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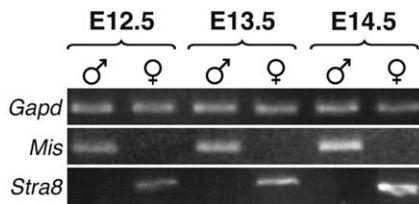
Fig. 1. Detection of sex-specific *Stra8* expression by RT-PCR. Expression of *Gapd* (ubiquitous control), *Mis* (testis-specific control), and *Stra8* in E12.5, E13.5, and E14.5 XY and XX gonads.

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
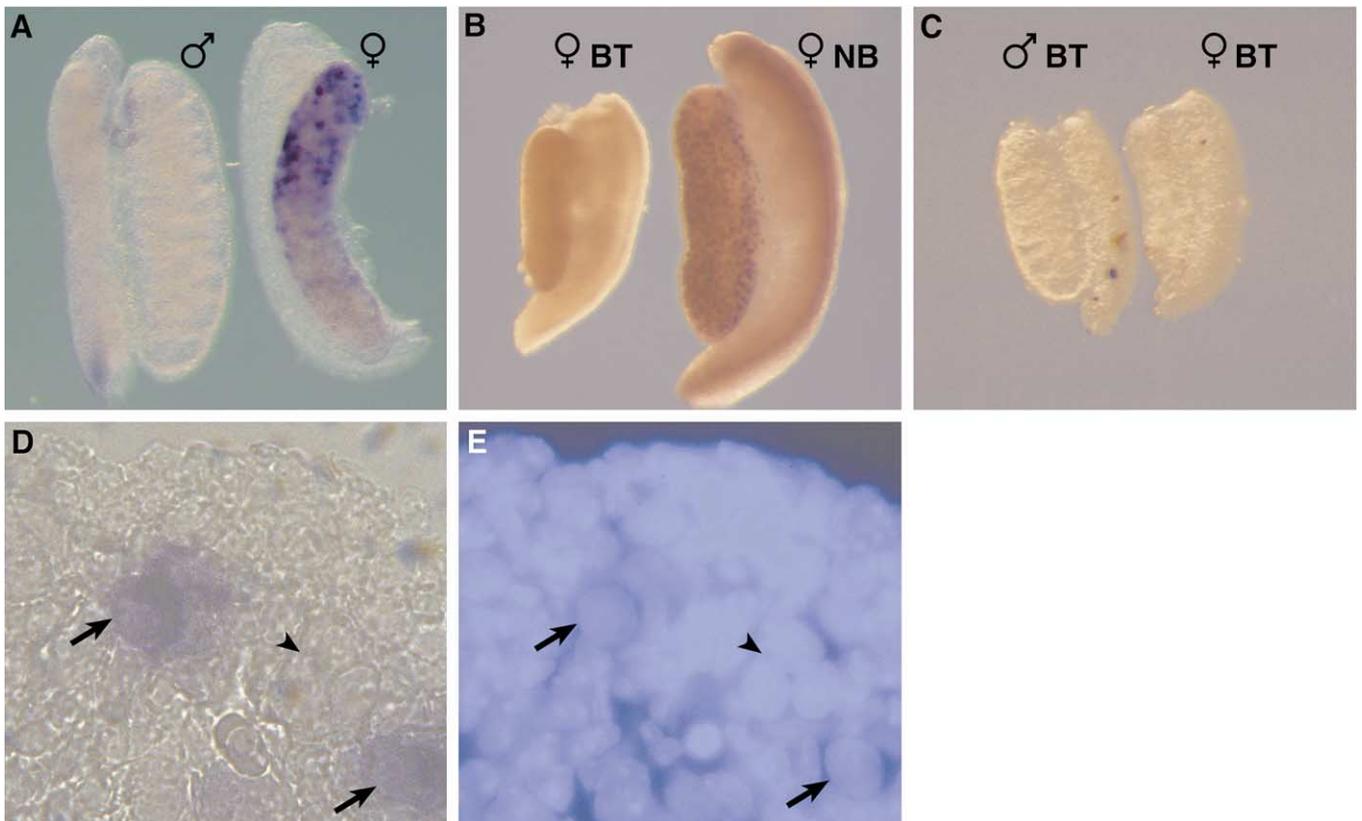
Fig. 2. Expression of *Stra8* in normal and germ cell-depleted embryonic gonads. (A) *Stra8* whole-mount in situ hybridizations on E13.5 gonads reveals *Stra8*-expressing cells (stained purple) in ovaries but not testes. (B) Detection of all germ cells by staining for endogenous alkaline phosphatase 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
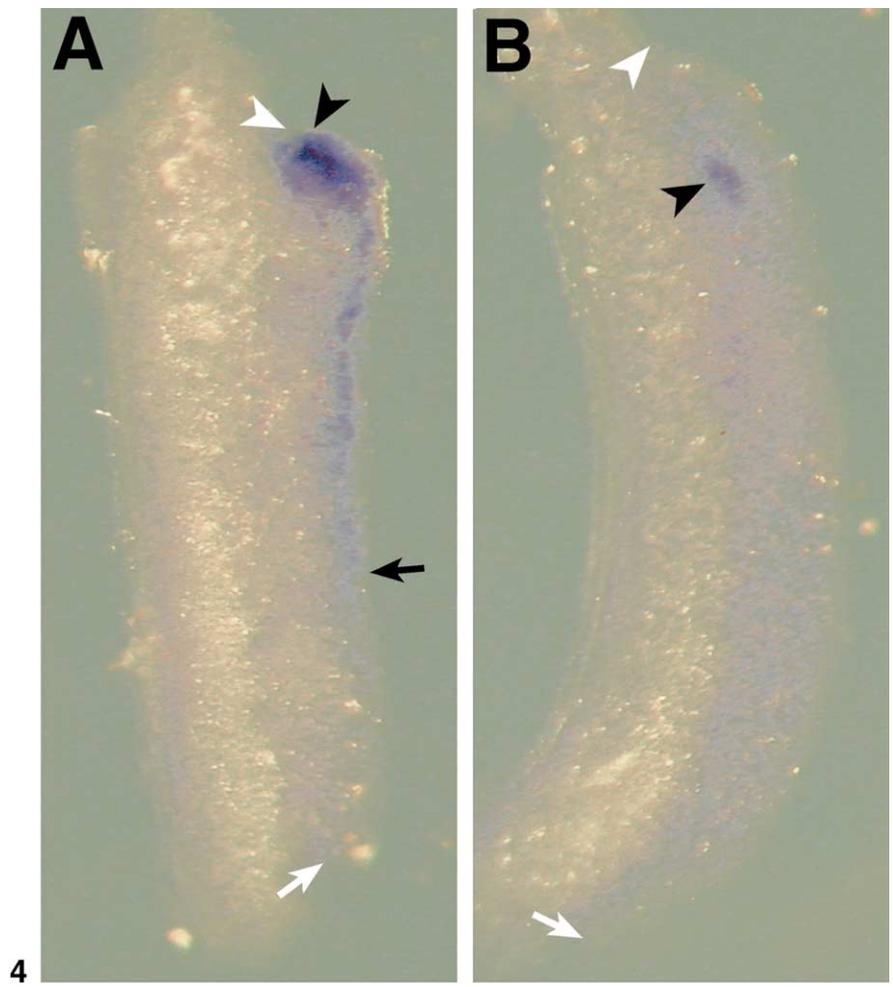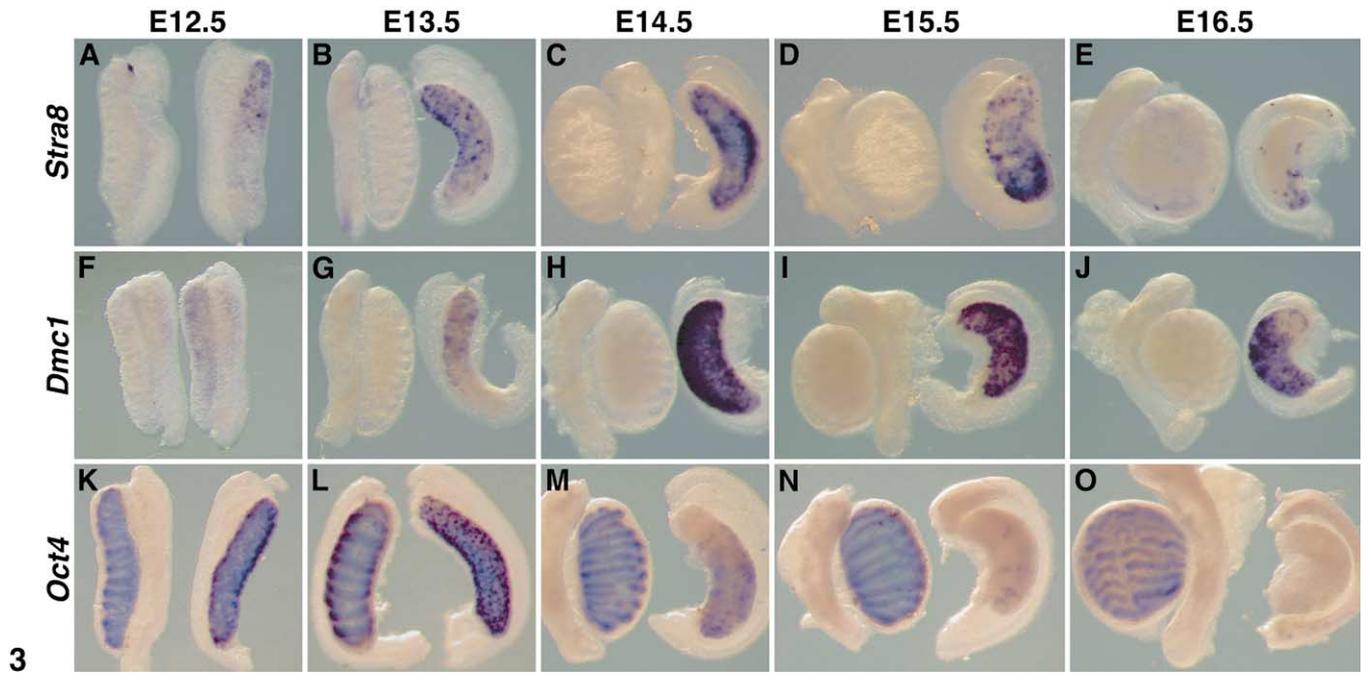
presence of male- specific germ cell transcripts in our sub-tracted cDNA libraries. Contrary to previous work (Oulad-Abdelghani et al., 1996), our RT-PCR analysis revealed that *Stra8* is expressed in embryonic ovaries and not in embryonic testes from E12.5 to E14.5 (Fig. 1).

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 hybrid-ization 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* ex-pression by depleting embryos of germ cells using the chemical busulfan (Merchant, 1975). Detection of germ cells by endogenous alkaline phosphatase activity con-firmed that most germ cells were eliminated in busulfan-treated E13.5 XX gonads (Fig. 2B). Similarly, *Stra8* stain-ing 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 histolog-ical 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 through-out normal E13.5 ovaries (Fig. 2B). In contrast, we ob-served 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 devel-opmental 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 devel-opment has been considered the defining hallmark of female germ cell sex determination, and both the morphological changes characteristic of meiotic germ cells and the expres-sion 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 embry-onic 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 ex-pressed 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 per-ceived 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 un-

detectable 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-
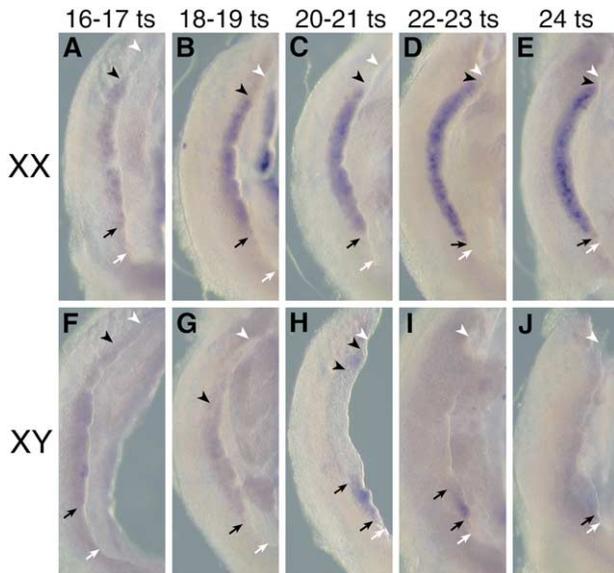
Fig. 5. Developmental time course of *follistatin* expression in XY and XX embryonic gonads from 16 to 24ts. Whole-mount in situ hybridization demonstrates that *follistatin* is expressed at similar levels in XX (A) and XY (F) genital ridges at 16-17ts. *Follistatin* expression is upregulated in the 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 (Gp-box)*, 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-
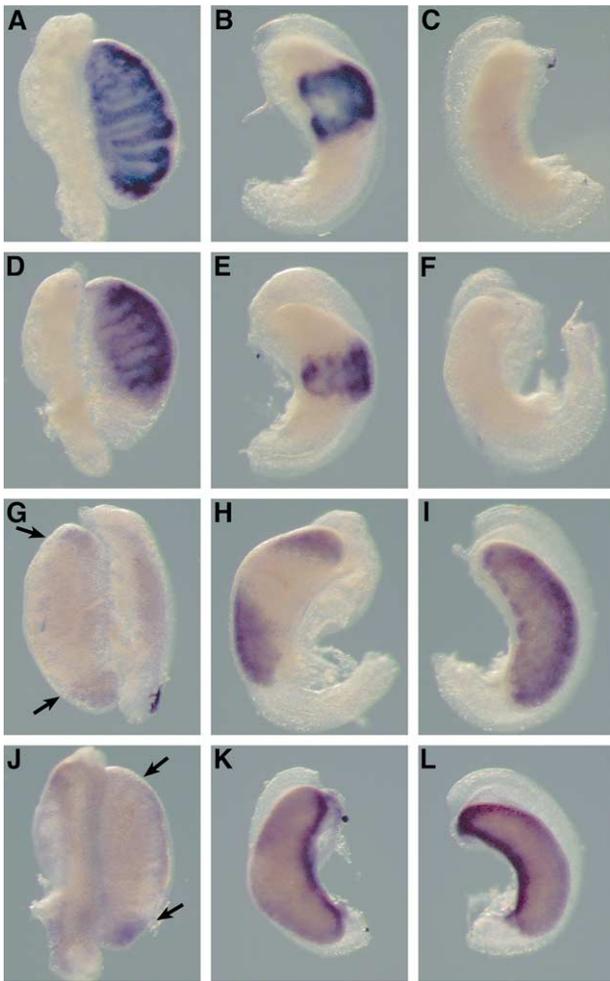
Fig. 6. Whole-mount in situ hybridizations examining somatic testis and ovary genes in XY^{ZAL} gonads. *Aard* expression in a very mildly feminized XY^{ZAL} testis (A), a severely feminized XY^{ZAL} ovotestis (B), and a completely sex-reversed XY^{ZAL} ovary (C). Expression of *Cbln1* in a mild XY^{ZAL} 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 distinguish-

able 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
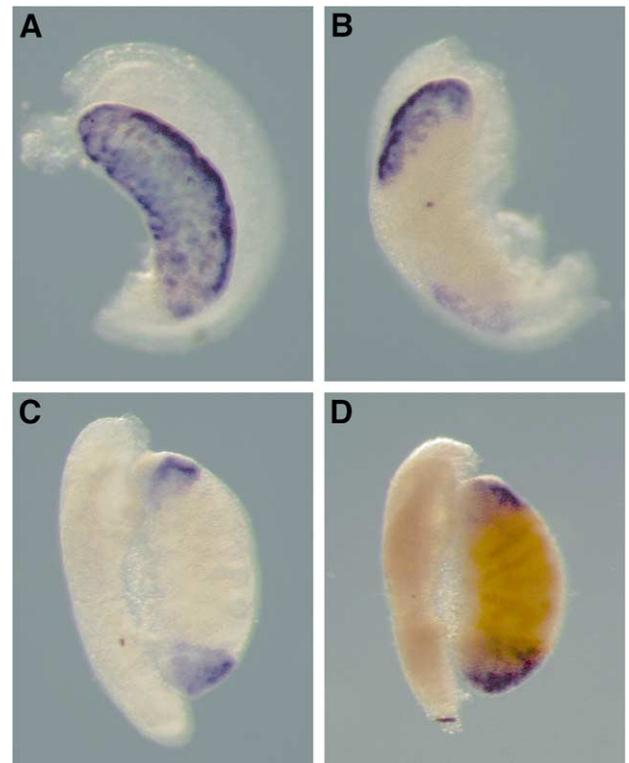

Fig. 7. Expression of *Stra8* in feminized E14.5 XY^{ZAL} gonads. Whole-mount in situ hybridization of *Stra8* on a fully feminized XY^{ZAL} ovary (A), a moderately feminized ovotestis (B), and a mildly feminized ovotestis (C). Double whole-mount in situ for *Stra8* and *Aard* on a mildly sex-reversed 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.

Acknowledgments

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