

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

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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 G<sub>0</sub>/G<sub>1</sub> mitotic cell cycle arrest, and meiosis begins after birth. In mice, the *Stimulated by Retinoic Acid Gene 8* (*Stra8*) 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 before meiotic initiation, whereas 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 cytochrome p450 from the 26 family (CYP26) 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. Because 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.

sex determination

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 G<sub>0</sub>/G<sub>1</sub> stage of the mitotic cell cycle (1). In this study, we address the question of why ovarian germ cells initiate meiosis embryonically, whereas 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 (A. E. Baltus, D.B.M., Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D.C.P., unpublished work). *Stra8* expression in ovarian germ cells occurs over a period of 4 days starting at E12.5, just 1 day before meiotic germ cells with characteristically condensed chromatin can be observed (2). *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) (2–4). In contrast, embryonic testes do not express *Stra8* (2). Instead, *Stra8* is first expressed in the mitotic germ cells of postnatal testes and

subsequently in the premeiotic germ cells (spermatogonia) of adult testes (2, 5).

*Stra8* expression is stimulated by RA in embryonic carcinoma cells and embryonic stem cells in culture (5). 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 (6). It is generated by a series of oxidative reactions from dietary vitamin A [retinol (ROL)] (6). Local levels of RA are regulated by retinaldehyde dehydrogenases, which catalyze the last step of RA synthesis and by the cytochrome p450, 26 family of enzymes (CYP26), which degrade RA (7–9). RA serves as a ligand to a family of nuclear hormone receptors known as RA receptors (RARs), which bind to RA response elements (RAREs) in the regulatory regions of target genes (10). Three major RAR isotypes have been identified (RAR- $\alpha$ , - $\beta$ , and - $\gamma$ ), and these exhibit overlapping expression patterns and functional redundancy in many tissues (10, 11).

RARs are expressed in the gonads of both sexes (12–14), 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 (15). Their testicular tubules are depleted of all meiotic cells, and the only remaining germ cells are undifferentiated spermatogonia and preleptotene spermatocytes (16, 17). Because 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 (17–19). Second, RA treatment of embryonic rat ovaries in culture hastens meiotic prophase (20). 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 whether RA is required for *Stra8* expression in embryonic ovaries, we assayed the gene's expression in gonads cultured in the presence of RAR panantagonist BMS-204493. This compound, which antagonizes all three RAR isotypes, prevents RA signaling by stabilizing interactions between RARs and corepressor proteins, thereby silencing expression of genes

A preliminary report of these findings was presented at the Germ Cells meeting, October 2004, Cold Spring Harbor, NY.

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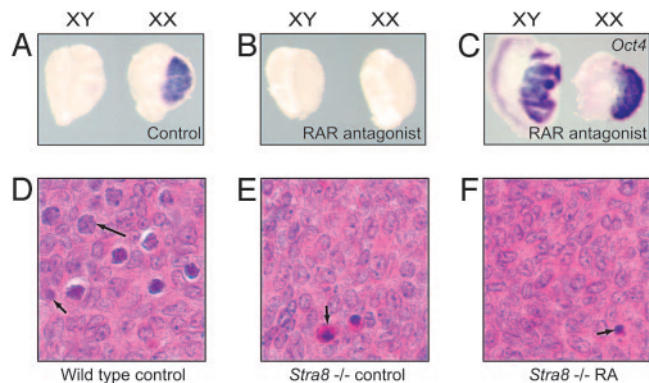
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Abbreviations: En, embryonic day *n*; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; CYP, cytochrome p450; ROL, retinol; VAD, vitamin A-deficient; CYP26, cytochrome p450 from the 26 family of enzymes.

See Profile on page 2471.

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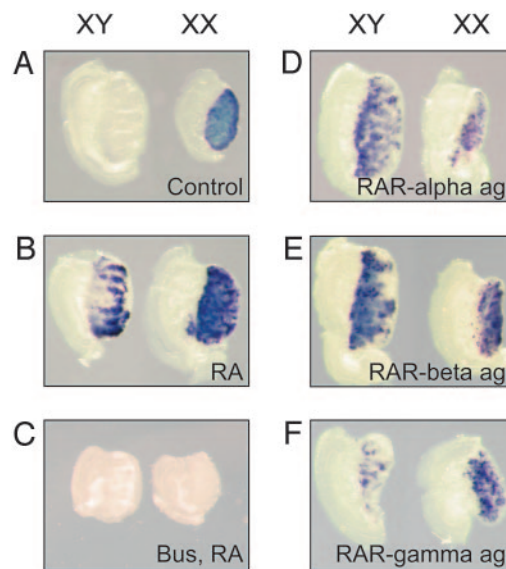
**Fig. 1.** RAR signaling is required for *Stra8* expression in embryonic ovaries. (A and B) Whole-mount *in situ* hybridization with *Stra8* probe in gonads dissected at E11.5 and cultured for 2 days (A) in control medium or (B) with RAR panantagonist BMS-204493. (C) Whole-mount *in situ* hybridization with *Oct4* probe in gonads dissected at E11.5 and cultured for 2 days with BMS-204493. (D–F) Photomicrographs of hematoxylin/eosin-stained sections of (D) wild-type ovary dissected at E11.5 and cultured for 4 days in control medium or (E and F) *Stra8*<sup>-/-</sup> ovaries cultured (E) in control medium or (F) with RA. The long arrow indicates representative cell with meiotic prophase condensation (D). We observed a few pyknotic nuclei in all samples (D–F, short arrows).

normally up-regulated by RA (21). Ovaries dissected at E11.5 and cultured for 2 days in control medium expressed *Stra8*, whereas 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), although 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* (A. E. Baltus, D.B.M., Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D.C.P., unpublished work), imply that meiotic initiation there requires RAR signaling. To confirm that RA acts through *Stra8* to induce meiotic prophase condensation in embryonic ovaries, we cultured *Stra8*<sup>-/-</sup> ovaries with and without RA. As expected, germ cells in wild-type ovaries dissected at E11.5 and cultured for 4 days in control medium displayed chromatin condensation (Fig. 1D), indicative of early meiotic prophase (20, 22). No such condensation was observed in *Stra8*<sup>-/-</sup> 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*<sup>-/-</sup> 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 (2), despite the fact that testicular cells express RARs during embryonic development (13). Perhaps the germ cells of embryonic testes are not exposed to RA *in vivo*. To determine whether exogenous RA can induce *Stra8* expression in embryonic testes, we assayed the gene's expression in gonads cultured with RA added to the medium. As expected, testes dissected at E12.5 and cultured for 2 days in control medium expressed no *Stra8* (Fig. 2A). By contrast, testes cultured with 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 (23)

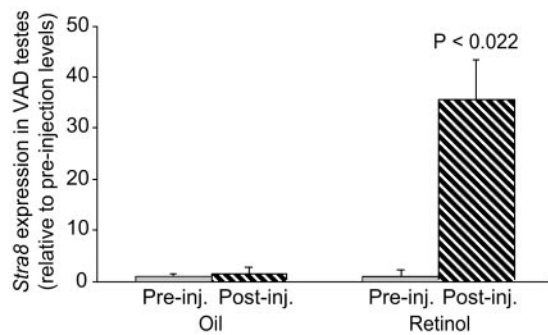


**Fig. 2.** RA and agonists to all three RARs induce *Stra8* expression in testes. Whole-mount *in situ* hybridization with *Stra8* probe in gonads dissected at E12.5 and cultured for 2 days (A) in control medium, (B) with RA, (C) with RA after busulfan treatment, (D) with RAR- $\alpha$  agonist BMS-194753,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

and then cultured with 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. (Although 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<sup>v</sup>/W<sup>v</sup>* embryos, which are severely depleted of germ cells due to a point mutation in the c-kit tyrosine kinase receptor (24). Like busulfan-treated testes, testes from *W<sup>v</sup>/W<sup>v</sup>* embryos failed to express *Stra8* when cultured with 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 (13, 25), 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–F). These results suggest that, in embryonic testes, all three RAR isotypes are capable of activating *Stra8* transcription.

**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 male mice but to increase after injection of such mice with ROL (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 ROL in oil or with oil alone. The second testis from each animal was harvested 24 h later, and *Stra8* transcript levels, pre- and postinjection, were compared by quantitative RT-PCR. *Stra8* expression increased dramatically in the testes of VAD males after injection of ROL but not after injection of oil alone (Fig.



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

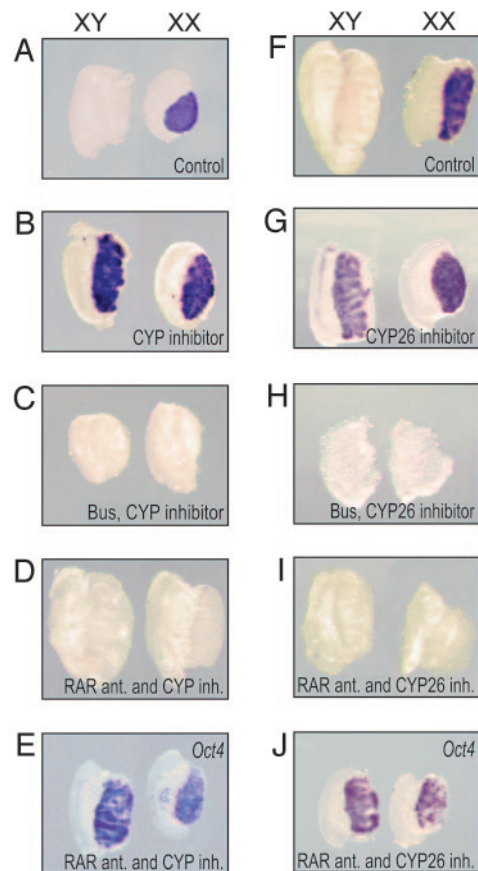
3). This result demonstrates that RA signaling can induce *Stra8* expression in adult testes *in vivo* and thus represents a shared element of the regulatory pathway required for meiotic initiation in adult testes and embryonic ovaries.

**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, metabolize RA more efficiently, or both. Lower levels of RA synthesis in embryonic testes have not been reported. However, several CYP enzymes, including some that may degrade RA, are known to be expressed in embryonic testes but not embryonic ovaries (26–28). One of these CYP enzymes could metabolize RA and thereby shield testicular germ cells from RA signaling.

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 nonspecific CYP inhibitor (29, 30). As expected, testes dissected at E12.5 and cultured for 2 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 2 days in the presence of both ketoconazole and RAR panantagonist 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 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 particularly specific and efficient (31). Although ketoconazole is a nonspecific inhibitor of CYP enzymes, the compound R115866 is a highly selective inhibitor of CYP26-mediated metabolism of RA *in vivo* (32). To test whether CYP26-mediated RA metabolism in embryonic testes prevents *Stra8* expression, we dissected embryonic gonads



**Fig. 4.** CYP26 activity prevents *Stra8* expression in embryonic testes. (A–D and F–I) Whole-mount *in situ* hybridization with *Stra8* probe in gonads dissected at E12.5 and cultured for 2 days in control medium (A and F), with CYP inhibitor ketoconazole (B), with ketoconazole after busulfan treatment (C), with RAR panantagonist BMS-204493 and ketoconazole (D), with CYP26 inhibitor R115866 (G), with R115866 after busulfan treatment (I), or with BMS-204493 and R115866. (E and J) *In situ* hybridization with *Oct4* probe in gonads dissected at E12.5 and cultured for 2 days with BMS-204493 and ketoconazole (E) or with BMS-204493 and R115866 (J).

at E12.5 and cultured them for 2 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 panantagonist 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 (26, 33, 34). The *Cyp26b1* gene was shown previously to be expressed in somatic cells of embryonic testes (26). 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.

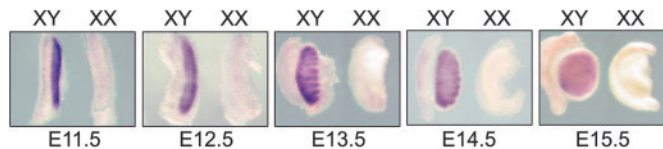


Fig. 5. Developmental time course of *Cyp26b1* expression in embryonic gonads as revealed by whole-mount *in situ* hybridization.

5). No 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<sup>ZAL</sup> embryos are partially sex-reversed, with ovotestes (2, 35, 36). An XY<sup>ZAL</sup> ovotestis is typically comprised of a central region exhibiting testicular histology and two polar regions displaying ovarian histology (35, 36). Whole-mount *in situ* hybridization to XY<sup>ZAL</sup> 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, whereas *Cyp26b1* expression is limited to the testicular (central) region. Thus, *Stra8* and *Cyp26b1* appear to be expressed in nonoverlapping 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 up-regulated in response to 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.

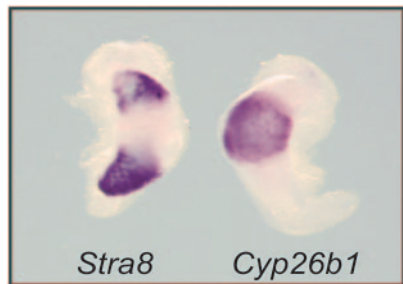
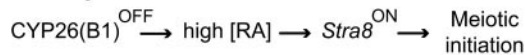


Fig. 6. *Stra8* and *Cyp26b1* expressed in nonoverlapping domains in E14.5 XY<sup>ZAL</sup> ovotestes. *Stra8* is expressed in the polar ovarian regions. *Cyp26b1* is expressed in the central testicular region.

**Embryonic ovary**



**Embryonic testis**

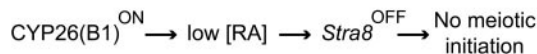


Fig. 7. A proposed model for regulation of meiotic initiation in embryonic gonads.

Conversely, testis-wide induction of *Stra8* by RA may contribute to the spermatogenic synchronization observed in VAD animals after administration of ROL (17).

In the 1970s, Byskov and Saxen (37) 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. However, other investigators noted that ectopic germ cells localized not in ovaries but in the adrenal glands, in the mesonephros, or even in embryonic lung aggregates, also initiated meiosis (1, 22, 38). These observations led investigators to argue that, if a meiosis-inducing substance existed, it must be present in all of these somatic contexts (22, 38). 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 (39, 40), mesonephros (41), and lung (42). 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, although 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 (22, 38). 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, RARs are readily detected in germ cells but are expressed at very low levels, if at all, in somatic cells (12). Indeed, the promoter of the *Stra8* gene contains two putative 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 (i) induces *Stra8* expression in embryonic ovaries and (ii) is eliminated by CYP26 activity in embryonic testes. Although 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 (39, 40). Could diffusion of RA from the embryonic adrenal gland account for the anterior-to-posterior wave of *Stra8* expression observed in the developing ovary (2)? Second, by studying mice carrying a *lacZ* transgene controlled by RAREs (43), we detected RA in the mesonephroi of both sexes at E11.5 and E14.5 (Fig. 8, which is published as supporting information on the PNAS web site), 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 (22) suggested that the somatic cells of the embryonic testis inhibit germ cells from entering meiotic prophase. Our findings substantiate this hypothesis and identify CYP26-catalyzed degradation of RA as a central mechanism of meiotic inhibition. McLaren and Southee (22) 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. 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 (14). Similarly, in embryonic testes, *Cyp26b1* is expressed in cells outside the developing testis cords (26). 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 (14).

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 (44, 45). 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 Laboratories) were used in all embryonic gonad culture experiments, except where mutant strains are specified. *Stra8*<sup>-/-</sup> embryos were generated by mating heterozygotes (A. E. Baltus, D.B.M., Y. C. Hu, M. L. Goodheart, A. E. Carpenter, D. G. de Rooij, and D.C.P., unpublished work). XY<sup>ZAL</sup> embryos with ovotestes were obtained by crossing Zalende/Ei males (The Jackson Laboratory) with C57BL/6 females to generate F<sub>1</sub> hybrids. F<sub>1</sub> males were then backcrossed to C57BL/6 females to create N<sub>2</sub> XY<sup>ZAL</sup> embryos with feminized gonads, as reported (2, 36). Embryos carrying a *RARE-lacZ* transgene were obtained from matings of *RARE-lacZ*<sup>+/-</sup> males (43) with CD-1 females. *W<sup>v</sup>/W<sup>v</sup>* (The 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'-TTACGTCCATCGTGGACAG-CAT-3' and 5'-TGGGCTGGGTGTTAGTCTTAT-3'). The second pair of primers amplifies a 342-bp segment of a Y-chromosome repeat sequence (5'-CAGTTACCAATCAACA-CATCAC-3' and 5'-CTGGAGCTCTACAGTGATGA-3').

**Germ Cell Depletion.** At 9.5 days postcoitum, pregnant females were injected i.p. with 200  $\mu$ l of 8 mg/ml busulfan (Sigma) in 50% dimethyl sulfoxide (23). Embryos from these busulfan-treated mothers were dissected at E11.5 or E12.5.

**Embryonic Gonad Cultures and Treatments.** Pregnant mice were killed by cervical dislocation, and embryonic gonads were dissected out in PBS solution. Gonads were cultured atop 1.5- to 1.7-ml agar blocks, as described (46). The following compounds were dissolved in ethanol and stored in aliquots at  $-80^{\circ}\text{C}$  before use: all-trans RA (Sigma); RAR panantagonist 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, New Brunswick, NJ) were dissolved in dimethyl sulfoxide and stored at room temperature before use. All compounds were added to the culture media surrounding the agar blocks at least 1 hour before freshly dissected gonads were deposited on the blocks. The concentrations of compounds in culture media were as follows: BMS-204493, 5  $\mu$ M; all-trans RA, 0.7  $\mu$ M; BMS-194753, 0.5  $\mu$ M; BMS-213309, 0.5  $\mu$ M; BMS-270394, 0.5  $\mu$ M; ketoconazole, 0.7  $\mu$ M; and R115866, 0.7  $\mu$ M. 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 PBS solution and cultured as described above. Gonads were then fixed in Bouin's solution at  $4^{\circ}\text{C}$  overnight, sectioned, and stained with hematoxylin/eosin.

For *in situ* hybridizations, embryonic gonads were dissected in PBS solution and fixed in 4% paraformaldehyde at  $4^{\circ}\text{C}$  overnight. Whole-mount *in situ* hybridizations were performed as reported (47). Digoxigenin-labeled RNA probes for *Stra8* and *Oct4* (2) were detected by using an alkaline phosphatase-conjugated antidigoxigenin antibody and staining with BM purple alkaline phosphatase substrate (Roche Applied Science, Indianapolis).

**Analysis of *Stra8* Expression in VAD Testes.** Adult female mice (129/SvJ, The Jackson Laboratory) were maintained on a VAD diet (Harlan Teklad, Indianapolis) for at least 2 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 preinjection control in RT-PCR analysis. Incisions were sealed and the animals allowed to recover for 24 h. Five animals with similarly deficient spermatogenesis (as judged by preinjection testicular histology) were selected for subsequent analysis. Three (experimental) animals were injected s.c. with 100  $\mu$ l of 15 mg/ml all-trans ROL acetate (Sigma) in 10% ethanol and sesame oil. Two (control) animals were injected with 100  $\mu$ l of 10% ethanol and sesame oil. The experimental and control animals' remaining testes were harvested 24 h after injection and placed in TRIzol for RNA extraction. Quantitative RT-PCR analysis was performed on a Prism 7000 System (Applied Biosystems) by using SYBR-green labeling. Each RNA sample was analyzed, in triplicate with *Stra8* primers that amplify a 151-bp product (primers: 5'-GTTTCCTGCGTGTTCCA-CAAG-3' and 5'-CACCCGAGGCTCAAGCTTC-3') and control *Rps2* primers that amplify a 112-bp product (primers: 5'-CTGACTCCCGACCTCTGGAAA-3' and 5'-GAGCCTGGGTCTCTGAACA-3'). *Stra8* expression was normalized to *Rps2* expression and compared in pre- and postinjection testes from each mouse.

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