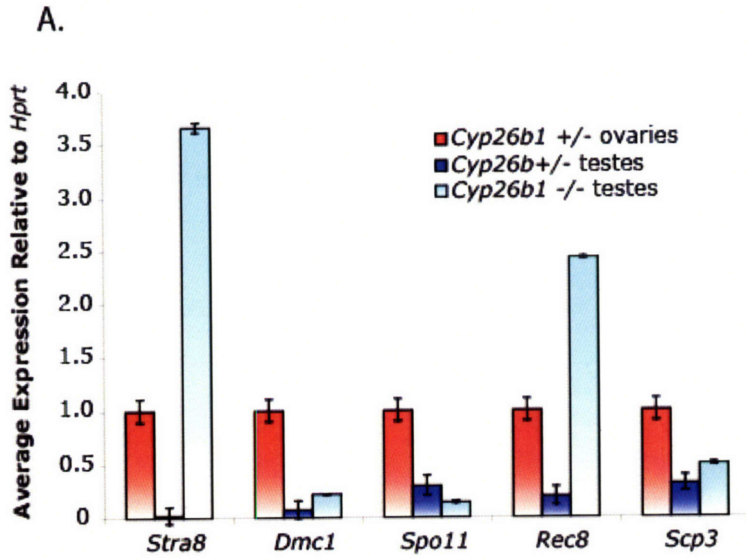
While RA is not sufficient for meiotic prophase *in vivo*, it is able to induce increased mRNA levels of several meiotic markers. An *in vitro* culture of wild type embryonic testes with RA led to increased expression levels of *Scp3, Dmc1* and *Stra8* and these levels appeared close to the expression levels observed in ovaries (Bowles et al., 2006). Our results show that RA is sufficient to upregulate mRNA levels of these markers in embryonic testes but the expression levels are not as dramatic as previously shown *in vitro* for *Scp3* and *Dmc1*. This is due to a difference in normalization methods of

Figure 1. RA is not sufficient to induce functional meiosis in *Cyp26b1-/-* testes.

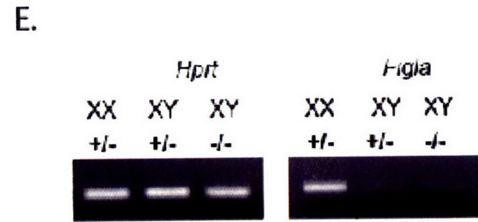
A. Quantitative RT-PCR analysis of meiotic markers in E14.5 *Cyp26b1-/-* and *Cyp26b1+/-* testes and ovaries. Only *Stra8* and *Rec8* have highly upregulated expression levels in comparison to ovaries, where meiosis is taking place. *Spo11* is not upregulated in *Cyp26b1-/-* testes. B. Whole mount *in situ* hybridization with *Stra8* probe shows that *Stra8* is not downregulated in E15.5 *Cyp26b1-/-* testes, while it is downregulated in *Cyp26b1+/-* ovaries and not present in *Cyp26b1+/-* testes. C. Immunohistochemical staining showing that REC8 (green) is not loaded on the DNA in E15.5 *Cyp26b1+/-* or *Cyp26b1-/-* XY germ cells (red = germ cell marker GCNA), while it is loaded on the chromosomes in XX *Cyp26b1+/-* germ cells. D. Immunohistochemical staining showing that SCP3 (green) is not loaded on the DNA at E15.5 in XY *Cyp26b1+/-* or *Cyp26b1-/-* germ cells (red = germ cell marker GCNA), while it is loaded on the chromosomes in XX *Cyp26b1+/-* germ cells. E. RT-PCR showing that E14.5 *Cyp26b1 -/-* testes fail to express *Figla,* transcription factor necessary for primordial follicle formation.

F. Immunohistochemical staining showing that cytoplasmic protein, MSY2, is not expressed in E16.5 *Cyp26b1+/-* or *Cyp26b1-/-* testes, while it is expressed in *Cyp26b1+/-* ovaries (red = germ cell marker GCNA, green = MSY2, blue = DAPI; arrowheads = auto fluorescent cells; arrows = examples of MSY2-positive germ cells).

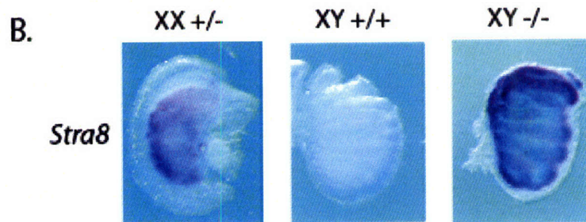
A.



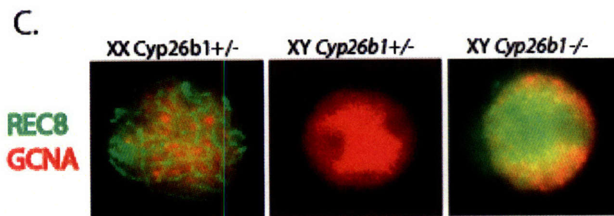
E.



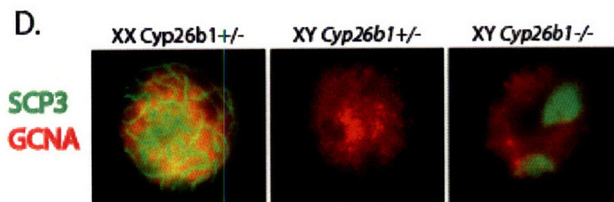
B.



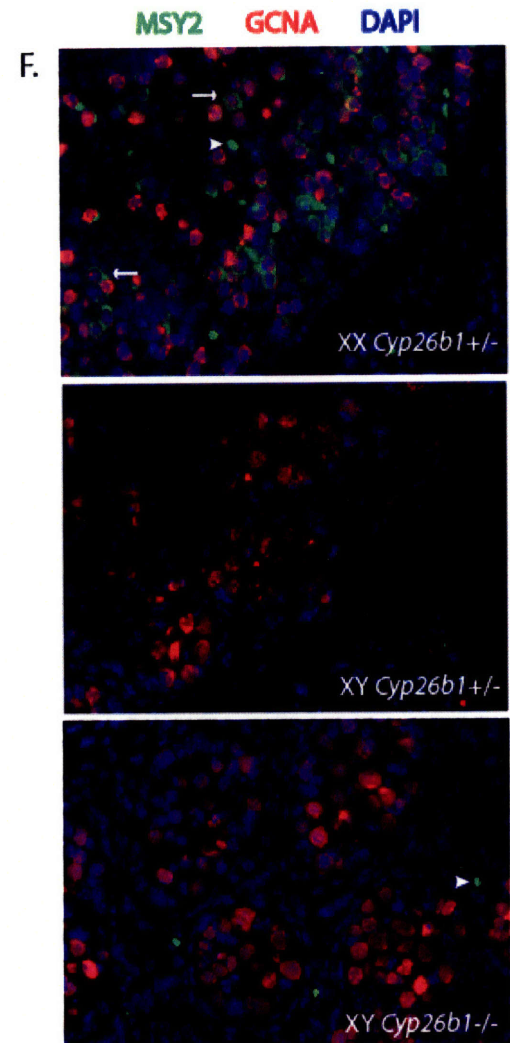
C.



D.



F.



quantitative RT-PCR – the *in vitro* experiments were normalized to *mouse vasa homologue (Mvh)*, while our results from *Cyp26b1-/-* testes are normalized to *hypoxanthine guanine phosphoribosyl transferase 1* (*Hprt*). Using *Mvh* as a normalization factor makes levels of upregulation of meiotic markers appear higher, because its own expression is down-regulated by RA *in vivo* (Suppl. Fig. 2). In the absence of a marker of germ cells, whose expression reliably represents germ cell number, we have normalized to *Hprt,* a housekeeping gene present in all cells.

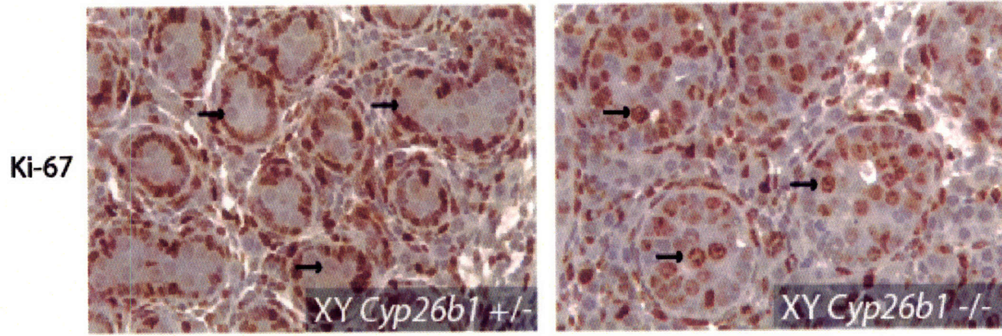
We found that only two factors, *Stra8* and *Rec8,* had expression levels significantly higher in *Cyp26b1-/-* testes than in wild type ovaries. *Scp3* and *Dmc1* had higher levels than control testes but not as high as observed in embryonic ovaries. To determine if RA is sufficient to induce meiotic markers independently or if their upregulation is dependent on *Stra8* expression, we generated *Cyp26b1-/- Stra8-/-* double knockout testes. Only *Rec8* maintained increased expression levels in the double knockout embryos, while all other markers appeared to require *Stra8* (data not shown). Thus, RA is only sufficient to induce *Rec8* and *Stra8*, while *Stra8* expression is required for *Dmc1* and *Scp3* upregulation.

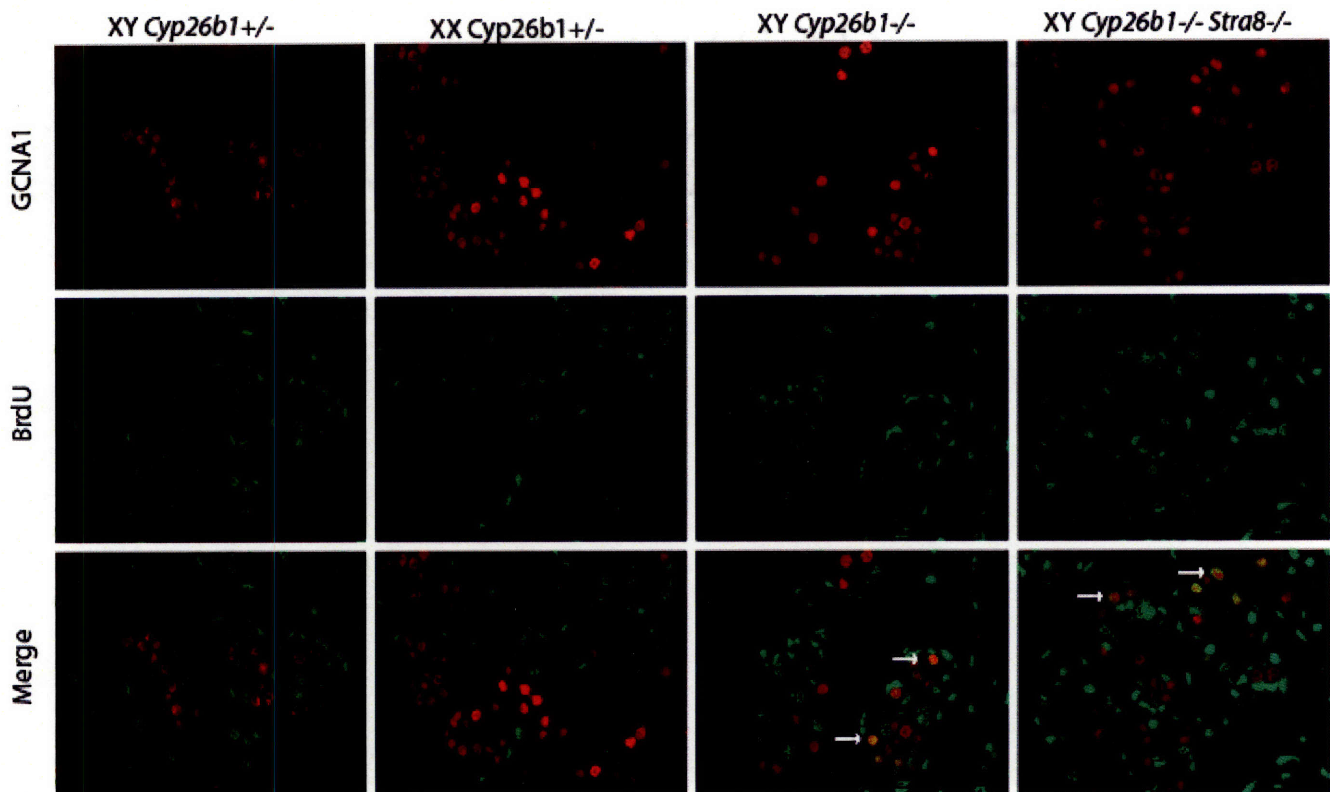
Since embryonic testicular germ cells do not enter meiosis but arrest in G0/G1, we used a Ki-67 antibody, which stains cycling but not resting cells (Scholzen and Gerdes, 2000), to determine if *Cyp26b1-/-* testicular germ cells stopped cycling by E16.5. In control embryonic testes, germ cells do not stain with Ki-67, while somatic cells continue to proliferate and stain with Ki-67. In *Cyp26b1-/-* testes, Ki-67 stained

germ cells as well as somatic cells suggesting that *Cyp26b1-/-* testicular germ cells failed to arrest in G0/G1 (Fig. 2A). To ensure that these cells did not just delay G0/G1 arrest, we used BrdU incorporation, which labels cells during S phase at E18.5. By E18.5, all germ cells in wild type ovaries entered diplotene arrest and all germ cells in wild type testes are arrested in G0/G1. We used GCNA antibody, which specifically labels germ cells, and BrdU-antibody to label cells replicating their DNA during S phase. As expected, at E18.5, wild type ovaries did not express BrdU and GCNA in the same cells (Fig. 2B). Similarly, wild type testes presented no cells staining with both GCNA and BrdU (Fig. 2B). However, *Cyp26b1-/-* testes revealed a number of cells with overlapping staining for GCNA and BrdU indicating that germ cells were still replicating their DNA at E18.5 (Fig. 2B). To confirm that this staining was not indicative of pre-meiotic DNA replication, we also tested *Cyp26b1-/- Stra8-/-* embryos. *Cyp26b1-/- Stra8-/-* embryonic testes showed an overlapping BrdU and GCNA expression identical to single knockout *Cyp26b1-/-* testes (Fig. 2B). Thus, RA induces proliferation in testicular germ cells. This proliferation is not caused by ectopic *Stra8* expression or expression of meiotic markers downstream of *Stra8*.

Only a few mouse mutants display the failure of male germ cells to arrest in G0/G1 during embryonic development. One of these mutants is a germ cell-specific conditional knockout of *phosphatase and tensin homologue* (*PTEN*). *PTEN*-deficient germ cells in embryonic testes fail to arrest in G0/G1 and form teratomas after birth (Kimura et al., 2003). The germ cell proliferation paradoxically coincides with progressive embryonic germ cell death. This is similar to the phenotype of *ter* mice,

Figure 2. RA causes XY germ cell proliferation and failure to arrest in G0/G1. A. E16.5 testicular germ cells (black arrows) in *Cyp26b1+/-* testes no longer stain with antibody against Ki-67, a marker of cycling cells. In E16.5 *Cyp26b1-/-* testes, many germ cells (black arrows) stain with Ki-67 antibody. B. At E18.5, *Cyp26b1+/-* testes, all germ cells are arrested in G0/G1 and do not incorporate BrdU. In *Cyp26b1+/-* ovaries, germ cells are arrested in diplotene and do not incorporate BrdU. However, in *Cyp26b1-/-* testes or in *Cyp26b1-/- Stra8-/-* testes, many germ cells are replicating their DNA and incorporating BrdU (white arrows), [red = germ cell marker GCNA, green = BrdU, yellow = GCNA and BrdU overlap].


A.
Ki-67
XY Cyp26b1 +/-
XY Cyp26b1 -/-

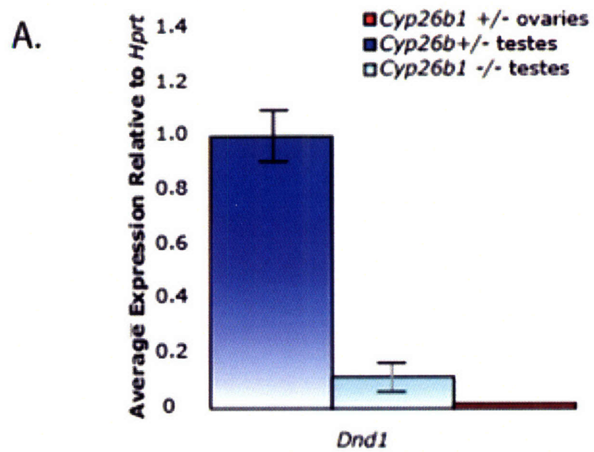

B.
XY Cyp26b1+/-
XX Cyp26b1+/-
XY Cyp26b1-/-
XY Cyp26b1-/- Stra8-/-
GCNA1
BrdU
Merge

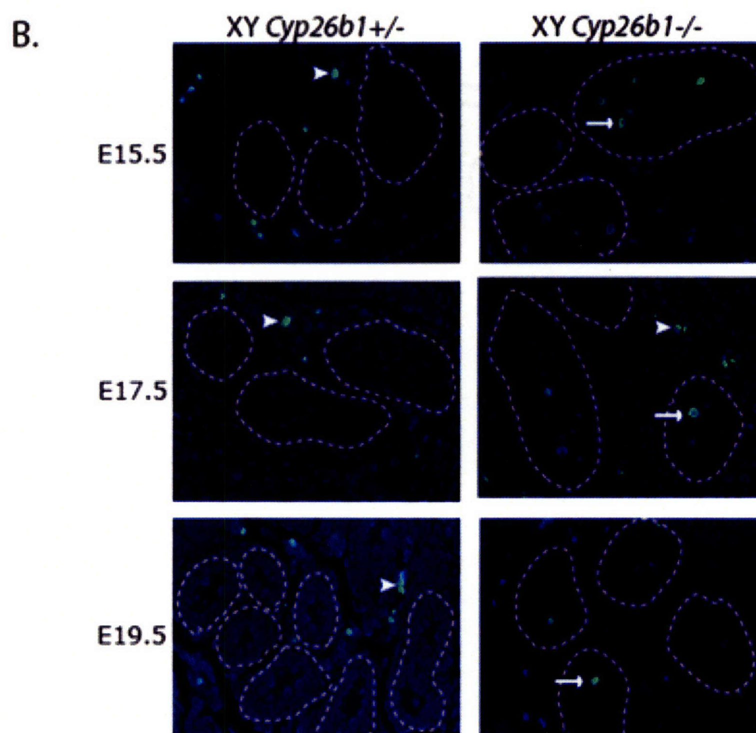
where early germ cell death is observed on all mouse backgrounds (Sakurai T, 1995), but in the inbred 129 strains, some surviving PGCs form teratomas after birth (Kimura et al., 2003). Interestingly, *Cyp26b1-/-* testes express dramatically lower levels of *Dnd1,* the gene affected by the *ter* mutation (Youngren et al., 2005) (Fig. 3A).
It has been hypothesized that different *Dnd1* levels between male and female gonads may reflect the sex-specific differences in susceptibility to teratocarcinogenesis (Youngren et al., 2005).

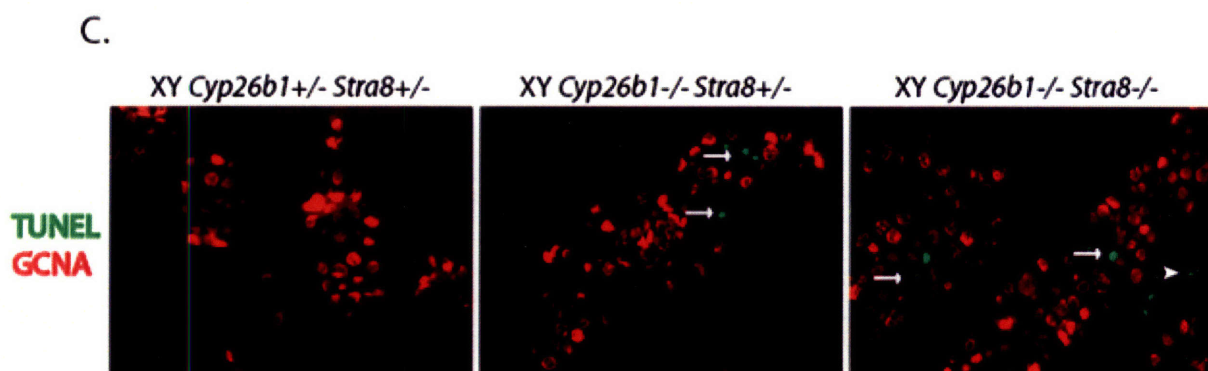
To see if cell death can also be detected along with continued germ cell proliferation, we used the TUNEL assay, which detects the late stages of apoptosis. We observed TUNEL-positive cells inside *Cyp26b1-/-* testicular cords from E15.5 until birth. However, several condensed nuclei indicative of cell death were observed in *Cyp26b1 -/-* testes already at E14.5, suggesting that cell death process occurs gradually over several days (Fig. 3B). TUNEL positive cells were also observed inside the cords of *Cyp26b1-/- Stra8-/-* testes suggesting that cell death was not caused by the ectopic expression of meiotic markers (Fig. 3C).

During the G0/G1 arrest, wild type embryonic testicular germ cells initiate *de novo* DNA methylation (Coffigny et al., 1999). Timing of this process is sex-specific because ovarian germ cells do not undergo *de novo* DNA methylation until after birth. It is not known what regulates this process in males or if arrest in G0/G1 is required for this process to occur. We tested for the presence of DNA methylation in E16.5 *Cyp26b1-/-* embryonic testes and found that *Cyp26b1-/-* germ cells do not stain with the 5-

Figure 3. RA causes apoptosis inside testes cords. A. Quantitative RT-PCR analysis showing that E14.5 *Cyp26b1-/-* testes express dramatically lower levels of *Dnd1* than *Cyp26b1+/-* testes. B. TUNEL assay staining apoptotic cells (white arrows) inside testes cords (outlined with dotted line) with fluorescein from E15.5 through birth (blue = DAPI, green = dying cells; arrowheads = autofluorescent cells outside testes cords). C. E16.5 *Cyp26b1-/-* testes and *Cyp26b1-/- Stra8-/-* testes display TUNEL-positive apoptotic cells among germ cells inside testes cords (red = germ cell marker GCNA, green/white arrows = dying cells, arrowhead = autofluorescent cells outside cords).


A.
Cyp26b1 +/- ovaries
Cyp26b+/- testes
Cyp26b1 -/- testes
Average Expression Relative to Hprt
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Dnd1


B.
XY Cyp26b1+/-
XY Cyp26b1-/-
E15.5
E17.5
E19.5


C.
XY Cyp26b1+/- Stra8+/-
XY Cyp26b1-/- Stra8+/-
XY Cyp26b1-/- Stra8-/-
TUNEL
GCNA

methylcytosine antibody (Fig. 4A) suggesting that methylation is inhibited or delayed by RA. Since methyltransferase *Dnmt3a* is required for DNA methylation in male germ cells (Bourc'his and Bestor, 2004), we also tested for DNMT3A expression in *Cyp26b1-/-* gonads. *Cyp26b1-/-* testicular germ cells fail to upregulate DNMT3A (Fig. 4B), suggesting that absence of RA from embryonic testes is essential for re-establishing DNA methylation during embryonic development.

RA has been previously shown to inhibit mesonephric cell migration and cord formation, as well as Sertoli cell differentiation in rat testes *in vitro* (Li and Kim, 2004). Mesonephric cell migration takes place in the embryonic testes but not the embryonic ovary from E11.5 through E16.5 (Martineau et al., 1997), and it induces the formation of testicular cords, which appear at E12.5 (Tilmann and Capel, 1999). Microscopic examination of *Cyp26b1-/-* testes reveals that testicular cords and the characteristic coelomic vessel form, and are indistinguishable from wild type testes at E12.5 (data not shown). Presence of cords in the *Cyp26b1-/-* testes suggests that early mesonephric cell migration took place. However, little is known about the cord maintenance and cord remodeling during the later stages of embryonic development or about the function of mesonephric cell migration beyond E12.5. While testicular cords form in *Cyp26b1-/-* testes, their maintenance during later embryonic development is abnormal. By E15.5, disorganization of the cords becomes obvious. Some *Cyp26b1-/-* testes have fewer cords and the cords appear larger. Somatic cells can be seen inside the cords and Sertoli cells appear to detach from basal lamina. At E18.5, in some tubules, germ cells appear to move from the center of the tubule to the periphery, replacing the Sertoli cells (Figure 4C).

To examine if Sertoli and Leydig cells are differentiating normally, we tested expression levels of several somatic cell markers (Fig. 4D). Expression levels of Sertoli cell markers, *Sox9*, *Dhh* and *Fgf9,* appear indistinguishable in wild type and *Cyp26b1-/-* testes at E14.5 suggesting that the Sertoli cells differentiate normally. Markers of Leydig cells showed more dramatic changes. One of the earliest Leydig cell-specific markers, *Scc* (Yao et al., 2002), showed a lower level of expression in E14.5 *Cyp26b1-/-* testes than in controls. *Cyp19* and *Cyp17* also exhibited lower levels in *Cyp26b1-/-* testes, indicating that differentiation of embryonic Leydig cells is impaired. Expression of *Pdgfra,* which is required for embryonic Leydig cell differentiation (Brennan et al., 2003), was also lower in *Cyp26b1-/-* testes in comparison to control testes. Orphan nuclear hormone receptor *Sf1,* expressed in both the Sertoli cells and the embryonic Leydig cells (Hatano et al., 1994), also revealed lower level of expression in *Cyp26b1-/-* testes than in control testes, again indicating that embryonic Leydig cell differentiation is perturbed by RA.

Interestingly, *Dax1,* an orphan nuclear hormone receptor with complex roles in ovarian and testicular development, has significantly higher level of expression in *Cyp26b1-/-* testes than in control testes or ovaries (Fig. 4D). *Dax1* is expressed in both Sertoli and Leydig cells in the testes, where it is required for early cord formation (Meeks et al., 2003). It also plays a role in the somatic cells of the embryonic ovary, where it increases in expression after E12.5. Its expression in the testes is decreased at the same time (Swain et al., 1996). It has been shown that *Dax1*-deficient testes fail to upregulate

Figure 4. RA prevents male-specific DNA methylation and DNMT3A expression in germ cells. A. E15.5 *Cyp26b1+/-* testicular germ cells stain with methylcytosine antibody, while *Cyp26b1-/-* germ cells show little or no methycytosine staining. B. DNMT3A antibody labels *Cyp26b1+/-* testicular germ cells while it is absent in *Cyp26b1-/-* testicular germ cells. C. At E18.5, there is an abundance of germ cells in *Cyp26b1-/-* testes, however, cords are disorganized and many germ cells line the edges of the cords (red = germ cell marker GCNA, blue = DAPI). D. Quantitative RT-PCR showing that markers of Leydig cells but not Sertoli cells have lower expression levels in *Cyp26b1-/-* testes at E14.5. *Dax1* is highly upregulated in E14.5 *Cyp26b1-/-* testes.

XY *Cyp26b1+/-* XY *Cyp26b1-/-*

A.

MeC

B.

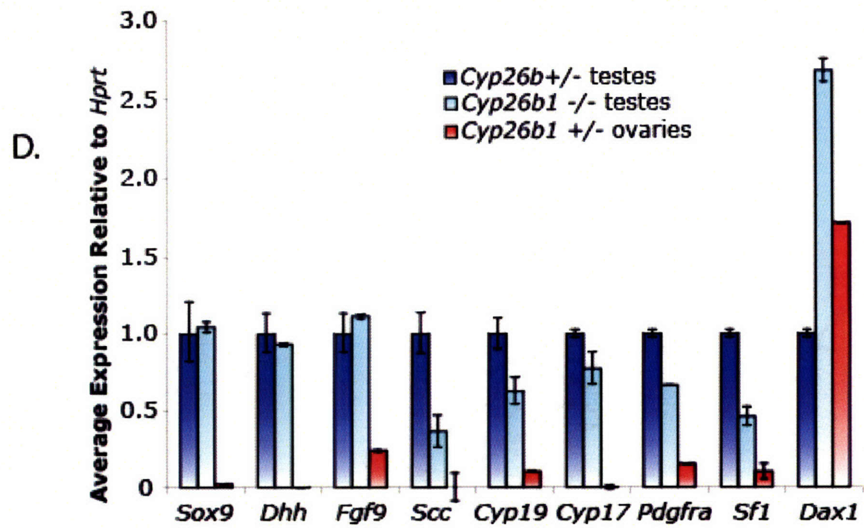
DNMT3A

C.

XY *Cyp26b1+/-* XY *Cyp26b1-/-*

GCNA
DAPI

D.



Cyp26b1 (Bouma et al., 2005), suggesting that a negative feedback loop exists between RA and *Dax1* signaling in embryonic testes.

It is difficult to definitively tease apart the different effects of RA on testicular development. Do somatic cell defects cause the germ cell defects or vice versa? Experimental evidence suggests that RA likely affects germ cells and somatic cells separately. First, germ cell presence is not essential for the normal somatic development of the embryonic testes (Merchant, 1975), suggesting that germ cell death and other germ cell defects observed in *Cyp26b1-/-* testes do not cause the initial somatic defect in embryonic Leydig cell differentiation. Second, primordial germ cells cultured with retinoic acid, in the absence of any feeder cells, proliferate, and do not arrest in G0/G1 (Koshimizu et al., 1995). RA can thus induce germ cell proliferation and failure to enter G0/G1 arrest independently from its effects on somatic cells.

It has been reported that a small population of germ cells enters meiosis in wild type embryonic testes (McLaren, 1984; Yao et al., 2003). We also observed a small population of germ cells, which load SCP3 and REC8 on their chromosomes in wild type embryonic testes (~1-2% as visualized in cell spreads, Suppl. Table 1). We observed that in some *Cyp26b1-/-* testes, this small population can be slightly increased (approximately 2-5%), but it never reaches the levels observed in wild type ovaries where over 90% of germ cells load SCP3 and REC8 on their chromosomes by E15.5. If RA can slightly increase the number, why is it not sufficient to induce full-scale meiosis in all male germ cells? It is possible that the *in vivo* timing or physiological levels of RA are not sufficient

for meiosis in embryonic *Cyp26b1-/-* testes. Re-aggregation experiments have shown that E11.5 testicular germ cells can be placed into a meiosis-inducing environment, where they will enter meiosis. However, E12.5 testicular germ cells placed in the same environment continue to develop as prospermatogonia (McLaren and Southee, 1997). Thus between E11.5 and E12.5, XY germ cells lose their ability to enter meiosis. It is possible that in *Cyp26b1-/-* testes, RA acts on germ cells after most testicular germ cells have lost their sexual plasticity. Thus beyond upregulating expression of a few meiotic genes, RA is not sufficient for germ cells to proceed through meiotic prophase as they would in the ovary. This is also consistent with the finding that *in vivo,* RA does not inhibit the formation of testis cords, in contrast to what has been demonstrated *in vitro.* Testis cord formation takes place between E11.5 and E12.5, precisely at the time when testicular germ cells lose their sexual plasticity and may thus no longer respond to RA.

Why don't *Cyp26b1-/-* testicular germ cells successfully proceed through meiotic prophase? Clearly, RA is sufficient to induce expression of several markers of meiosis, suggesting that some portion of the meiotic program is turned on. However, meiotic marker localization is inconsistent with functional meiotic prophase and indicates problems preceding pachytene. Additional germ cell defects observed in *Cyp26b1-/-* testes, such as proliferation and cell death, also appear in *Cyp26b1-/- Stra8-/-* mice, suggesting that meiotic marker upregulation does not cause these defects. Thus, the role of CYP26B1 is not as a meiosis-inhibiting substance (since *Cyp26b1-/-* germ cells fail to proceed normally through meiotic prophase) but rather a mitosis-inhibiting substance.

Recent observations indicate that RA is necessary for meiotic entry in embryonic ovaries, because it stimulates *Stra8* expression (Koubova et al., 2006; Bowles et al., 2006) and suggest that RA is sufficient to induce "full-scale meiosis" in embryonic testes and that "CYP26B1 holds the key to preventing oogenesis in males by retarding meiosis *in vivo"* (Bowles et al., 2006). Our experiments show that i) RA is not sufficient to induce "full-scale" meiosis in embryonic testes *in vivo,* as judged by a thorough examination of expression and protein localization of meiotic markers in *Cyp26b1-/-* testes; ii) instead of progression through meiotic prophase, RA causes testicular germ cell proliferation and failure to enter male-specific G0/G1 arrest; iii) RA causes *Dnd1* downregulation concurrent with gradual cell death inside cords; iv) RA inhibits male germ cells from initiating *de novo* DNA methylation; v) CYP26B1 has an important novel function in protecting somatic cells from endogenous levels of RA, which causes defects in Leydig cell differentiation and disrupts testis cord maintenance *in vivo.*

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Methods and Materials

Mice:

Cyp26b1 null mice were made by targeted deletion of *Cyp26b1* exons 3 through exon 6 by homologous recombination. A *Cyp26b1/PGK-Neo* targeting construct was generated using PCR products amplified with Advantage HF2 polymerase (Clontech) from BAC RP24-470013. All PCR products were sequenced to ensure absence of point mutations. The v6.5 ES cells (Rideout et al., 2000) were electroporated with 50μg of linearized targeting construct and selection was performed with Geneticin (Invitrogen). ES colonies were screened by PCR specific for correct targeting of both homology arms (right arm: nrg5/6/7/8R: cctagtccctagaaccctcatggacatt; nra5/6/7/8bL: catcgcattgtctgagtaggtgtcattc; and left arm, lgn3aL: ttgagaggaagtctggctgagagagaag; lan1r: ccagactgccttgggaaaagtactgata). Clones which tested positive by both PCR assays were further confirmed by Southern blot analysis (5' left probe primer 1L: gcaggcagctggagaact; right probe primer 1R: atgcatagggtctggagt; 3' left probe primer 1L: cctaggcaaatgatcctcca; right probe primer 1R: gagaagaccgcattcaccat) using EcoRV and Nde1 enzymes. Correctly targeted ES clones were injected into BalbC or C57Bl/6 blastocysts and transferred to pseudopregnant Swiss Webster females. Germline transmission was obtained from one clone. *Cyp26b1-/+* and *Cyp26b1-/-* embryos were genotyped by hot start PCR assays (95C for 4min, 95C for 30sec, 68C for 4min, go to step 2 30x, 68 for 4 min, 4C forever, end) using Advantage 2 polymerase (Clontech) with three primers (lgn3aR: tgccttgggaaaagtactgataacttcg; lan1bL gactgcaggggaaattctcacatttaca; ro3ge: gagaagacattctccacaaactgctggt) as well as independently by Transnetyx (Memphis, TN). *Cyp26b1-/-* embryos and

Cyp26b1-/- Stra8-/- embryos were obtained by breeding *Cyp26b1+/-* and *Stra8+/-* mice (Baltus et al., 2006).

Quantitative RT-PCR:

Total RNA was prepared by Trizol (Gibco BRL) extraction from pooled E14.5 gonads (without mesonephroi) and DNase-treated using DNA-free kit (Ambion). It was reverse-transcribed using random decamers by RETROscript (Ambion). Target cDNA was analyzed using SYBR Green PCR Core Reagents (Applied Biosystems). All expression targets were tested in triplicate on 7500 Fast Real-Time PCR System (Applied Biosystems). Data was analyzed using comparative Ct method, error bars indicate standard deviation. Because no known germ cell marker can accurately represent germ cell number, *hypoxanthine guanine phosphoribosyl transferase 1* (*Hprt)* was used for normalization.

Primers were designed using PCR Primer Bank (Wang and Seed, 2003):

Hprt -f: tcagtcaacgggggacataaa; *Hprt -r:* gggggctgtactgcttaaccag

Stra8 -f: ctgttgccggacctcatgg ; *Stra8 –r:* tcacttcatgtgcagagatgatg

Rec8 -f: ctacctagcttgcttctttccca; *Rec8 -r:* gcctctaaaaggtgtcgaatctg

Dmc1hA: ccctctgtgtgacagctcaac; *Dmc1hB*: ggtcagcaatgtcccgaag

Spo11-f: cgtggcctctagttctgaggt ; *Spo11-r:* gctcgatctgttgtctattgtga

Scp3 -f: agccagtaaccagaaaattgagc; *Scp3 -r:* ccactgctgcaacacattcata

Dnd1 -f: gaggtgtatatcggacgacttcc; *Dnd1 -r:* ggttcaaaccactgaaggtcatc

Mvh -f: gcttcatcagatattggcgagt; *Mvh -r:* gcttggaaaaccctctgctt

Dazl -f: atgtctgccacaacttctgag *; Dazl -r:* ctgatttcggtttcatccatcct

Sox9-f: agtacccgcatctgcacaac; *Sox9-r:* tacttgtaatcggggtggtct

Dhh- f: cttggcactcttggcactatc; *Dhh- r:* gacccccttgttaccctcc

Fgf9 –f: atggctcccttaggtgaagtt; *Fgf9 –r:* tcatttagcaacaccggactg

Scc-f: aggtccttcaatgagatccctt; *Scc-r:* tccctgtaaatggggccatac

Cyp17a1-f: cagagaagtgctcgtgaagaag; *Cyp17a1-r:* aggagctactactatccgcaaa

Cyp19-f: caagtcctcaagcatgttcca *Cyp19-r:* aaggctcgggttgttgttaaata

PdgfraF: tccatgctagactcagaagtca; *PdgfraR:* tcccggtggacacaatttttc

Sf1-f: cccaagagttagtgctccagt; *Sf1-r:* ctgggcgtcctttacgagg

Dax1-f: gggcagcatcttatacagcttg; *Dax1-r:* cactctgggtacagtaggacag

Cell spreads:

Gonads were removed from E15.5 embryos of both sexes and dissected free of mesonephroi. They were incubated in 50 ul of trypsin-EDTA solution at 37 °C for 5 min and then washed briefly in PBS. To obtain single cells, trypsin-digested gonads were pipetted repeatedly and then centrifuged, followed by resuspension in hypotonic solution (0.5% sodium chloride). Cell suspensions were placed on the slides coated with VECTABOND reagent (Vector Laboratories) and kept in a humid chamber at 37 °C until the cells had settled. The slides were then fixed in 2% paraformaldehyde and 0.03% SDS for 15 min at 4°C, washed three times in 0.4% Photoflo (Kodak) for 1 min and air dried. Slides were stored at -80 °C before use.

For fluorescence immunostaining, slides were removed from storage at -80 °C, washed twice in PBS and treated with blocking buffer (10% goat serum, 10% donkey serum, 1% bovine serum albumin, 0.05% Tween-20 and 0.05% Triton X-100 in PBS) for 30 min. Slides were then incubated with a 1:500 dilution of rabbit anti-rat SCP3 (courtesy of C. Heyting) or rabbit anti-REC8 (courtesy of C. Heyting) in anti-GCNA IgM supernatant (courtesy of G. Enders) overnight at 4 °C, washed with PBS and incubated with Texas Red– or fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:400 dilutions; Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After extensive washing with PBS, slides were mounted using VECTASHIELD medium with 4,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

***In situ* hybridization and immunohistochemistry:**

Embryonic gonads for whole mount *in situ* hybridization were dissected in PBS solution and fixed at 4C in 4% paraformaldehyde over night. Whole mount *in situ* hybridization was performed as previously described (Wilkinson and Nieto, 1993) using digoxigenin-labeled RNA probe for *Stra8* (Menke et al., 2003). Probe was detected by alkaline phosphatase-conjugated antidigoxigenin antibody and staining with BM purple alkaline phosphatase substrate (Roche Applied Science).

Embryonic gonads for immunohistochemistry were dissected in PBS solution, fixed in 4% paraformaldehyde overnight at 4C, embedded in paraffin and sectioned. Sections were stained with GCNA anti-rat IgM (courtesy of G. Enders, undiluted supernatant), anti-human Ki-67 (BD Biosciences, 1: 200), anti-rabbit MSY2 (courtesy of

R. Schultz, 1: 200), anti-DNMT3A (IMGENEX, 1: 100) and 5-methylcytosine antibody (Calbiochem, 1: 1000). Colorimetric staining was obtained using ABC kit (Vector Laboratories, Inc) followed by development with DAB peroxidase substrate kit (Vector Laboratories, Inc). Fluorescent staining was obtained using Texas-Red or FITC-conjugated secondary antibodies (Jackson Immunoresearch Laboratories, 1:500) and mounted in Vectashield Medium with DAPI (Vector Laboratories).

BrdU labeling:

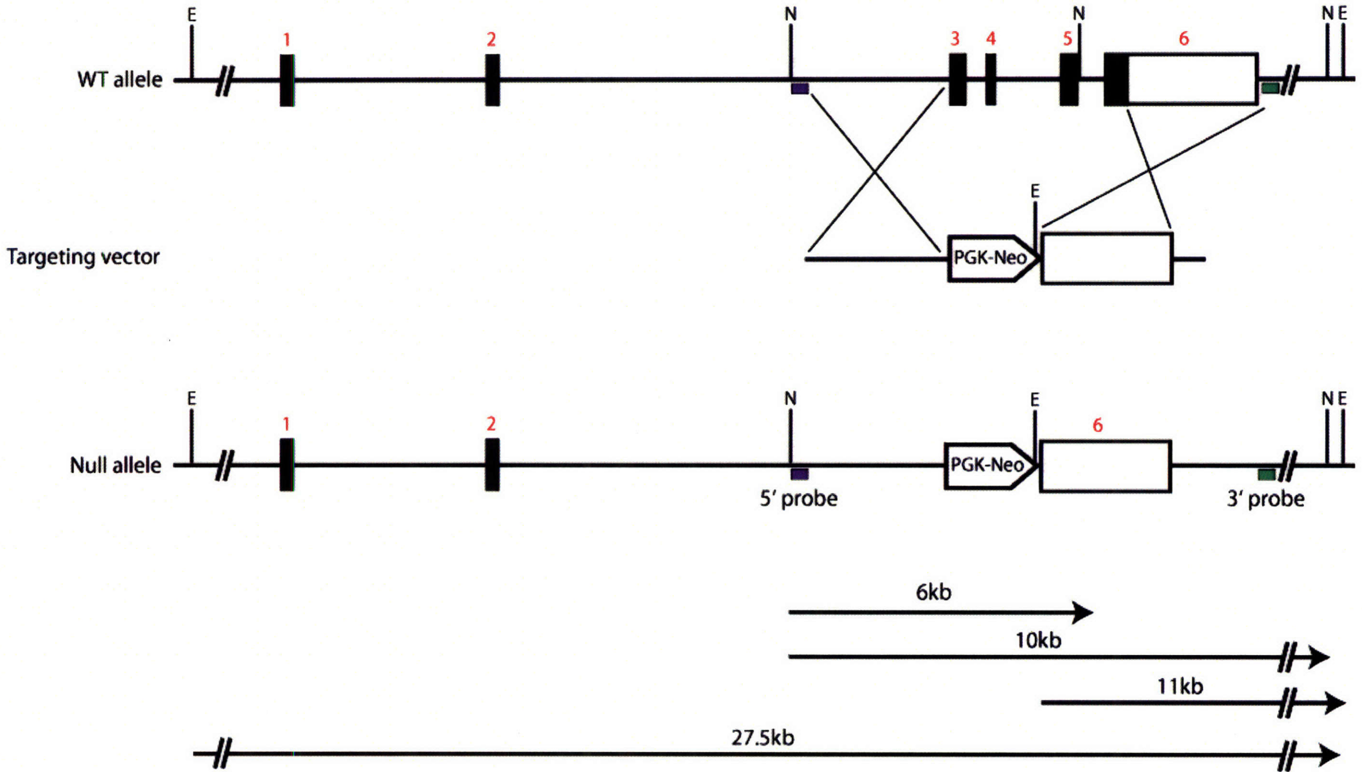
Pregnant females were injected with 5-bromo-2-deoxyuridine (BrdU) solution (50mg/kg) at 18.5 days *post coitum*. After 6 hours, embryonic gonads were dissected from pregnant females, and fixed in 4% paraformaldehyde at 4C overnight. BrdU incorporation was detected using BrdU anti-rat Fc IgG (Jackson Immunoresearch Laboratories, 1:500) and GCNA anti-rat IgM (courtesy of G. Enders, undiluted).

TUNEL assay:

Detection of apoptosis was achieved using *In situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science) on paraffin sections of embryonic testes of different ages. GCNA and TUNEL staining was obtained using Fluorescein FragEL DNA Fragmentation Detection Kit (CalBiochem) and GCNA anti-rat IgM (courtesy of G. Enders, undiluted).

Supplementary Figure 1

A



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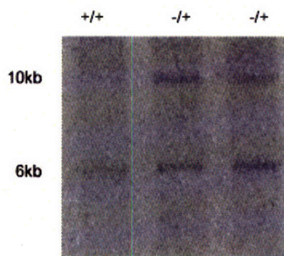

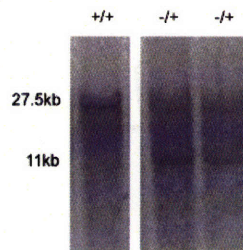

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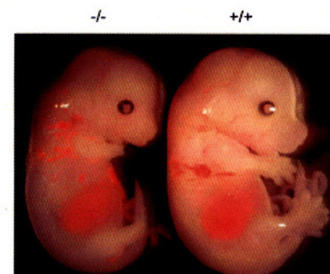
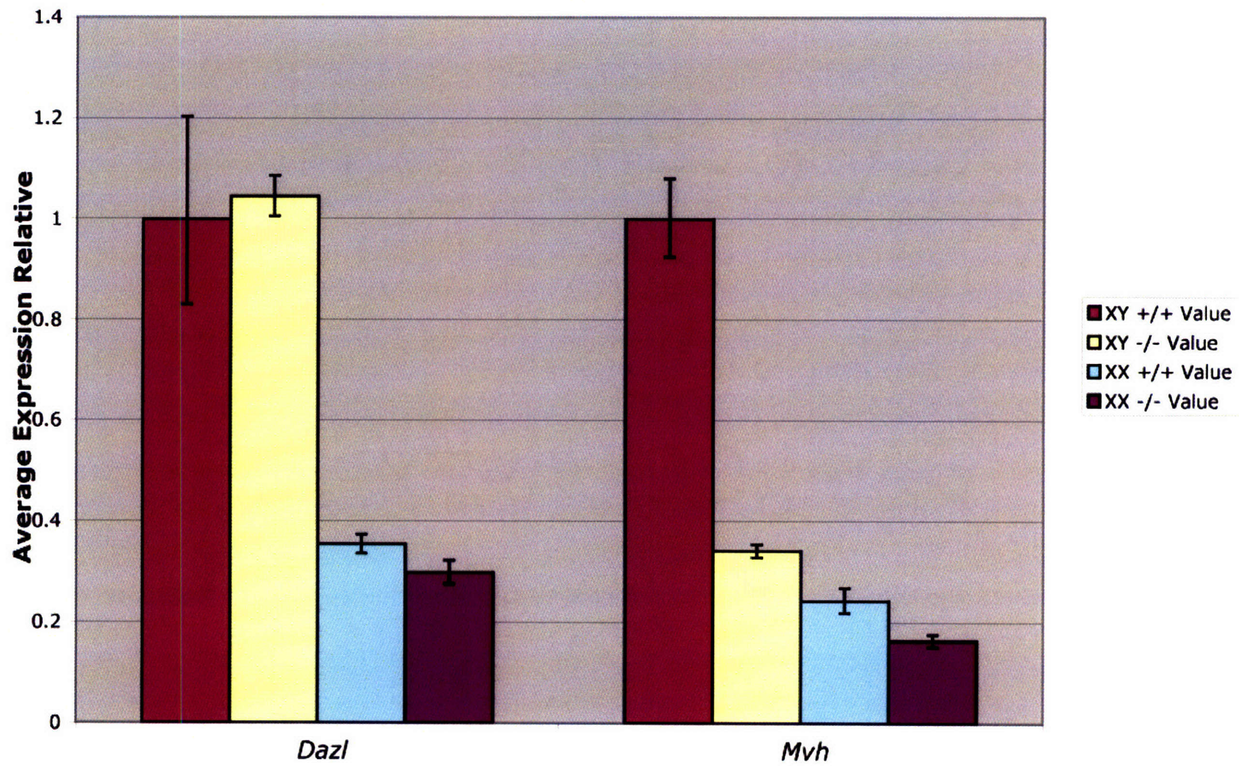


Suppl. Fig. 1A. Targeted disruption of the *Cyp26b1* locus on chromosome 6. Homologous recombination removes exons 3, 4, 5 and portion of exon 6) and replaces it with PGK-Neo selection cassette. E, EcoRV; N, Nde1. B. Correctly targeted ES cell clones were confirmed by Southern blot analysis with EcoRV digested DNA and 3' probe, and Nde1-digested DNA and 5' probe. C. E14.5 *Cyp26b1-/-* embryos exhibit identical limb and chin defects previously reported (Yashiro et al., 2004).

Supplementary Figure 2



Suppl. Fig. 2. *Mouse vasa homologue (Mvh)* expression levels are regulated by RA, because its expression levels drop in *Cyp26b1-/-* testes, while another germ cell marker, *deleted in azoospermia-like (Dazl)* maintains same levels of expression between *Cyp26b1-/-* and *Cyp26b1+/-* testes.

Supplementary Table 1

SCP3

Mice ID	Show loading	No. GCNA(+) cells counted
#5 XY Cyp26b1+/-	3 (1.4%)	208
#1 XY Cyp26b1-/-	19 (5%)	375
#6 XX Cyp26b1+/-	441 (91.8%)	480

REC8

Mice ID	Show loading	No. GCNA(+) cells counted
#13 XY Cyp26b1 +/-	9 (0.81%)	1111
#11 XY Cyp26b1-/-	25 (4.1%)	607
#10 XX Cyp26b1+/-	721 (92.8%)	777

Suppl. Table 1. Example of germ cell counts from cell spreads at E15.5. Only about 1-2% of XY *Cyp26b1+/-* germ cells (GCNA-positive) load SCP3 or REC8 on their chromosomes. 4-5% of XY *Cyp26b1-/-* germ cells exhibit loading of SCP3 and REC8, representing only a modest increase. In contrast, over 91% of XX *Cyp26b1+/-* germ cells load SCP3 and REC8 on their chromosomes.

Methods and Materials and Supplementary Figures - References:

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CHAPTER 4

Conclusions and future directions

In this thesis, I provide evidence that retinoic acid (RA) is required for meiotic initiation in embryonic ovaries, because it induces *Stra8,* a gene necessary for pre-meiotic DNA replication (Baltus et al., 2006). Furthermore, I show that embryonic testes do not express *Stra8* because of testis-expressed cytochrome p450 protein, CYP26B1, which metabolizes RA. These results suggest for the first time that differential RA levels between the embryonic testis and the embryonic ovary play a role in germ cell sexual development.

The realization that RA signaling is sex-specific in embryonic gonads opened a new window into the study of germ cell development. In order to explore the effects of RA on embryonic testis development *in vivo,* I generated *Cyp26b1-/-* mice. In the second part of my thesis, I explore the role of CYP26B1 in embryonic testes. Surprisingly, RA is not sufficient to drive testicular germ cells through functional meiotic prophase and while expression of some meiotic markers is elevated, their localization is abnormal and the germ cells never reach diplotene. Instead of meiosis, XY germ cells respond to RA by proliferation and death. Thus RA is not sufficient to "sex reverse" the embryonic germ cells, but it disrupts normal male development.

Our experiments with *Cyp26b1-/-Stra8-/-* embryos provide further insight into the effects of RA on testes. Except for the upregulation of several meiotic markers, all other effects of RA on testis development are still observed – dying cells inside testis cords, germ cells proliferation and failure to arrest in G0. These results suggest that RA has multiple effects on embryonic germ cells beyond upregulating *Stra8* expression. Since

Stra8 is first expressed at E12.5, RA must act on germ cells prior to E12.5. The window of its activity is likely even more narrow, because gonads of both sexes briefly express *Cyp26b1* mRNA prior to E12.0, when it is rapidly downregulated in XX gonads (Bowles et al., 2006). While nothing is known about CYP26B1 protein levels, its mRNA expression pattern suggests that the timing of RA signaling in the gonads is tightly regulated.

It has been previously thought that prior to E12.5, germ cells of both sexes are identical and if exposed to different environments (e.g. in extragonadal locations), they will begin to develop as oocytes regardless of their sex chromosomes (McLaren, 1981; Upadhyay and Zamboni, 1982; McLaren, 1984; McLaren and Southee, 1997). In these studies, germ cell sex was scored by the chromatin condensation typically observed in embryonic ovarian germ cells in meiotic prophase, and nuclear morphology similar to prospermatogonia, which is indicative of G0 arrest in embryonic testicular germ cells. These experiments were qualitative, making it hard to determine if and how many germ cells looked like neither the meiotic nor the G0-arrested germ cells. Also, it is not known if germ cells exhibiting chromatin condensation progressed normally through a functional meiotic prophase including chromosome cohesion, recombination and synapsis. We also do not know if these cells downregulated markers of pluripotency.

Despite these unanswered questions, the experiments of McLaren and colleagues solidified several core assumptions about the development of embryonic germ cells: First, XX and XY germ cells are sexually plastic prior to E12.5. Second, a single sex

determining switch accounts for the two germ cell paths – meiosis or G0 arrest. Third, once XY germ cells condense their chromatin (indicative of meiosis), they are "sex-reversed" because entry into meiotic prophase is all that separates them from turning into oocytes or following through other events associated with oogenesis until birth.

One unanticipated result of our studies is the discovery that testicular and ovarian germ cells do not behave according to the assumptions presented above. In response to RA, ovarian germ cells express *Stra8* and enter meiotic prophase while *Cyp26b1-/-* testicular germ cells proliferate and die. Since RA must act prior to E12.5, and since we found that XX and XY germ cells respond to RA differently, this result suggests that at the time of RA signaling, XX and XY germ cells are no longer sexually plastic, contradicting the first assumption that prior to E12.5, the XX and XY germ cells behave similarly.

There is evidence from other studies that XX and XY germ cells must already differ prior to E12.5. For example, testicular but not ovarian germ cells deficient for the germ cell-specific gene *Mvh* exhibit a mitotic proliferation defect between E11.5 and E12.5 (Tanaka et al., 2000). Germ cells in embryos lacking functional *Fgf9*, which is expressed in somatic cells, also behave differently – testicular germ cells die prior to E12.5, while ovarian germ cells are unaffected (DiNapoli et al., 2006). Finally, re-aggregation experiments have also shown that testicular germ cells at E12.5 are no longer able to enter meiosis, even if placed in an ovarian environment (McLaren and Southee, 1997). Ovarian germ cells can, however, still arrest in G0 if placed in a testicular

environment at E12.5 (McLaren and Southee, 1997). Thus by E12.5, XX and XY germ cells already appear to differ in their potential to condense their chromatin, implying that they must be different from each other.

The second assumption, that a single sex determining switch accounts for the two germ cell paths, meiosis or G0 arrest, is also not supported by the result from *Cyp26b1-/-* testes. Since *Cyp26b1-/-* testicular germ cells proliferated instead of proceeding through meiotic prophase or G0 arrest, it suggests that the bimodal switch between male and female germ cells is not the only possibility and that neither one of these two paths is a default path. An alternative explanation is that a single sex determining switch (which does not directly determine G0 or meiotic prophase) has taken place already. While the initial switch may be bimodal, it is no longer possible to reverse decision by the time RA signaling occurs. This hypothesis, however, suggests that the initial switch defines some other yet undefined events prior to the germ cell's decision to enter meiosis or G0 arrest.

The third assumption that once XY germ cells entered meiosis, they were fully "sex-reversed" reflects the underlying belief that entry into meiotic prophase was all that separated male germ cells from turning into oocytes – at least during embryonic development. This is exemplified by a conclusion in a recent report claiming that "CYP26B1 holds the key to preventing oogenesis in males by retarding meiosis in vivo" (Bowles et al., 2006). Such statements underline the fundamental belief that the germ cell's sexual development is a linear event. Once the decision to enter meiosis has been

made, all other events associated with oogenesis (such as downregulation of pluripotency markers or follicle formation) will automatically follow until at least birth.

To test if the expression of meiotic markers is required for the downregulation of pluripotency markers, I looked for the downregulation of pluripotency markers in *Stra8-/-* ovaries. In these mice, germ cells do not enter meiosis and fail at pre-meiotic DNA replication (Baltus et al., 2006). If the germ cell's sexual development was a linear event triggered by entry into meiosis, these germ cells should fail at all later aspects of oogenesis. I found that despite the lack of meiotic entry, *Stra8-/-* germ cells downregulate *Oct4* and *Dppa3,* both markers of pluripotency (Fig. 1). Thus, even if meiotic initiation fails to take place, the ovarian germ cells differ from their testicular counterparts, which maintain expression of pluripotency markers much longer. This suggests that the events of meiotic entry and downregulation of pluripotency markers are genetically separable.

Consistent with these findings is the observation that in *Stra8-/-* ovaries, follicles can be seen around birth (Baltus, unpublished observations). Thus, follicle formation and downregulation of pluripotency markers, both features of oogenesis, take place even in the absence of meiosis. These results suggest that female germ cell sex determination cannot be described solely as meiotic entry followed by a linear sequence of events leading to oogenesis. XX germ cells that fail to enter meiosis do not behave like male germ cells and exhibit key features of the oogenesis pathway such as early downregulation of pluripotency markers and follicle formation. Unfortunately, the analysis of the *Cyp26b1-/-* testicular germ cells was not as useful in evaluating the third

assumption. Two events associated with embryonic testicular germ cell development, i.e. G0 arrest and *de novo* DNA methylation, were both perturbed in *Cyp26b1-/-* mice. However, the question still remains – is it possible that germ cells require not one, but multiple sexual differentiation signals to develop as oocytes or prospermatogonia?

The idea of cell-autonomous clock has been suggested in several contexts of embryonic germ cell development. First, in connection with the sudden expression of conserved germ cell-specific markers upon the germ cell entry into the gonad (Wang et al., 1997). Second, germ cells' entry into meiotic prophase has also been hypothesized to result from intrinsic timing mechanism present in all germ cells (McLaren, 1981; McLaren, 1984; McLaren and Southee, 1997).

While seemingly simple, the idea of germ cell clock is very hard to explain on the molecular level. There is also no direct evidence to support the existence of this clock during either germ cell entry into the gonad or during germ cell entry into meiosis. Furthermore, we now know that germ cells in embryonic ovaries need signaling of at least one extrinsic factor – RA. We also know that germ cells in ovaries do not enter meiosis at once and that meiotic entry occurs in an ovarian wave (Menke et al., 2003; Yao et al., 2003; Bullejos and Koopman, 2004). This wave has never been observed in extragonadal germ cells. In fact, studies of extragonadal germ cells suggested that germ cells in these sites enter meiosis simultaneously with the germ cells present in the ovary (Upadhyay and Zamboni, 1982; Zamboni and Upadhyay, 1983). Since germ cells in ovaries are now known to enter meiosis over a period of several days, it puts the precise

timing observed in extragonadal germ cells into question. However, it is not known if chromosomal condensation, a marker of meiotic prophase used in studies of germ cell behavior in extragonadal sites, occurs itself in an anterior-to-posterior wave in embryonic ovaries.

It has been suggested that CYP26B1 is the meiosis-preventing factor in the testes (Bowles et al., 2006). Our results, however, suggest otherwise. In the absence of CYP26B1, testicular germ cells still fail at meiosis and proliferate, suggesting that CYP26B1 is a mitosis-inhibiting factor instead. Why do they fail at meiosis? It is likely that another factor is responsible for preventing meiosis in embryonic testes. It has been suggested that this factor is a short range diffusible substance (McLaren and Southee, 1997), a description which was not consistent with CYP26B1 (Bowles et al., 2006). Independent of the RA signal, which is necessary for meiotic prophase, another factor(s) must be absent in embryonic testes for XY germ cells to successfully proceed through meiosis. The identity of such a factor remains unknown.

Our discovery provides the first few insights into the complex role of RA signaling in embryonic germ cell development. Many fascinating questions remain to be answered. What happens to ovarian germ cells, which are prohibited from entering meiosis? We do not yet know if they enter G0 arrest as their male counterparts do or if they continue proliferating similarly to germ cells in *Cyp26b1-/-* testes. Staining *Stra8 -/-* germ cells with Ki-67, a marker of cycling cells, would help us understand whether these cells arrest in G0.

Another intriguing question is what else RA is regulating in embryonic gonads besides *Stra8.* The germ cell proliferation observed in *Cyp26b1-/-Stra8-/-* embryonic testes suggests that other potent targets might exist in this tissue. Knowledge of their identity would improve our understanding of the regulation of G0 arrest in XY germ cells as well as the sex-specific timing of *de novo* DNA methylation. Microarray analyses of RNA from *Cyp26b1-/-* testes, *Cyp26b1-/-Stra8-/-* testes, and RAR-inhibited ovaries would yield data sets useful in answering these questions.

Finally, we do not know why only germ cells but not somatic cells respond to RA by up-regulating *Stra8.* Finding germ cell-specific factors, which also regulate *Stra8* expression *in vivo,* would be a useful tool to improving our understanding of this fascinating question. One such factor is *Dazl* (Lin et al., unpublished), which is required in XX germ cells to express *Stra8.* DAZL is a translational regulator suggesting that an intermediate between RA signaling (which acts by transcriptional regulation) and *Dazl* must exist in order to allow *Stra8* expression in germ cells. The identity of this mediator is currently unknown. In addition, *Dazl* is germ cell-specific, which only re-emphasizes the tricky question of how germ-cell specific expression of *Dazl* is established in the first place.

The objective of my work was to examine the role (if any) of retinoic acid in embryonic germ cell development. A more specific question, however, is the question of how do germ cells transition from mitosis into meiosis. We now know that germ cell

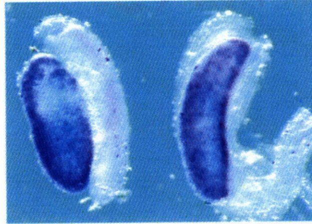
entry into meiosis cannot be a cell-autonomous event, because it requires RA signaling from the soma. We also know that RA is not sufficient for meiosis in embryonic testes and that surprisingly, it causes testicular germ cells to proliferate, suggesting a role in regulating the G0 arrest in testicular germ cells. Much remains to be learned about the cell cycle regulation in the germ cells of both sexes and the work presented here provides both a modest beginning and clues that there is much yet to be learned about germ cell development.

Figure 1. Whole mount *in situ* hybridization showing that pluripotency markers *Oct4* and *Dppa3* have similar expression patterns in E14.5 and E16.5 *Stra8-/-* and *Stra8+/-* ovaries.

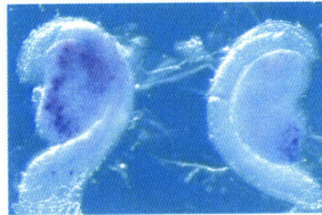
Dppa3 probe

Stra8 -/- *Stra8 +/-*

E14.5



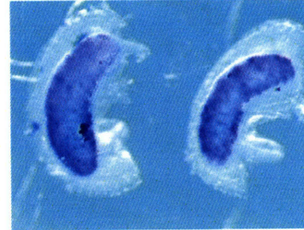
E16.5



Oct4 probe

Stra8 -/- *Stra8 +/-*

E14.5



E16.5

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APPENDIX 1

Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave

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Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave

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Abstract

Differentiation of mouse embryonic germ cells as male or female is dependent on the somatic environment of the gonad rather than the sex chromosome constitution of the germ cell. However, little is known about the initiation of germ cell sexual differentiation. Here, we traced the initiation of germ cell sexual differentiation in XX gonads using the *Stra8* gene, which we demonstrate is an early molecular marker of female germ cell development. *Stra8* is upregulated in embryonic germ cells of XX gonads prior to meiotic entry and is not expressed in male embryonic germ cells. A developmental time course of *Stra8* expression in germ cells of XX gonads has revealed an anterior-to-posterior wave of differentiation that lasts approximately 4 days, from embryonic days 12.5 to 16.5. Consistent with these results, we find that embryonic ovarian germ cells upregulate the meiotic gene *Dmc1* and downregulate the *Oct4* transcription factor in an anterior-to-posterior wave. In complementary experiments, we find that embryonic XX gonads upregulate certain gene markers of somatic female differentiation in an anterior-to-posterior pattern, while others display a center-to-pole pattern of regulation. Thus, sexual differentiation and meiotic entry of germ cells in embryonic XX gonads progress in an anterior-to-posterior pattern that may reflect local environmental cues that are present in the embryonic XX gonad.
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Keywords: Germ cell; *Stra8*; *Dmc1*; *Oct4*; *Adamts19*; *Follistatin*; Embryo; Sexual differentiation; Meiosis; Ovary

Introduction

The events that underlie sexual differentiation of the mammalian gonad are critical for the generation of functional gametes in the adult animal. Recent studies have elucidated some of the cellular and molecular events that occur during differentiation of the testicular soma (Tilmann and Capel, 2002). However, many aspects of gonadal development, including the sexual differentiation of embryonic germ cells, remain largely unexplored.

Primordial germ cells of the mouse are first identifiable at approximately E7.2 at the base of the allantois (Ginsburg et al., 1990). The germ cells of XY and XX embryos are morphologically indistinguishable as they migrate from this extraembryonic location and enter the bipotential gonad from E10 to E11.5. Although sexual differentiation of the somatic elements of the XY gonad is visually evident by E12.5, it is not until E13.5 that the first meiotic germ cells appear in XX gonads and morphological differences between germ cells of XY and XX gonads become apparent (McLaren, 2000). The entry of ovarian germ cells into meiotic prophase continues over the next 2 days with the majority of germ cells entering meiosis by E15.5 (Peters, 1970; Peters et al., 1962). Over the same time period, testicular germ cells cease their mitotic proliferation and arrest as prospermatogonia (McLaren, 1984).

The differentiation of germ cells as male or female is dependent on the somatic environment rather than the sex chromosome content of the germ cell. This conclusion was initially based on observations of XX↔XY chimeric gonads and on mouse models of sex-reversal (McLaren and

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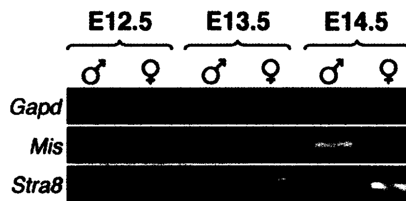


Fig. 1. Detection of sex-specific *Stra8* expression by RT-PCR. Expression of *Gapd* (ubiquitous control), *Mis* (testis-specific control), and *Stra8* in E12.5, E13.5, and E14.5 XY and XX gonads.

Monk, 1981; Palmer and Burgoyne, 1991; Taketo-Hosotani et al., 1989). These studies revealed that XX embryonic germ cells in a testicular environment will develop as male prospermatogonia, and similarly, XY germ cells in ovarian surroundings will enter meiosis and develop as oocytes. Moreover, the discovery that XY and XX germ cells that mistakenly migrate into the embryonic adrenal gland develop as oocytes suggested that germ cells will cell-autonomously differentiate as female unless masculinized by a testicular environment (Zamboni and Upadhyay, 1983).

Although meiotic germ cells are not observed in XX gonads until E13.5, experimental evidence indicates that germ cells developing in XY gonads differ from those in XX gonads by E12.5. Germ cells isolated from XY gonads at E11.5 will enter meiosis and develop as oocytes if grown in lung reaggregates (McLaren and Southee, 1997), in reconstituted ovaries (Adams and McLaren, 2002), or in tissue culture (Nakatsuji and Chuma, 2001). However, by E12.5, most germ cells isolated from XY gonads are masculinized and will not enter meiosis if grown under these conditions. In contrast, E12.5 germ cells from XX gonads have not yet committed to female development and can still be influenced in vitro to develop as male. It is only at E13.5 that most XX germ cells are thought to have committed to female development (Adams and McLaren, 2002).

While in vitro results suggest that germ cells of XY and XX gonads differ by E12.5, little is known about sexually dimorphic gene expression differences that develop in germ cells at this stage. Here, we report the identification of the earliest known gene marker of female germ cell sexual differentiation. An expression analysis that included this gene has revealed unexpected regional differences in the timing of germ cell sexual differentiation within XX gonads.

Materials and methods

Mice and embryo collection

Mouse embryos derived from matings between male and female C57BL/6 mice (Taconic Farms Inc., Germantown, NY) were used in all experiments except those using feminized XY^{ZAL} gonads. Timed matings were performed with noon on the day a vaginal plug was found designated as E0.5. Germ cell-depleted embryonic gonads were generated by injecting pregnant females at E9.5 with 0.2 ml of 6.6 mg/ml busulfan in 50% dimethyl sulfoxide (53 mg/Kg body weight) (Merchant, 1975).

For the generation of embryos with sex-reversed XY^{ZAL} gonads, male Zalende/Ei mice from the Jackson Laboratory (Bar Harbor, ME) were bred with C57BL/6 females to generate F_1 hybrids. F_1 stud males were backcrossed to C57BL/6 females to create N2 XY^{ZAL} embryos with partially or fully feminized gonads as previously reported (Eicher and Washburn, 1983). All sex-reversal analyses were performed by using these N2 progeny.

Embryos for in situ hybridization were collected at E11.5–E16.5, and genital ridges were dissected out in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde at 4°C overnight. Tissues were dehydrated into 100% methanol and stored at −20°C until used. Embryos younger than E12.5 were sexed by isolating DNA and using a PCR assay that amplifies intronic sequence from the Y-linked gene *Uty* (primer sequences: T35L-5′-GGAATGAATGT-GTTCCATGTCT-3′; T35R-5′- CTCATGTAGACCAA-GATGACC-3′). Gonads isolated from E12.5–E16.5 were sexed visually with the exception of embryonic gonads derived from XY^{ZAL} matings, which were sexed by using the *Uty* PCR assay.

RT-PCR

Total RNA was isolated from XY and XX genital ridges by using Trizol (GIBCO- BRL) as directed by the manufacturer. Total RNA (1 μg) was reverse transcribed with oligo $d(T)_{18}N$ using Superscript II (GIBCO-BRL) in a total reaction volume of 25 μl. PCR was performed by using 1 μl of RT as template in a total volume of 20 μl [10 mM Tris– HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPS, 0.5 μM primers, 0.5 U Taq polymerase]. PCR cycling conditions for all primers were as follows: 94°C (30 s), 60°C (30 s), 72°C (1 min) for 25–30 cycles. *Stra8* primers: 6695 (5′-GAGGT-CAAGGAAGAATATGC-3′) and 6698 (5′- CAGAGA-CAATAGGAAGTGTC-3′), *Gapd* primers: 6787 (5′-GTGTTCCTACCCCCAATGTG-3′) and 6788 (5′-GT-CATTGAGAGCAATGCCAG- 3′), *Mis* primers: 4616 (5′-TTGCTGAAGTTCCAAGAGCC-3′) and 4617 (5′-TTCTCTGCTTGGTTGAAGGG-3′).

In situ hybridization

Digoxigenin whole-mount in situ hybridizations were performed essentially as previously reported (Wilkinson and Nieto, 1993). Digoxigenin riboprobes were generated by amplifying cDNA fragments by RT-PCR from *Stra8* (NM_009292: bases 766-1279), *Dmc1* (NM_010059: bases 602-1245), *Oct4* (NM_013633: bases 183-1286), *Aard* (AY134665: bases 576-1154), *Cbln1* (NM_019626: bases

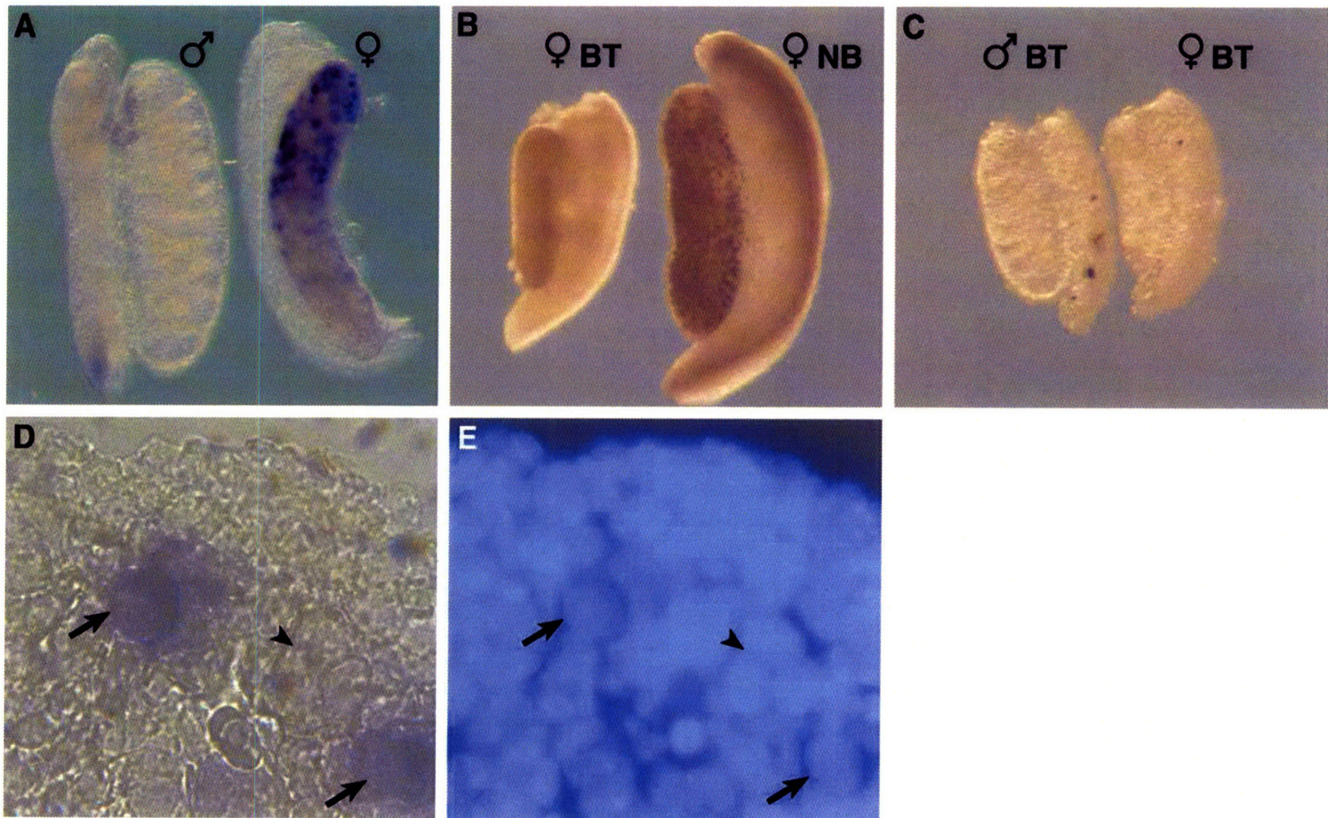


Fig. 2. Expression of *Stra8* in normal and germ cell-depleted embryonic gonads. (A) *Stra8* whole-mount in situ hybridizations on E13.5 gonads reveals *Stra8*-expressing cells (stained purple) in ovaries but not testes. (B) Detection of all germ cells by staining for endogenous alkaline phosphatase activity demonstrates the absence of germ cells in E13.5 busulfan-treated ovaries and an even distribution of germ cells in normal untreated ovaries (BT, busulfan treated; NB, no busulfan). (C) No signal is observed in *Stra8* whole-mount in situ hybridizations of E13.5 busulfan-treated testes and ovaries. Paraffin sections of *Stra8* E13.5 ovarian whole-mount in situ hybridizations were counterstained with DAPI and observed under bright-field (D) and fluorescent (E) illumination. *Stra8*-expressing germ cells (arrows) are located next to a cluster of *Stra8*- negative germ cells (arrowhead).

994-1760), *Adamts19* (AY135183: bases 3129-4036), and *follistatin* (AK083556: bases 1484-2330), and inserting them into TA cloning vector pCR2.1-TOPO or pCR4-TOPO (Invitrogen). Plasmids were then linearized and transcribed with T3 or T7 RNA polymerase in the presence of Dig-labeling mix (Roche) to create digoxigenin riboprobes. Fluorescein- labeled *Stra8* riboprobe was created by using Fluor-labeling mix (Roche).

Double in situ hybridization was performed by hybridizing embryonic gonads simultaneously with fluorescein-labeled *Stra8* riboprobe and digoxigenin-labeled *Aard* riboprobe. Fluorescein riboprobe signals were detected with anti-Fluor-AP (Roche) and BM Purple (Roche). Anti-Fluor-AP was inactivated with 0.1 M Glycine–HCl (pH 2.2), and digoxigenin riboprobe signals were detected by incubation with anti-Dig-AP (Roche) followed by INT/BCIP (Pharmingen).

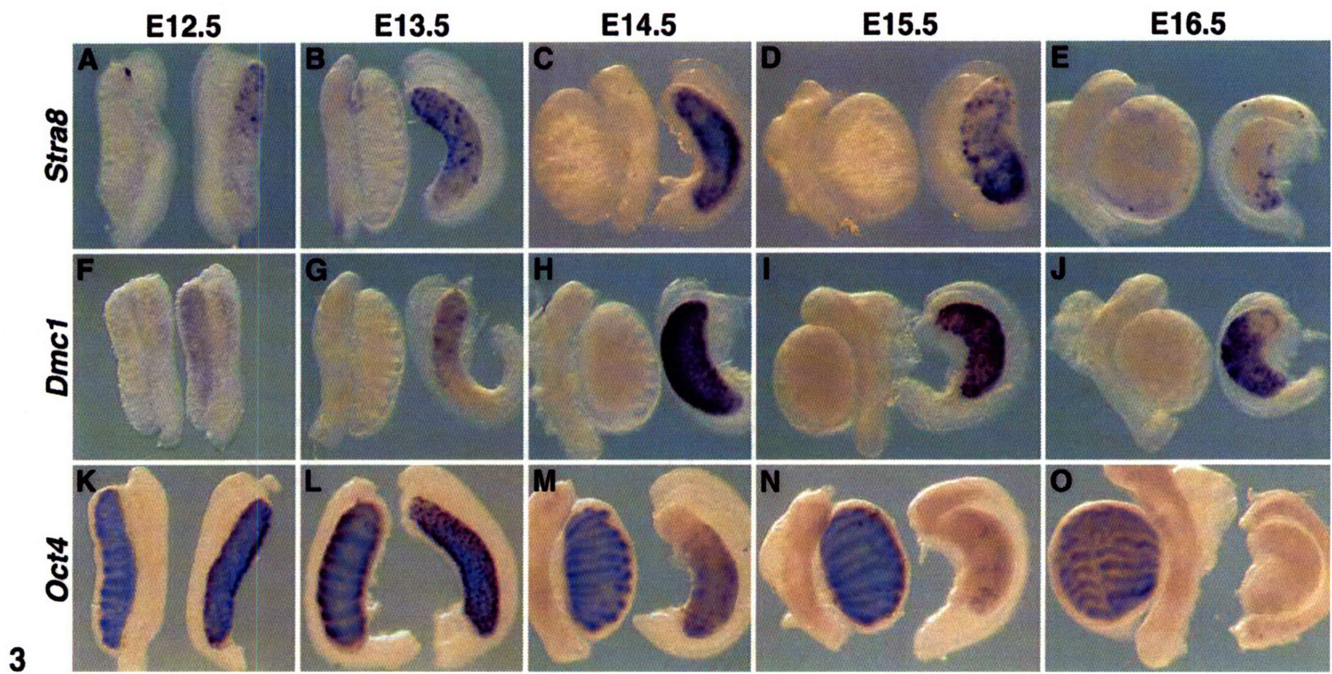
Alkaline phosphatase staining of germ cells

Embryonic gonads were dissected out in PBS and placed in 70% ethanol at 4°C overnight. Gonads were rinsed three times in NTMT [100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl, 0.1% Tween 20], after which endogenous AP activity was detected by incubation at room temperature in NTMT containing 0.337 mg/ml NBT and 0.175 mg/ml BCIP (Roche).

Results

Stra8 is expressed in embryonic germ cells of XX gonads

The *Stra8* gene (Stimulated by Retinoic Acid gene 8) encodes a predicted 393- amino-acid protein of unknown function, and was originally identified in a screen to detect genes that are upregulated in P19 embryonal carcinoma cells in response to retinoic acid (Bouillet et al., 1995). A subsequent study reported that *Stra8* is specifically expressed in male germ cells of embryonic and adult mice (Oulad-Abdelghani et al., 1996). While performing a series of cDNA subtractive hybridizations to identify genes that are expressed differentially between XY and XX embryonic gonads (Menke and Page, 2002), we tested the *Stra8* gene as a potential control for the


E12.5
E13.5
E14.5
E15.5
E16.5
Stra8
Dmc1
Oct4
A
B
C
D
E
F
G
H
I
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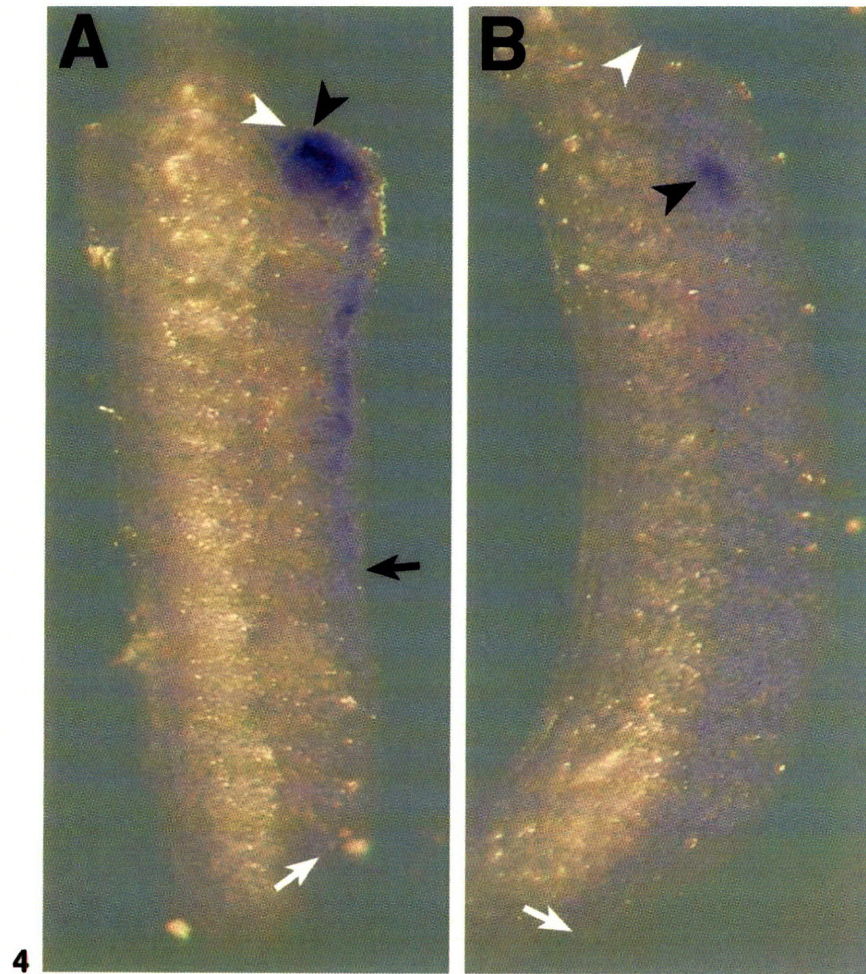
A
B
4

presence of male- specific germ cell transcripts in our subtracted cDNA libraries. Contrary to previous work (Oulad-Abdelghani et al., 1996), our RT-PCR analysis revealed that *Stra8* is expressed in embryonic ovaries and not in embryonic testes from E12.5 to E14.5 (Fig. 1).

Since our RT-PCR analysis of *Stra8* was at odds with published embryonic in situ hybridization results (Oulad-Abdelghani et al., 1996), we examined the expression of *Stra8* in greater detail. Whole-mount mRNA in situ hybridization of E13.5 XX and XY gonads revealed *Stra8*-positive cells only in XX gonads (Fig. 2A). We believe the prior report that *Stra8* is expressed in embryonic male germ cells at E12.5 and E14.5 may have resulted from improperly sexed tissue sections (Oulad-Abdelghani et al., 1996).

We investigated the germ cell dependence of *Stra8* expression by depleting embryos of germ cells using the chemical busulfan (Merchant, 1975). Detection of germ cells by endogenous alkaline phosphatase activity confirmed that most germ cells were eliminated in busulfan-treated E13.5 XX gonads (Fig. 2B). Similarly, *Stra8* staining was completely absent in busulfan-treated XX gonads (Fig. 2C). In contrast to *Stra8,* the expression of two genes expressed in somatic cells of XX gonads, *follistatin* and *Adamts19*, was not affected by busulfan (data not shown; Menke and Page, 2002). Therefore, *Stra8* expression is dependent on the presence of germ cells.

We definitively established that *Stra8* is expressed in germ cells of XX gonads by sectioning *Stra8* E13.5 whole-mount in situ hybridized gonads. *Stra8* staining colocalized with cells containing large round nuclei, a distinctive feature of embryonic germ cells (Fig. 2D and E). These nuclei are readily distinguished from the smaller nuclei of surrounding somatic cells. Groups of *Stra8*-expressing germ cells were sometimes seen in close proximity to groups of *Stra8*-negative germ cells. This is consistent with prior histological evidence that ovarian germ cells generally develop as clusters, with some clusters exhibiting more advanced states of differentiation than others (Pepling and Spradling, 1998; Peters, 1970).

Sexual differentiation of ovarian germ cells occurs in an anterior-to-posterior wave

Visualization of germ cells by alkaline phosphatase staining revealed an even distribution of germ cells throughout normal E13.5 ovaries (Fig. 2B). In contrast, we observed more *Stra8*-positive germ cells in the anterior portion of E13.5 ovaries than in posterior regions (Fig. 2A). We therefore determined the distribution of *Stra8*-positive cells along the anteroposterior axis of ovaries at different developmental stages (Fig. 3). At E11.5 and E12, we observed no *Stra8* staining in XX or XY gonads (data not shown). We first detected *Stra8*-positive cells in E12.5 XX gonads (Fig. 3A). These cells were located almost exclusively in the anterior half of the gonad. By E14.5, *Stra8* staining was observed throughout the ovary (Fig. 3C). However, at E15.5, *Stra8*-expressing cells were located predominantly in the posterior half of the ovary (Fig. 3D). Only a small number of *Stra8*-positive cells remained in the ovary at E16.5, primarily at the posterior most pole (Fig. 3E). *Stra8* was not detected in E17.5 or postnatal ovaries by RT-PCR (data not shown). Thus, *Stra8* is expressed in embryonic ovarian germ cells in an anterior-to- posterior wave that spans approximately 4 days, from E12.5 to E16.5.

This pattern of *Stra8* expression suggests that ovarian germ cells differentiate in an anterior-to-posterior wave. Historically, initiation of meiosis during embryonic development has been considered the defining hallmark of female germ cell sex determination, and both the morphological changes characteristic of meiotic germ cells and the expression of meiotic genes have been used as markers of germ cell sexual identity (Chuma and Nakatsuji, 2001; McLaren and Southee, 1997). Therefore, if differentiation of embryonic ovarian germ cells occurs in an anterior-to-posterior wave, meiotic genes should first be upregulated in germ cells located in the anterior portion of XX gonads. We performed whole-mount in situ hybridizations for a meiosis-specific gene, *Dmc1,* to test this hypothesis. *Dmc1* is expressed during meiotic prophase and is required for double-strand break repair during male and female meiotic recombination (Pittman et al., 1998; Yoshida et al., 1998). *Dmc1* expression appears to be upregulated somewhat later than *Stra8*. At E12.5, we detected very faint staining for *Dmc1* in the anterior portion of XX gonads (Fig. 3F). By E13.5, we observed stronger expression of *Dmc1* with a distinct anterior bias (Fig. 3G). Intense *Dmc1* expression was found at E14.5 and E15.5 with positive cells observed throughout the ovary (Fig. 3H and I). At E16.5, we perceived a reduction of *Dmc1* in anterior regions (Fig. 3J). Thus, *Dmc1* is also expressed in an anterior-to-posterior wave.

The differentiation of female germ cells is associated with not only the upregulation of particular genes, but also the downregulation of others. The POU transcription factor

Fig. 3. Developmental time course of *Stra8, Dmc1,* and *Oct4* expression in embryonic gonads reveals an anterior-to-posterior wave of female germ cell differentiation in XX gonads. Whole-mount in situ hybridization for *Stra8* on testes and ovaries at E12.5 (A), E13.5 (B), E14.5 (C), E15.5 (D), and E16.5 (E). Whole-mount in situ hybridization for *Dmc1* on testes and ovaries at E12.5 (F), E13.5 (G), E14.5 (H), E15.5 (I), and E16.5 (J). Whole-mount in situ hybridization for *Oct4* on testes and ovaries at E12.5 (K), E13.5 (L), E14.5 (M), E15.5 (N), and E16.5 (O). In all panels, testes are located on the left and ovaries on the right. Gonads are oriented with their anterior pole toward the top of each panel.

Fig. 4. Whole-mount in situ hybridization of *Adamts19* in XY and XX embryonic genital ridges at ~24ts reveals an anterior expression bias. (A) *Adamts19* anterior expression (black arrowhead) expands posteriorly along the ventral region of XX gonads (black arrow). (B) Anterior expression of *Adamts19* is reduced in XY gonads (black arrowhead) and is ultimately lost. All genital ridges are positioned with the anterior towards the top of each panel. White arrowheads indicate the anterior tip of the gonad. White arrows on the right indicate the posterior tip of the gonad.

Oct4 is required for the maintenance of pluripotency during early embryogenesis (Nichols et al., 1998), but by E9.0, expression of *Oct4* has been lost in all cells of the developing embryo except for the primordial germ cells (PGCs) (Rosner et al., 1990; Scholer et al., 1990). As the germ cells differentiate, they too extinguish *Oct4* expression. In germ cells of XX gonads, *Oct4* expression is downregulated between E13.5 and E16.5 as ovarian germ cells enter meiosis; *Oct4* expression in embryonic testicular germ cells is maintained during this period (Pesce et al., 1998). We obtained corroborating evidence for the anterior-to-posterior wave of germ cell differentiation by investigating the expression of *Oct4*. We found that *Oct4* expression is first lost in anterior germ cells of XX gonads between E13.5 and E14.5 (Fig. 3K–M) and is subsequently lost in posterior germ cells (Fig. 3N and O). Therefore, *Oct4* is down regulated in an anterior-to-posterior wave that is complementary to that observed with *Stra8* and *Dmc1*.

Somatic ovarian differentiation

Observations by others have suggested that embryonic germ cells will cell- autonomously enter meiosis and develop as oocytes unless masculinized by an embryonic testicular environment. Our discovery that sexual differentiation of ovarian germ cells transpires in an anterior-to-posterior wave, rather than in a positionally unbiased manner, is therefore unexpected. We decided to explore the possibility that regional differences in the pattern of somatic ovary differentiation might be responsible for the wave of germ cell differentiation. We examined the expression of two markers of ovarian somatic differentiation, *Adamts19* and *follistatin* (Menke and Page, 2002), to determine whether somatic cells of XX gonads differentiate in an anterior-to-posterior pattern reminiscent of that observed in germ cells.

Both *Adamts19* and *follistatin* become differentially expressed before XX and XY genital ridges exhibit overt morphological differences. We staged these genital ridges by counting the number of tail somites behind the hind limb [eight tail somites (8ts) corresponds to ~E10.5, 18ts to ~E11.5, and 30ts to ~E12.5 (Hacker et al., 1995)]. We observed *Adamts19* expression at the anterior tips of XX and XY gonads beginning at ~19ts (data not shown). Prior to this stage, we did not detect the presence of *Adamts19* in XX or XY genital ridges. By the 24ts stage, *Adamts19* expression had spread along the ventral aspect of XX genital ridges toward the posterior pole; however, an anterior expression bias was still very apparent (Fig. 4A). At this stage, staining in XY gonads was quite faint and anterior expression was lost or greatly reduced in all XY gonads examined (Fig. 4B). By the 30ts stage (E12.5), *Adamts19* was no longer detected in XY gonads and expression in XX gonads was increased (data not shown; Menke and Page, 2002). Therefore, in XX gonads, *Adamts19* expression spreads in an anterior-to-posterior fashion.

The second ovarian somatic marker, *follistatin,* was undetectable in genital ridges at the 13ts stage. However, by the 16ts stage, we detected expression in XX and XY genital ridges (Fig. 5A and F). *Follistatin*-positive cells were scattered throughout most of the gonad, but the anterior and posterior tips were devoid of signal. At 18–19ts, sexually dimorphic expression of *follistatin* was quite evident (Fig. 5B and G). *Follistatin* expression had increased in XX genital ridges, though the anterior and posterior-most regions still lacked expression. At later stages, *follistatin* expression had spread to these areas as well (Fig. 5C–E). Therefore, in XX gonads, *follistatin* is upregulated in a center-to-pole pattern rather than an anterior-to-posterior pattern. In contrast to XX genital ridges, XY gonads displayed reduced *follistatin* expression at 18– 19ts (Fig. 5G). Remarkably, by 20–21ts, prominent *follistatin* expression was evident at the anterior and posterior poles of XY genital ridges and was completely absent in central regions (Fig. 5H). Anterior expression was lost in XY gonads by 22–23ts, but expression at the posterior pole was observed as late as 24ts (Fig. 5I and J). Therefore, *follistatin* expression is downregulated in XY genital ridges in a center-to-pole pattern.

Stra8 expression in XY^{ZAL} *embryonic gonads*

We investigated the relationship between somatic gonadal differentiation and the wave of female germ cell differentiation in more detail by observing the effects of partial somatic sex-reversal on *Stra8* expression. Certain *Mus musculus domesticus Y* chromosomes (Y^{DOM}) are not effective at masculinizing the gonads of XY embryos when bred onto a C57BL/6J strain background (Eicher and Washburn, 1983; Eicher et al., 1982). This effect, variously referred to as C57BL/6J-Y^{POS} sex-reversal or XY^{DOM} sex-reversal, probably results from the misregulation of the *Sry* gene of particular Y^{DOM} chromosomes (Albrecht and Eicher, 1997). The feminized embryonic XY^{DOM} gonads that result often develop as ovotestes that contain testicular tissue in central regions of the gonad and feminized regions that lack testicular cords at the anterior and posterior poles. In some instances, XY^{DOM} gonads are completely feminized and form ovaries. Germ cells in masculinized regions of partially sex-reversed XY^{DOM} gonads develop into male prospermatogonia, while germ cells in feminized regions differentiate as female and initiate oogenesis (Taketo-Hosotani et al., 1989).

We obtained fully and partially feminized XY embryonic gonads by breeding male Zalende/Ei mice that carry a Y^{DOM} sex-reversal chromosome (that we have designated Y^{ZAL}) with C57BL/6 females (see Materials and methods). We focused our analysis of XY^{ZAL} gonads at E14.5. This stage is ideal because feminized and masculinized regions are morphologically discernable, and it is at this stage that large numbers of posteriorly located germ cells are first observed to express *Stra8* in normal XX gonads. This allowed us to determine whether the presence of intervening masculinized tissue significantly delayed the sexual differ-

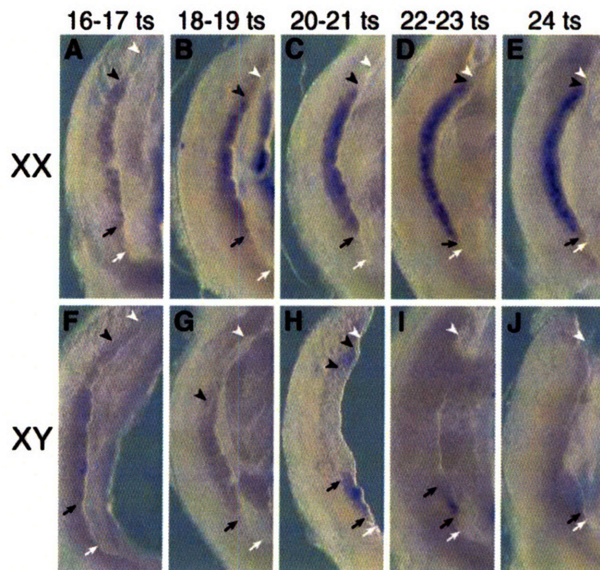

Fig. 5. Developmental time course of *follistatin* expression in XY and XX embryonic gonads from 16 to 24ts. Whole-mount in situ hybridization demonstrates that *follistatin* is expressed at similar levels in XX (A) and XY (F) genital ridges at 16-17ts. *Follistatin* expression is upregulated in the central portion of XX genital ridges by 18-19ts (B) and then spreads to the anterior and posterior tips of XX genital ridges (C), (D), and (E). *Follistatin* expression is reduced in XY genital ridges at 18-19ts (G). Expression is lost in the central region of XY gonads at 20-21ts, while it becomes upregulated at the anterior and posterior poles (H). Anterior expression in XY gonads is then lost (I), followed by the loss of posterior expression (J). All genital ridges are positioned with the anterior toward the top of each panel. White arrowheads indicate the anterior tip of the gonad. White arrows indicate the posterior tip of the gonad. Black arrowheads demarcate the anterior-most expression. Black arrows demarcate the posterior-most expression. In cases where expression is confined to the poles of the gonad, these expression domains are flanked with black arrowheads (anterior) or black arrows (posterior).

entiation of germ cells at the posterior pole of XY^{ZAL} ovotestes. We first characterized the expression of somatic markers of testicular and ovarian differentiation in E14.5 XY^{ZAL} gonads to confirm the expression of male and female genes in regions that morphologically appeared to be masculinized and feminized. The testis markers we analyzed include *Aard*, which is expressed in the testicular cords, and *Cbln1*, which is expressed in interstitial cells of embryonic testes (Menke and Page, 2002). As expected, we found that, in mildly and severely feminized XY^{ZAL} ovotestes, the expression of *Aard* and *Cbln1* was completely confined to masculinized regions that contained testicular cords (Fig. 6A, B, D, and E). These testis genes were not expressed in fully feminized XY^{ZAL} ovaries (Fig. 6C and F). Conversely, in feminized regions of severely and fully sex-reversed XY^{ZAL} gonads, the ovarian somatic markers *follistatin* and *Adamts19* (Menke and Page, 2002) were expressed strongly (Fig. 6H, I, K, and L). These ovarian markers were excluded from masculinized regions. When the feminized regions of XY^{ZAL} gonads were less extensive, ovarian markers were still expressed, but the levels of expression were more variable (Fig. 6G and J).

After testing the expression of somatic genes, we examined expression of *Stra8* in E14.5 XY^{ZAL} gonads. Since *Stra8* is a marker of female germ cell sexual differentiation, *Stra8* should be expressed in germ cells present in feminized regions of XY^{ZAL} gonads, but not in germ cells located in masculinized regions. As expected, *Stra8*-positive germ cells were observed throughout fully sex-reversed E14.5 XY^{ZAL} ovaries, just as in normal E14.5 XX ovaries (compare Fig. 3C with Fig. 7A). In severely feminized ovotestes, *Stra8*-positive cells were located at both the anterior and posterior poles and were not detected in central masculinized regions (Fig. 7B). When we examined mildly feminized E14.5 XY^{ZAL} ovotestes that had extensive masculinized regions, we still found intense *Stra8* expression at the anterior and posterior tips (Fig. 7C). The border between polar regions containing feminized *Stra8*-positive germ cells and the masculinized central portion of the ovotestes were sharply demarcated. Double in situ hybridization with *Stra8* (purple) and the cord marker *Aard* (orange–brown) on mildly feminized ovotestes highlighted the presence of well-organized testicular cords directly adjacent to feminized regions containing robust *Stra8* expression (Fig. 7D). Our results indicate that the presence of large tracts of masculinized gonadal tissue does not grossly delay the timing of *Stra8* expression in posteriorly located germ cells.

Discussion

The early stages of embryonic ovary differentiation have often been regarded as relatively static, and research on ovary development and on female germ cells has primarily focused on postnatal time periods. Our work demonstrates that, contrary to this notion, the early stages of ovary differentiation are characterized by active and highly patterned programs of germ cell and somatic cell differentiation.

Recent reaggregation experiments suggest that most germ cells in XX gonads are committed to female development by E13.5 (Adams and McLaren, 2002), yet it has remained unclear when germ cells of XX gonads begin to differentiate as female. We have now found that the *Stra8* gene, a marker of embryonic female sexual differentiation, is expressed in a subset of germ cells of the XX gonad by E12.5. This expression of *Stra8* highlights the relatively early onset of female differentiation in certain germ cells, and provides a solid point of reference in our attempt to understand the origins of germ cell feminization. *Psx2 (Gpbox),* a germ cell expressed homeobox gene, has been reported to be upregulated in germ cells of XX gonads relative to those in XY gonads 1 day earlier than *Stra8* (Takasaki et al., 2000). However, *Psx2* expression in XX gonads is estimated to exceed that in XY gonads by only two-fold at E11.5 and by five-fold at E12.5. Therefore, the expression of *Psx2* is not exclusive to germ cells of embryonic XX gonads. Moreover, the location of *Psx2* on the mouse X chromosome raises the possibility that differential expres-

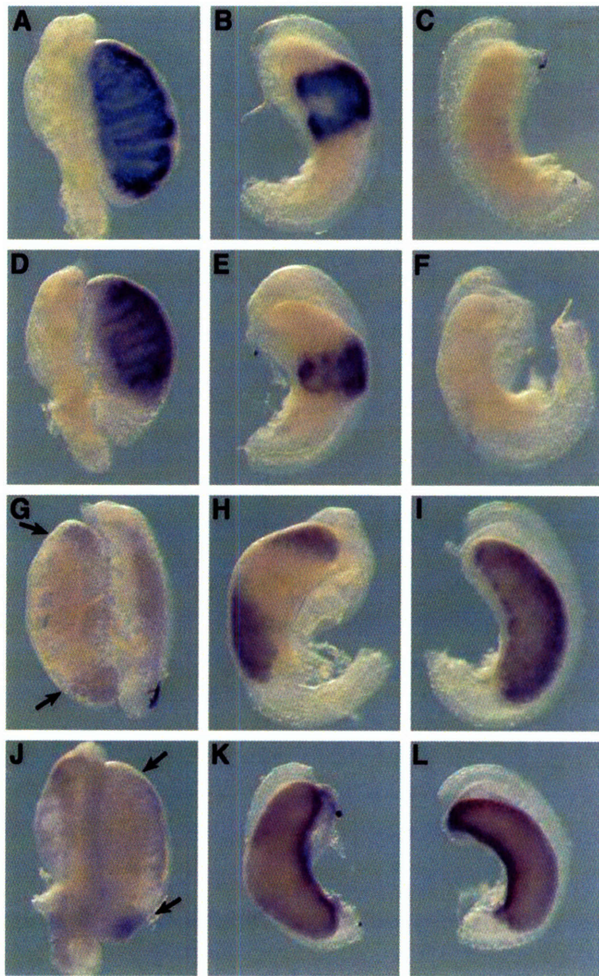

Fig. 6. Whole-mount in situ hybridizations examining somatic testis and ovary genes in XY^{ZAL} gonads. *Aard* expression in a very mildly feminized XY^{ZAL} testis (A), a severely feminized XY^{ZAL} ovotestis (B), and a completely sex-reversed XY^{ZAL} ovary (C). Expression of *Cbln1* in a mild XY^{ZAL} ovotestis (D), a severely feminized XY^{ZAL} ovotestis (E), and a completely sex-reversed XY^{ZAL} ovary (F). Expression of *follistatin* in a mild XY^{ZAL} ovotestis (G), a severely feminized XY^{ZAL} ovotestis (H), and a completely sex- reversed XY^{ZAL} ovary (I). Expression of *Adamts19* in a mild XY^{ZAL} ovotestis (J), a severely feminized XY^{ZAL} ovotestis (K), and a completely sex-reversed XY^{ZAL} ovary (L). Gonads are positioned such that the anterior pole is at the top of each panel. Arrows in (G) and (J) demarcate the boundary between feminized and masculinized regions.

sion of *Psx2* may result from reactivation of the inactive X chromosome in XX germ cells, a process which is initiated at approximately E11.5 and which is known to occur independently of germ cell sexual differentiation (Jamieson et al., 1998; McLaren and Monk, 1981). In contrast to *Psx2, Stra8* clearly identifies feminized embryonic germ cells. It is the earliest unambiguous marker of female germ cell sexual differentiation identified to date.

Stra8 is upregulated in germ cells of XX gonads in an anterior-to-posterior wave that runs from E12.5 to E16.5 (Fig. 3). This wave of expression is notable not only for its well-defined anterior-to-posterior progression, but also for its duration. The wave is initiated in XX gonads just as XX and XY genital ridges become morphologically distinguishable and ends 4 days later, by which time the appearance of XX and XY gonads differs dramatically. Since meiotic germ cells are not observed in XX gonads until E13.5, the expression of *Stra8* at E12.5 indicates the transcript is produced by premeiotic germ cells. Although we have not yet determined whether *Stra8* expression in ovarian germ cells is maintained during the initial stages of meiotic prophase, a prior analysis of *Stra8* expression in newborn and adult mouse testes demonstrated that *Stra8* is specifically expressed in a subset of spermatogonia and possibly preleptotene spermatocytes (Oulad-Abdelghani et al., 1996). It therefore seems likely that expression of *Stra8* reflects a male or female germ cell's commitment to progress into meiosis. However, the function of the STRA8 protein remains unknown.

Previous work regarding sexual differentiation of mouse germ cells demonstrated that germ cell sex is determined by the somatic environment rather than by the sex chromosome content of the germ cell. More specifically, embryonic germ cells are thought to differentiate cell-autonomously as meiotic oocytes unless masculinized by an embryonic testicular environment. An ovarian environment does not appear to be required for the initial stages of germ cell feminization and meiotic entry. Thus, one might have predicted that germ cells in embryonic XX gonads would enter meiosis and

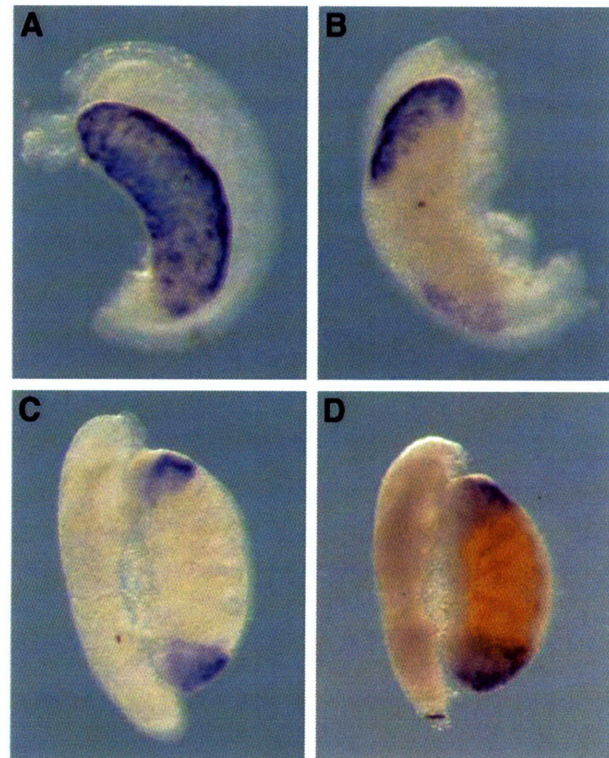

Fig. 7. Expression of *Stra8* in feminized E14.5 XY^{ZAL} gonads. Whole-mount in situ hybridization of *Stra8* on a fully feminized XY^{ZAL} ovary (A), a moderately feminized ovotestis (B), and a mildly feminized ovotestis (C). Double whole-mount in situ for *Stra8* and *Aard* on a mildly sex-reversed ovotestis (D). *Stra8* expression (purple) is limited to feminized regions at the poles. Centrally located testicular cords are revealed by expression of the testis gene *Aard* (orange–brown).

differentiate into oocytes in a positionally unbiased manner. Our discovery that sexual differentiation of germ cells occurs as a wave in the embryonic ovary was therefore unexpected. Our expression analysis of the meiosis-specific gene *Dmc1* confirmed the anterior-to-posterior pattern of germ cell differentiation in XX gonads. As with our *Stra8* analysis, we found that *Dmc1*-positive germ cells are initially located in anterior regions of embryonic XX gonads (Fig. 3). The anterior-to-posterior wave observed with *Stra8* appears to be more sharply defined than that observed with *Dmc1*. This might reflect a difference in relative lengths of time that *Stra8* and *Dmc1* are expressed in a given cell. A shorter window of expression for *Stra8* (as opposed to *Dmc1*) could produce an anterior- to-posterior wave that is visually more evident.

We observed a complementary anterior-to-posterior downregulation of *Oct4* in germ cells of XX gonads. *Oct4* is required for the maintenance of pluripotency during early embryogenesis (Nichols et al., 1998), and it has been proposed that later in development the expression of *Oct4* maintains PGCs in an undifferentiated state (Pesce et al., 1998). The strong correlation between *Oct4* downregulation and germ cell differentiation is consistent with the supposition that *Oct4* may be involved in the regulation of meiotic entry. Regardless of the precise role of *Oct4* in germ cell development, the pattern of *Oct4* downregulation in germ cells of embryonic XX gonads is consistent with an anterior-to-posterior wave of differentiation.

The conclusion that embryonic germ cells will enter meiosis and develop into oocytes by default was founded on the observations that XX and XY germ cells ectopically located in the adrenal gland develop as oocytes and that embryonic germ cells from XX or XY genital ridges will enter meiosis when grown in culture if isolated prior to E12.5. In both of these instances, the germ cells are believed to progress through meiosis on a schedule similar to that of germ cells within a normal XX gonad. However, the presence of an anterior-to-posterior wave of germ cell sexual differentiation within XX gonads suggests that germ cell differentiation in the XX gonad may not be purely cell-autonomous.

What mechanism might regulate the anterior-to-posterior wave of germ cell sexual differentiation in XX gonads? Perhaps the wave of differentiation reflects the timing of germ cell entry into the gonad. In this scenario, the first primordial germ cells to migrate into the gonad would tend to arrive in anterior regions. However, studies of the migration of primordial germ cells have not detected such anterior-to-posterior partitioning of arriving germ cells (Molyneaux et al., 2001). A second possibility, which we favor, is that local environmental differences in the XX gonad may affect the timing of meiotic entry. These environmental influences may not be strictly required to achieve meiotic entry. Local differences within the XX gonad could reflect the pattern of somatic differentiation in XX gonads, or alternatively, could be generated by a gradient of a meiosis promoting substance that is produced from a fixed source in or near the anterior portion of the ovary.

It is well established that the presence of oocytes in embryonic XX gonads is required for proper organization and development of the somatic ovary (McLaren, 2000). In the complete absence of germ cells, an ovary fails to form. However, evidence that somatic cells of embryonic XX gonads influence the initial stages of oocyte development has been lacking. If somatic cells of the ovary are responsible for inducing the meiotic wave, then differentiation of the ovarian soma might also be expected to exhibit an anterior-to-posterior wave of differentiation. While our examination of somatic ovarian differentiation was limited to two genes, our analysis has demonstrated an underlying complexity to ovarian differentiation that has not been previously reported. While upregulation of *follistatin* in XX gonads occurs in a center-to-pole pattern, *Adamts19* expression spreads in an anterior-to-posterior pattern. This anterior bias for *Adamts19* in XX gonads is observable as late as E13.5 (Menke and Page, 2002). Though it is uncertain whether anterior-to-posterior differentiation of the XX somatic gonad is directly driving the wave of germ cell feminization, it is clear that sexual differentiation must be coordinated to ensure that the sex of the somatic gonad and that of the germ cells is concordant. If the somatic ovary differentiates in the anterior first, then germ cells in the anterior might be able to "safely" commit to the female developmental pathway before germ cells in the posterior can.

Our analysis of *Stra8* expression in ovotestes did not reveal major delays in the timing of female sexual differentiation in posteriorly located germ cells. This was true even in ovotestes with large expanses of well-organized testicular tissue separating small regions of feminized somatic tissue at the anterior and posterior poles. This suggests that any positional or timing information regarding female differentiation of posterior germ cells is not grossly perturbed when the somatic feminization of the gonad is discontinuous. However, it is also possible that in the absence of normal environmental cues, the posterior germ cells in ovotestes may simply enter meiosis at E14.5 by default. Additional experiments may identify the mechanisms that regulate the timing of female germ cell sexual differentiation and meiotic entry.

We did not attempt to monitor the progress of male germ cell differentiation in XY gonads because of the absence of highly specific markers of embryonic male germ cell development. The earliest marker of male germ cells that has been reported is *prostaglandin D2 synthase* (Adams and McLaren, 2002). However, this gene is also expressed in somatic cells of the embryonic testis. Interestingly, prostaglandin D2 can partially masculinize XX gonads grown in organ culture. This led Adams and McLaren to suggest that germ cells that are induced to differentiate as male may themselves promote masculinization of the gonadal soma. Since the testis-determining gene, *Sry,* exhibits a "center-to-pole" expression pattern in XY genital ridges, it is ex-

pected that differentiation of testicular cell types will generally follow a similar pattern (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). In agreement with this expectation, we have observed similar expression patterns with other testis genes (D.B.M. and D.C.P., unpublished observations). We also observed that the downregulation of *follistatin* in XY gonads occurs in a center-to-pole pattern that is complementary to the upregulation of *Sry*. This is consistent with the center-to-pole model of testis differentiation. Despite this evidence for center-to-pole differentiation, a recent study has observed anterior-to-posterior expression of the *patched* gene in differentiating XY genital ridges (Yao et al., 2002). It will be interesting to see whether the masculinization of germ cells in XY genital ridges transpires in either of these patterns.

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APPENDIX 2

How does calorie restriction work?

Jana Koubova and Leonard Guarente

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PERSPECTIVE

How does calorie restriction work?

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For almost 70 years, calorie restriction has been known to extend life span. Despite the extensive physiological characterization of this dietary regimen, the molecular basis for the slowing in aging remains unsolved. Recent findings have pinpointed a few molecular pathways that appear to regulate the aging process. In this review, we propose a molecular model for how calorie restriction works that incorporates these recent findings.

Calorie restriction (CR) refers to a dietary regimen low in calories without undernutrition. It was first noted in the 1930s that food restriction significantly extends the life span of rodents (McCay et al. 1935). This longevity results from the limitation of total calories derived from carbohydrates, fats, or proteins to a level 25%–60% below that of control animals fed ad libitum (Richardson 1985; Weindruch et al. 1986). The extension in life span can approach 50% in rodents (Sohal and Weindruch 1996). CR extends life span in a remarkable range of organisms, including yeast, rotifers, spiders, worms, fish, mice, and rats (Weindruch and Walford 1988). Emerging data show that its effect may also apply to nonhuman primates (Lane et al. 2001).

CR delays a wide spectrum of diseases in different experimental animals; for example, kidney disease, a variety of neoplasias, autoimmune disease, and diabetes (Fernandes et al. 1976; Sarkar et al. 1982; Fernandes and Good 1984; Kubo et al. 1984; Engelman et al. 1990; Shields et al. 1991; Johnson et al. 1997). CR reduces age-associated neuronal loss in most mouse models of neurodegenerative disorders such as Parkinson's disease (Duan and Mattson 1999) or Alzheimer's disease (Zhu et al. 1999). However, beneficial effects in a mouse model for amyotrophic lateral sclerosis were not observed (Pedersen and Mattson 1999). The CR regimen also prevents age-associated declines in psychomotor and spatial memory tasks (Ingram et al. 1987) and loss of dendritic spines necessary for learning (Moroi-Fetters et al. 1989) and improves the brain's plasticity and ability for self-repair (Mattson 2000).

Why does CR exert these effects? Because CR delays reproduction and promotes survival in times of scarcity, it may have been evolutionarily adaptive during boom/bust cycles (Harrison 1989; Holliday 1989). Despite the plausibility of this reasoning, several challenges to the significance of CR studies in the laboratory have been made. Perhaps the restricted animals live longer simply because controls are overfed to the point of ill health. However, regimens in which animals are fed controlled amounts of food rather than ad libitum still show beneficial effects of low calories (Weindruch and Walford 1988). Another objection is that inbred strains of rodents are not representative of animals in the wild. For example, lab strains are selected for rapid reproduction and large litters (Miller et al. 1999). It has been argued that these animals may accordingly have shorter life spans than wild strains. By this reasoning, CR may simply correct a defect that has been created by domestication. However, the generality of CR in many different organisms, as mentioned above, supports the argument against this criticism.

Even though benefits of CR have been known for many years, the mechanism(s) of its action remains unclear. Its complexity lies in multiple effects including metabolic, neuroendocrine, and apoptotic changes, which vary in intensity and exhibit striking differences among specific organ systems. Several major models to explain CR exist, but none satisfactorily integrates all of CR's effects. In this review, we address the question of how CR might function to extend life span. We begin with a summary of several aging theories and classical views about the action of CR. Then we discuss how CR extends the life span in *Saccharomyces cerevisiae*. We extrapolate these findings from yeast to mammals and consider metabolic, neuroendocrine, and apoptotic shifts that may trigger longevity in the higher organisms. We conclude with a model of CR that integrates its effects on mammals.

Mechanisms of aging and classical views on how CR works

Evolutionary biologists have argued that aging is a consequence of the inability of natural selection to cull out undesirable characteristics in a postreproductive phase of life. In the wild, for example, rodents may rarely experience a postreproductive period because of early death, for example, by predation. This implies that aging is multifactorial with many mechanisms contributing to the decline. Nonetheless, ideas that one or a few specific mechanisms may lie at the heart of aging have gained currency. A leading theory is that aging is caused by

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cumulative oxidative damage generated by reactive oxygen species (ROS) produced during respiration (Harman 1988). Oxidative damage to DNA, RNA, protein, and lipids has indeed been demonstrated to occur with aging (Fraga et al. 1990; Stadtman 1992; Head et al. 2002; Liu et al. 2002). Some investigators argue that this damage may limit life span. For example, overexpression of the enzyme superoxide dismutase (SOD), which reduces ROS, extends life span in *Drosophila* (Parkes et al. 1998) and in stationary phase yeast cells (Longo et al. 1999).

How might CR slow down the generation of damage in cells? It has been documented that oxidative damage is reduced in CR animals (Lee and Yu 1990). If CR were to slow metabolism, the production of ROS would decrease as a simple consequence. However, studies measuring metabolic rate in CR animals give conflicting results. The weight of evidence in rodents indicates that metabolism, as measured by oxygen consumption normalized to the reduced body mass of the animal, does not slow down (McCarter et al. 1985). Because the balance of existing data does not support a long-term overall reduction in metabolic rate, more subtle explanations must be adduced. One possibility is that a more efficient transport of electrons through the respiratory chain might reduce the production of ROS and slow aging (Weindruch et al. 1986; Duffy et al. 1989, 1990). Another is that an increased ability to detoxify ROS slows oxidative damage in CR. The data relating CR to detoxification of ROS is again conflicting. On the one hand, organisms tend to be more resistant to an acute challenge by an exogenous oxidative stressor. For example, life-long CR seems to increase expression of SOD in rat liver (Semsei et al. 1989). On the other hand, in genetically altered strains of mice, there is no consistent correlation in the expression levels of SOD and life span (Hauck and Bartke 2000).

Another theory suggests that lack of protein turnover may cause aging. Multiple studies of aging organisms have shown accumulation of aberrant (e.g., oxidatively damaged) proteins and a reduction in protein turnover (Lavie et al. 1982; Gracy et al. 1985). CR may slow down accumulation of these potentially harmful abnormal proteins by speeding up protein turnover (Taylor et al. 1989; Sohal and Weindruch 1996). How can CR accomplish this? As the body runs out of fat during CR, it may trigger the degradation of proteins, thereby increasing their turnover. Indeed, the age-associated accumulation of oxidized proteins declines with CR (Aksenova et al. 1998; Dhahbi et al. 1999), and the activity of the liver 20S proteosome may increase during CR (Scrofano et al. 1998). Microarray study of mouse skeletal muscle also showed an increase in protein synthesis and degradation during CR (Lee et al. 1999). However, the elevated turnover during CR is not uniform; although some damaged proteins were degraded, others continued to accumulate (Scrofano et al. 1998). All told, the data suggest an increase in protein turnover during CR, but whether this change has an impact on the rate of aging is uncertain.

The covalent modification of proteins by derivatives of glucose has also been shown to increase with age (Masoro et al. 1989; Smith et al. 1994; Cefalu et al. 1995; Sell et al. 1996). These modified adducts in macromolecules, termed advanced glycation end products (AGE), have been linked to age-related pathologies (Lee and Cerami 1992). A reduction of AGE during CR has been demonstrated (Masoro et al. 1989; Cefalu et al. 1995). The blood profile of CR animals predicts this reduction, because both glucose and insulin levels are reduced in CR animals (Masoro et al. 1983, 1992). However, a lower percentage of AGE during CR does not clearly explain the multiple other effects that are known to occur. It is unlikely that a decrease in AGE is responsible for the long life span in CR, because AGE is one of many degenerative changes in aging.

The same logic argues against any model proposing a single effect of CR on a mechanical aspect of the aging process, including the antioxidation model above (Fig. 1A). As we discuss below, studies on CR in yeast suggest that the increase in life span is a regulated response to food deprivation. Indeed, a regulated response to CR may account for the plethora of effects required to slow the wholesale decline in aging (Fig. 1B).

Regulation of yeast replicative life span by CR

In budding yeast, mother cells divide asymmetrically, giving rise to a newly made daughter cell and an aging mother cell. The mother cell adopts phenotypes of aging, including an enlarged size and sterility, and senesces after ~20 divisions. This aging has been linked to the repeated rDNA genes, which encode the large and small subunits of ribosomal RNA (Sinclair and Guarente 1997). Aging mother cells accumulate extrachromosomal rDNA circles, and mutations that slow the generation of these circles extend the life span. However, this rDNA instability has not been observed in other organisms, and is evidently an idiosyncratic feature of yeast aging.

The SIR2 gene regulates the life span in yeast mother cells; mutations that inactivate SIR2 shorten the life span, and overexpression of SIR2 extends it (Kaeberlein et al. 1999). SIR2 functions to silence chromatin by deacetylating the histones in targeted regions of the yeast genome, including the rDNA. The silenced chro-

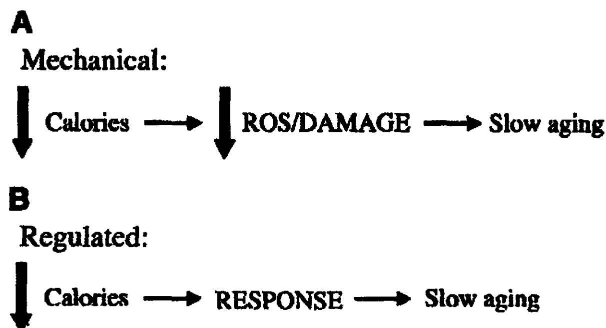

Figure 1. Two general mechanisms for how calorie restriction extends life span.

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matin is structurally less accessible to RNA polymerase and to recombinational enzymes, thereby reducing gene expression and stabilizing repeated DNA. The Sir2p deacetylase is unusual because it requires NAD as a cosubstrate (Imai et al. 2000; Landry et al. 2000; Smith et al. 2000). NADH, NADP, and NADPH neither activate nor inhibit the enzyme (Imai et al. 2000).

CR can be imposed in yeast by reducing the glucose concentration in the media from the usual 2% to 0.5% (Fig. 2; Lin et al. 2000). Because cells continue to feed on yeast extract plus peptone, which are rich in amino acids, nucleotides, and vitamins, the growth rate remains rapid as glucose levels are lowered. Thus, the reduction in glucose from 2% to 0.5%, although modest, likely imposes a state of partial energy (ATP) limitation. Other dietary restriction protocols, which also limit amino acids and other nutrients (Jiang et al. 2000, 2002), drastically slow the growth rate and may make it more difficult to impose energy limitation.

Under our conditions of CR, mother cells divide ~30% more times. This additional life span does not occur in a sir2 mutant or in strains in which NAD synthesis is reduced (Lin et al. 2000). Therefore, the activity of Sir2p is required to deliver the long life span by CR, and indeed, the silencing activity of Sir2p was shown to increase in CR cells.

How does CR activate Sir2p in yeast? When the glucose levels in the media are lowered, yeast cells respond by shunting more of the carbon to the TCA cycle to generate ATP by respiration (Lin et al. 2002). This comes at the expense of fermentation, which is the preferred pathway of carbon use when glucose levels are high. This metabolic shift makes sense because cells harvest much more ATP by metabolizing the glucose to CO_2 in the TCA cycle than by fermenting it to ethanol.

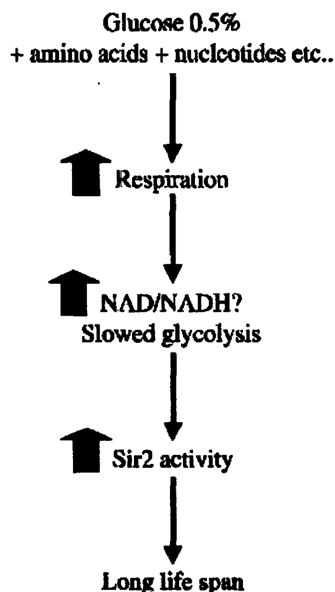

Figure 2. Calorie restriction triggers a regulatory response in yeast.

The shift toward respiration is necessary and sufficient to extend the life span in yeast. It is still not certain how this shift activates Sir2p to provide greater longevity. One possibility is that the activation of respiration converts more NADH to NAD and the resulting increase in the NAD/NADH ratio activates Sir2p. It has also been suggested that nicotinamide, which is generated during the deacetylation reaction and can inhibit Sir2p in vitro, is a negative regulator of Sir2p in vivo (Bitterman et al. 2002). There is still no direct evidence for either of these models. Another possibility is that the increase in respiration during CR slows glycolysis, and this metabolic change activates Sir2p. Any mechanism for this latter effect is at present unknown.

The important lesson from the yeast studies is that the extension in life span by CR is not a mechanical consequence of a reduction in ROS or AGE. Rather, the extension is a regulated response requiring SIR2 (Fig. 1). This regulation must involve a qualitative shift in metabolism that can be sensed by Sir2p. The deacetylase activity of the enzyme must then slow any degenerative processes that limit the life span.

Metabolic changes during CR in mammals

Studies suggest that there are two phases of CR in mammals, an adaptive period immediately after the regimen is imposed, and a steady state period, which can last the lifetime of the animal. During the adaptive phase, metabolism, as measured by oxygen consumption, declines (McCarter and McGee 1989). Glucose metabolism regulates secretion of insulin from the pancreatic β-cells. Interestingly, the β-cells in the pancreas that make insulin have been reported to sense glucose not by the production of ATP but by the conversion of NAD to NADH (Dukes et al. 1994; Eto et al. 1999; Antinozzi et al. 2002).

In the immediate response to low levels of glucose, the restricted animal quickly degrades glycogen stores upon the secretion of glucagon from the pancreas. When stored carbohydrates have been depleted, the animal starts to break down fats to compensate for the lack of glucose in the blood (Bertrand et al. 1980). As a result, loss of fat mass is one of the most striking phenotypic changes observed in calorically restricted animals (Barzilai and Gabriely 2001). The liver assumes a dominant role during CR by up-regulating the expression of enzymes involved in gluconeogenesis and down-regulating those in glycolysis (Dhahbi et al. 1999, 2001). Moreover, the liver makes ketones, such as acetoacetate, that result from the degradation of fat and proteins in the animal (Fig. 3).

After this adaptive period, the organism reaches a steady state in which ketones help meet energy needs of the brain. Thus, blood glucose falls precipitously during the adaptive period, and rises to a higher, but still below-normal level during the steady state (Greene et al. 2001). β-Cells sense the low glucose levels, and produce less insulin. The blood insulin level is lower than in control animals, and insulin sensitivity, that is, the efficacy of insulin in triggering glucose uptake by cells, is higher

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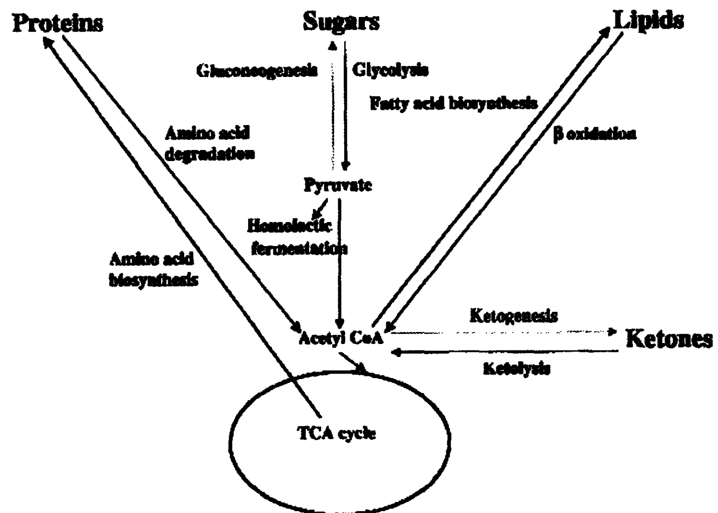

Figure 3. Metabolic pathways affected by calorie restriction in mammals. Red implies up-regulation, black implies down-regulation or no change.

(Reaven et al. 1983; Masoro et al. 1992; Cartee et al. 1994; Dhahbi et al. 2001).

Another metabolic alteration during CR in rodents, as well as in nonhuman primates, is a reduction in body temperature (Weindruch and Walford 1988; Duffy et al. 1990; Lane et al. 1996). This change may be due to an increase in the coupling of oxidative phosphorylation to ATP synthesis, perhaps by a reduction in levels of uncoupling proteins. These proteins span the inner membrane of the mitochondria and may allow proton leakage and thus hijack a fraction of the energy of electron transport to generate heat rather than ATP (Weindruch et al. 2001).

CR and the neuroendocrine system

The neuroendocrine system has long been thought to play a role in senescence (Landfield 1978; Finch et al. 1984; Sapolsky et al. 1986; Weindruch and Walford 1988). An attractive feature of this system is that it can coordinate effects on every tissue in the body (Nelson et al. 1995). Studies from *Caenorhabditis elegans* also provide strong evidence for the importance of hormones in aging, in particular for hormones involved in glucose metabolism. daf-2 is a worm ortholog to receptors for insulin and for its related peptide hormone, IGF-1 (Kimura et al. 1997). Mutant worms with reduced functional daf-2 (or several other components of the insulin/IGF-1 pathway, such as age-1 or pdk-1) live longer (Kenyon et al. 1993). Furthermore, the insulin/IGF-1 signaling pathway in worms is also responsible for triggering developmental arrest at the L2 larval stage in response to stress, such as the lack of food (Finch and Ruvkun 2001). These arrested L2 larvae, termed dauers, survive for long periods and can resume development to adulthood when conditions improve.

Many changes observed in the CR rodents trace directly or indirectly to changes in the neuroendocrine system. CR animals routinely have lower levels of the pituitary growth hormone (GH), thyroid stimulating hormone, IGF-1, and gonadotropins (Mobbs et al. 2001). Conversely, glucocorticoids, catecholamines, and glucagons increase (Klebanov et al. 1995; Mobbs et al. 2001). Mutations that affect expression of several hormones have also been shown to prolong life span. For example, Ames dwarf mice carry a loss-of-function mutation in the gene Pit^{dw} (Brown-Borg et al. 1996) that leads to abnormally low expression of GH (Sornson et al. 1996). Because GH normally turns on expression of IGF-1 in the liver, these mutant mice also have low levels of IGF-1 (Bartke et al. 1998; Coschigano et al. 2000). Ames dwarf mice live longer than wild type (Brown-Borg et al. 1996), whereas transgenic mice secreting large amounts of GH have a shortened life span (Laron 2002).

The synthesis and release of GH from the pituitary is controlled by the hypothalamus. This compartment of the brain releases hormones, such as growth-hormone-releasing hormone, that stimulate the release of GH from the pituitary. Interestingly, particular neurons in the hypothalamus may also stimulate the release of GH (Mobbs et al. 2001). The brain's ability to sense glucose is accomplished via a subset of hypothalamic neurons, which can sense the sugar and fire in response to threshold changes in its concentration. Amazingly, like the β-cells in the pancreas, these hypothalamic neurons sense glucose by the conversion of NAD to NADH (Yang et al. 1999). We speculate below that mammalian Sir2 proteins in the hypothalamus (and pancreas), because they are NAD-dependent deacetylases, sense CR and mediate the down-regulation of GH (and insulin).

Molecular analysis has the potential to strengthen the clues provided by the physiological and cellular studies above. Transcription profiling provides useful information in the brain. Expression of 6347 murine genes in 5- and 30-month-old mice, as well as CR versus control animals, was analyzed by microarrays (Lee et al. 2000). Several interesting patterns of gene expression were observed. First, genes reflecting oxidative stress and inflammation were induced in 30-month-old control mice. This induction did not occur in age-matched CR animals. Second, genes involved in protein synthesis were

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the largest class reduced in expression during CR. A reduction in protein synthesis in the brain is not surprising, because the CR brain derives much of its energy from ketones, which are less energy-rich than glucose. Third, the expression of genes for growth and neurotrophic factors increased during CR. This includes genes encoding neuroplasticity factors such as neuroserpin. This finding may help explain the improved psychomotor performance observed in CR animals (Ingram et al. 1987).

Links between CR, aging, and apoptosis

Several recent studies suggest that apoptosis may limit mammalian life span. Mice with a targeted disruption in the p66shc gene exhibit a longer life span than wild-type animals (Migliaccio et al. 1999). Importantly, cells derived from the p66shc mice are resistant to DNA-damage-induced apoptosis in culture. Further, p66shc is one of the down-stream targets of the key regulator of damage-induced apoptosis, the tumor suppressor p53 (Trinei et al. 2002). In the cell culture studies, p66shc cells were resistant to oxidative stress or ionizing radiation, which both kill cells by the p53-dependent cell death pathway. This finding suggests that apoptosis may be a two-edged sword, providing critical tumor surveillance during the reproductive years, but contributing to organ dysfunction and aging in a postreproductive period.

A second finding may also implicate apoptosis in mammalian aging. The yeast SIR2 gene appears to promote survival in a wide range of organisms. In yeast this gene promotes long life span in mother cells, and is also crucial to the generation of the long-surviving, specialized cell type termed spores. In *C. elegans*, an organism that diverged from the yeast lineage a billion years ago, the SIR2 ortholog sir-2.1 also promotes long life in adult animals and regulates the formation of dauers during development (Tissenbaum and Guarente 2001). Recently, it has been shown that a cytoplasmic Sir2p homolog can promote survival in the protozoan parasite *Leishmania* by preventing apoptosis (Vergnes et al. 2002). The mammalian ortholog of SIR2, SIRT1, represses the activity of p53 and therefore down-regulates apoptosis (Luo et al. 2001; Vaziri et al. 2001). If the survival function of SIR2 genes observed in yeast, worms, and protozoans extends to mammals, apoptosis may thus be important in limiting mammalian life span.

Furthermore, a hyperactive allele of p53 has been described that confers enhanced tumor surveillance on transgenic mice (Tyner et al. 2002). Interestingly, these mice develop early organ degeneration and signs of premature aging. These phenotypes further support the idea that apoptosis may limit mammalian life span, because its enhancement apparently speeds up the aging process.

The above studies raise the possibility that any process extending mammalian life span would have to slow down apoptosis. However, in some organs with rapidly dividing cells, apoptosis actually increases during CR, for example, in the liver (James et al. 1998) and the gut (Holt et al. 1998). This increase, along with the known shrinkage of cells during CR (Birchenall-Sparks et al. 1985), may both contribute to the down-sizing of these organs in the restricted animal. The increased rate of apoptosis may minimize the risk of cancer during CR (James et al. 1998). The increase in apoptosis in these organs appears at odds with any central role for SIR2. However, it is possible that neuroendocrine changes are dominant in up-regulating apoptosis in this subset of organs.

The brain is the one organ that does not shrink during CR (Keenan et al. 1995; Weindruch and Sohal 1997). Is it possible that the link between apoptosis and life span discussed above is due to effects on neurons? Could p66shc KO mice live longer because neuronal death is slowed? It would be of interest to determine whether CR slows cell death of neurons. This may be difficult to visualize in animals, because apoptosis is transient and the number of apoptotic cells at any given time will be low. However, it may be possible to test whether interventions that slow aging, such as CR, result in less apoptosis when neuronal cells are harvested and cultured.

Model for CR effects on mammalian aging

We have described metabolic, hormonal, and other changes, which coalesce to slow aging and extend the life span of animals during CR. Several classical models for CR propose a mechanical basis for the slowing of aging and extension of life span. For example, the accumulation of damage by oxidation or glycation may be expected to slow down as a consequence of reducing calories in the diet. But experiments in yeast show that the added life span during CR is not a mechanical output of low calories, but a process that is highly regulated. In this organism, CR triggers a metabolic shift toward respiration that activates the regulator SIR2. Could the extension in mammalian life span by CR also be a regulated process?

We can model the chain of events that follows the imposition of CR in mammals (Fig. 4). We speculate that the altered physiology resulting from lower levels of calories induces primary changes in the neuroendocrine system. As mentioned above, glucose-sensing neurons in the hypothalamus, as well as β-cells in the pancreas, somehow recognize a slowing in the rate of conversion of NAD to NADH during CR. This metabolic change likely results in a reduction in the secretion of growth hormone from the pituitary and insulin from the pancreas. Low GH would in turn reduce levels of IGF-1 made by the liver. In addition to affecting IGF-1, the lowering of other pituitary hormones, the gonadotropins, slows reproductive capabilities of the animal.

We speculate that mammalian Sir2 proteins may play roles at two critical positions in the pathway of CR effects on aging. The first would occur during the sensing of CR, leading to changes in levels of hormones in the blood stream. Because Sir2 proteins are NAD-dependent deacetylases, they are well suited to this regulatory function and may play key roles in the pituitary and pancreas in sensing the conversion of NAD to NADH and resetting the levels of insulin and IGF-1 that are released. Such a mechanism would bespeak a conserved role of

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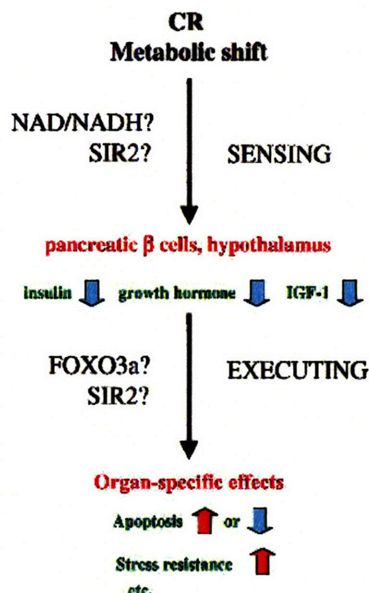

Figure 4. Model of how calorie restriction may extend life span in mammals. Effects occur at two levels: (1) sensing CR to adjust hormonal levels and (2) executing a slowing of aging on all organs. Roles for Sir2 genes are proposed at both levels, as discussed in the text.

Sir2 proteins as sensors of CR from yeast to mammals. The resulting endocrinological changes would allow the animal to mount a coordinated regulatory response to CR in different tissues.

Any hormonal changes must execute their effects by slowing aging in the animal. This execution phase is likely mediated, at least in part, by the insulin and IGF-1 signaling pathways in receptor-bearing cells. The precise effects of the hormone–receptor interaction may vary from organ to organ, because different cells bear different constellations of regulators. In general, however, longevity-promoting effects are expected to result from decreasing the insulin and IGF-1 pathways. In both *C. elegans* and mammals, these pathways impinge on transcription factors of the forkhead family. The worm factor is Daf-16 and the mammalian homologs are FOXO1–FOXO3. Decreased signaling by insulin/IGF-1 activates forkhead transcription factors, which in turn increase resistance to stress in mammalian cells (Nemoto and Finkel 2002; Tran et al. 2002) and worms (Guarente and Kenyon 2000). An increase in stress resistance is a hallmark of CR in a wide variety of organisms.

Changes that are not mediated by hormones may also be important. For example, metabolic changes on their own may directly slow aging in organs. In this regard, mammalian Sir2 proteins may play a pivotal role in some organs by recognizing the altered metabolism. If the metabolic shift during CR increases the activity of SIR2 in tissues with nondividing cells, it may directly slow apoptosis and age-dependent degeneration of organs such as the brain and perhaps the heart. Finally, metabolism-mediated changes in cells may synergize with changes in the levels of circulating insulin/IGF-1 hormones. In this regard, it is interesting that the worm sir-2.1 extends life span by down-regulating the insulin/IGF-1 responsive signaling pathway. It is tempting to speculate that mammalian Sir2 proteins play a second role during this execution phase of CR by modulating the insulin and IGF-1 signaling pathways in hormone-responsive cells.

Extension of life span by CR in mammals is a multidimensional phenomenon, which has ramifications ranging from endocrinology to metabolism to cell biology. In this review, we have discussed a regulatory model for how CR could extend life span in mammals. The studies in yeast imply that the extension of life span by CR is a regulated process. It is important therefore to consider regulatory mechanisms in any discussion of how CR slows aging in mammals. We have proposed one such model of how a coordinated global response to metabolic changes could work.

Acknowledgments

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