FGF9 Suppresses Meiosis and Promotes Male Germ Cell Fate in Mice

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SUMMARY
Sex determination of mammalian germ cells occurs during fetal development and depends on signals from gonadal somatic cells. Previous studies have established that retinoic acid (RA) triggers ovarian germ cells to enter meiosis and thereby commit to oogenesis, whereas in the developing testis, the enzyme CYP26B1 degrades RA and germ cells are not induced to enter meiosis. Using in vitro and in vivo models, we demonstrate that fibroblast growth factor 9 (FGF9) produced in the fetal testis acts directly on germ cells to inhibit meiosis; in addition, FGF9 maintains expression of pluripotency-related genes and upregulates markers associated with male germ cell fate. We conclude that two independent and mutually antagonistic pathways involving RA and FGF9 act in concert to determine mammalian germ cell sexual fate commitment and support a model in which the mitosis/meiosis switch is robustly controlled by both positive and negative regulatory factors.

INTRODUCTION
Germ cells play a uniquely important role in biology, providing a mechanism for sexual reproduction and for passing genetic legacy from one generation to the next. This role calls for a mode of cell division, meiosis, that results in formation of haploid gametes. How germ cells switch from replicative cell division (mitosis) to meiosis, and how germ cells in the fetus choose between spermatogenesis and oogenesis, remain key questions in reproductive biology.

Much of what is known about mammalian germ cells derives from studies in the mouse. Germ cells arise in mice before gastrulation, around 7 days post coitum (dpc), migrate for several days as the embryo elongates and organizes its body plan, and colonize the newly formed gonadal primordia around 10.5 dpc. Over the following days, they begin their complex path of differentiation into oocytes or sperm. The choice of germ cell sexual fate is dictated by cues from the somatic cells of the gonad (Adams and McLaren, 2002; McLaren, 1981; McLaren and Southee, 1997; Palmer and Burgoyne, 1991). Whether XX or XY in chromosomal constitution, germ cells are triggered to enter meiosis in a developing ovary, thereby committing to oogenesis. In a developing testis, germ cells do not enter meiosis but instead go into a state of mitotic quiescence (G0/G1 arrest) and express markers characteristic of commitment to the spermatogenic fate. The period from colonization of the gonad until either entry into meiosis or mitotic arrest is critical: the sexual fate of the germ cells must be in accord with the sex of the gonad, or infertility and/or cancer will result (Skakkebaek et al., 1998).

Recent studies have demonstrated that the signaling molecule retinoic acid (RA) triggers fetal germ cells to enter meiosis (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). The meiosis-inducing role of RA has been demonstrated also in rats (Li and Clagett-Dame, 2009) and likely also operates in chickens and amphibians (Smith et al., 2008; Wallacides et al., 2009). RA is produced in the mesonephros and is postulated to diffuse or flow into the adjacent gonad (Bolles et al., 2006). In the developing ovary, high levels of RA induce markers of meiosis (Bowles et al., 2006; Koubova et al., 2006), including the premeiotic marker Stimulated by retinoic acid (Stra8), an essential gatekeeper of meiosis and a probable direct target of RA signaling (Baltus et al., 2006; Menke et al., 2003; Oulad-Abdelghani et al., 1996). In the developing testis, germ cells are not triggered to enter meiosis because RA is degraded by a P450 enzyme, CYP26B1 (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). In Cyp26b1 null testes, XY germ cells enter meiosis and progress to at least the pachytene stage of meiosis I during fetal development (Bowles et al., 2006; MacLean et al., 2007), confirming the importance of the RA/CYP26B1 signaling system in vivo.

The existence of an ovarian meiosis-inducing factor (Byskov, 1974; Byskov and Saxen, 1976) and/or a testicular meiosis-inhibiting factor (Francavilla and Zamboni, 1985; McLaren, 1984) had been predicted for some decades (for review, see Bowles and Koopman, 2007). Although RA showed many of the properties predicted for a meiosis-inducing factor, the finding that the role of a meiosis inhibitor in the testis might be carried out by a cytoplasmic enzyme (CYP26B1) was contrary to the prediction that it would be a secreted factor. That expectation was based on the observation that some XY germ cells lodged ectopically in the XY mesonephros are spared entry into meiosis while all ectopic XX germ cells in an XX mesonephros do enter meiosis (McLaren, 1984). It was further
supported by the recent observation that XY germ cells in cultured mouse testes occasionally enter meiosis when all secretion is inhibited by use of the compound Brefeldin A (Best et al., 2008). Together, these observations support the concept that meiosis in the testis is actively suppressed through the action of a secreted factor, in addition to the absence of RA.

We hypothesized that the growth factor FGF9 might play a role in this system, based on a number of published observations. Fgf9 is initially expressed in gonads of both sexes, but its expression is greatly upregulated in the developing testis shortly after SRY and SOX9 are activated in pre-Sertoli cells (Colvin et al., 2001a, Nef et al., 2005). FGF9 has a known role in somatic sex determination: deletion of Fgf9 leads to male-to-female sex reversal (Colvin et al., 2001a; Kim et al., 2006; Schmahl et al., 2004). In addition, it has been reported that in XY but not XX Fgf9 null embryos, most germ cells die by 12.5 dpc, suggesting a sex-specific role for FGF9 in germ cell survival (DiNapoli et al., 2006). Some of the surviving XY germ cells expressed the meiotic marker γH2AX at 14.5 dpc, as might be expected in an ovarian environment, given the likely loss of Cyp26b1 expression and consequent exposure to RA in XY Fgf9 null gonads. Although the reason for the loss of germ cells in XY Fgf9 null gonads has remained obscure, these results suggest profound effects of FGF9 on germ cell biology and leave open the possibility of an additional role for FGF9 in directly influencing germ cell sexual fate.

In this study, we show that FGF9 plays a critical and direct role in germ cell sex determination. We show, using ex vivo gain-and-loss-of-function studies in gonads and isolated germ cells, and in vivo analysis of Cyp26b1−/−, Fgf9−/−, and double-knockout embryos, that FGF9 acts directly on germ cells to antagonize their entry into meiosis, making them less responsive to RA. We show that FGF9 signaling plays a further role, maintaining pluripotency of germ cells and actively promoting a male fate. Our data suggest a model whereby germ cell sexual fate is determined by the relative abundance of FGF9 and RA. This system, incorporating both positive and negative regulatory cues, imparts stability at the crucial stages of germ cell sexual determination.

**RESULTS**

**Germ Cells in Cyp26b1 Null Testes Differ from Germ Cells in Wild-Type Ovaries**

Initial analyses of Cyp26b1 null testes indicated that XY germ cells exposed to aberrantly high levels of endogenous RA progress into meiosis and through the pachytene stage of meiosis I, as assessed by expression of the premeiotic marker, Stra8, the meiotic marker SyCP3 (Synaptonemal complex protein 3), and by histological and cytogenetic criteria (Bowles et al., 2006; MacLean et al., 2007). We reasoned that if a diffusible meiosis-inhibiting substance were present in the XY gonad, then some aspects of this progression might differ between germ cells in Cyp26b1 null XY gonads and those in Cyp26b1 null XX gonads, even though both are exposed to RA.

To test this hypothesis, we used quantitative real-time RT-PCR (qRT-PCR) to compare the levels of Stra8 gene expression in wild-type XX and XY gonads, and XX and XY Cyp26b1 null gonads at 12.5 dpc (Figure 1A), a day earlier than previous, nonquantitative analyses (Bowles et al., 2006). First, as expected, Stra8 expression was more robust in XY Cyp26b1 null relative to XY wild-type testes. Second, Stra8 expression levels were higher in XX Cyp26b1 null relative to XX wild-type ovaries (Figure 1A), consistent with our previous observation that Cyp26b1 is expressed at 11.5 dpc before subsiding in developing ovaries (Bowles et al., 2006); it remains to be determined whether the higher levels of Stra8 in the Cyp26b1 null ovaries result in premature entry into meiosis in these germ cells. Third, although Stra8 was upregulated in XY Cyp26b1 null gonads compared with wild-type XY gonads, the level of expression was significantly lower than that seen in XX Cyp26b1 null gonads (Figure 1A). Because germ cells are exposed to RA in both XY and XX gonads when CYP26B1 is absent, this last observation suggests that some factor(s) in the testicular environment act to antagonize the initial meiosis-inducing actions of RA. It has been suggested that NANO2 prevents Stra8 expression (Suzuki and Saga, 2008), but we did not find Nanos2 to be upregulated in the XY Cyp26b1 null testis at 12.5 dpc (Figure S1A available online).

We next compared expression of markers of more advanced stages of meiosis in wild-type XX and XY gonads, and XY Cyp26b1 null gonads, at 15.5 dpc. Expression of SyCP3, DMC1 (dosage suppressor of mck1 homolog, meiosis-specific homologous recombination [yeast]), and Spo11 (sporulation protein, meiosis-specific, SPO11 homolog) was high in XX gonads and low in XY gonads, reflecting the advanced progression of meiosis in germ cells of the ovary but not the testes at that stage (Figure 1B). However, expression of these markers in XY Cyp26b1 null gonads was intermediate between the levels observed in wild-type XX and XY gonads (Figure 1B), further supporting the concept of a meiosis inhibitor that operates independently of CYP26B1 in the testis.

We also examined a range of male germ cell fate markers in XY Cyp26b1 null gonads at 15.5 dpc. No expression of male germ cell fate markers Nanos2, Dmnt3L, or Tdrd1 was detected in germ cells of the XY Cyp26b1 null gonads (see Figure S1B), which is not surprising given that the germ cells in these gonads are well advanced in meiosis at this stage (Figure 1B, and see below).

Finally, we compared expression of the pluripotency markers Oct4 and Sox2 in wild-type and Cyp26b1 null gonads at 15.5 dpc. These markers are expressed by germ cells from the time of their specification, and Oct4 is normally detectable in XY germ cells in a testicular environment until at least 17.5 dpc (Maldonado-Saldívar et al., 2007; Western et al., 2005). In contrast, Oct4 and Sox2 are normally downregulated as germ cells enter meiosis in XX gonads and expression is undetectable by 14.5–15.5 dpc (Maldonado-Saldívar et al., 2007; Pesce et al., 1998; Western et al., 2005). Unexpectedly, we found that expression of pluripotency markers Oct4 and Sox2 was not downregulated in XY Cyp26b1 null gonads relative to wild-type XY gonads at 15.5 dpc (Figure 1C). Immunohistochemical analyses using antibodies to the meiotic marker γH2AX (a phosphorylated histone variant) (Hunter et al., 2001) and the meiotic structural protein SYCP3 confirmed that germ cells in XY Cyp26b1 null testes, like those in XX gonads, were in an advanced stage of meiosis, and yet retained expression of OCT4 in their germ cells (Figures 1D, S1C, and S1D).
Responsive to RA in Mouse Cultured Gonads

FGF9 Acts to Inhibit Meiosis by Making Germ Cells Less Responsive to RA in Mouse Cultured Gonads

Together, these findings indicate that, although genetic ablation of Cyp26b1 in XY gonads results in levels of RA sufficient to induce the premeiotic marker Stra8 and to trigger progression through meiosis, germ cells in these gonads do not show a profile of marker expression typical of germ cells in an ovarian environment. These observations point to the existence of additional factor(s) produced in the developing testes that have at least two activities, one involved in impeding the female pathway of germ cell differentiation (i.e., initiation of meiosis), and another in promoting male germ cell behavior (i.e., retention of markers such as OCT4).

FGF9 Regulates Sex-Specific Germ Cell Fate

Figure 1. XY Germ Cells in the Cyp26b1 Null Embryo Are Distinct from Both XY Germ Cells and XX Germ Cells

(A) qRT-PCR analysis of expression of Stra8 in 12.5 dpc urogenital ridge (UGR) from genotypes of Cyp26b1−/−-knockout litters as indicated. Bars indicate the mean ± 1 SEM, n = 5, 11, 5, 7, 6, and 5 individual embryos, respectively. Mvh (also known as Ddx4) was used as the normalization control.

(B) qRT-PCR analysis of Sycp3, Dmc1, and Spo11 expression at 15.5 dpc in XY wild-type (WT), XY Cyp26b1 null (KO), and XX WT gonads. Bars indicate the mean ± 1 SEM, n = 4 individual embryos. Mvh was used as the normalization control.

(C) qRT-PCR analysis of Oct4 and Sox2 expression at 15.5 dpc in three genotypes as in (B). Bars indicate the mean ± 1 SEM, n = 4 individual embryos. Mvh was used as the normalization control.

(D) Confocal images of gonad tissue at 15.5 dpc in three genotypes as in (B). MVH marks germ cell cytoplasm, γH2AX marks germ cells in meiosis, SYCP3 shows meiotic chromosome architecture, and OCT4 marks pluripotent germ cells. Scale bar, 10 μm. See also Figure S1.

One possible explanation for this effect is that FGF9 drives Cyp26b1 expression in somatic cells of the developing testis: like Fgf9, Cyp26b1 is expressed initially in gonads of both sexes and then upregulated in the testis (Bowles et al., 2006). However, addition of FGF9 to mouse UGR organ cultures did not upregulate Cyp26b1 expression over a 24 hr period (Figure 2D). Likewise, manipulation of RA levels in cultured gonads, or in vivo by genetic ablation of Cyp26b1, had no effect on FGF9 expression (Figures S2A and S2B). Hence, Stra8 expression is antagonized in FGF9-treated XX UGRs independently of the RA/CYP26B1 pathway.

We also carried out a series of experiments involving chemical agonists and antagonists in XY and XX UGR explant cultures, to examine further the relationship between FGF9 and RA in this system. Inhibition of CYP26B1 using the P450 inhibitor ketocnazole, which would be expected to augment endogenous RA activity, led to greatly enhanced Stra8 expression, consistent with previous findings (Bowles et al., 2006; Koubova et al., 2006) (Figures 2E and 2F, columns 5 and 1). In XY UGR explants, simultaneous inhibition of CYP26B1 and FGF receptors caused even greater upregulation of Stra8, indicating that RA is more capable of inducing Stra8 expression in the absence of FGF signaling (Figure 2E, columns 6 and 5). In XY UGR explant culture, no such additive effect was observed, presumably because there is little endogenous FGF9 present in such cultures (Figure 2F, columns 6 and 5). Importantly, treatment of XY or XX cultures with FGFR antagonist alone did not augment Stra8 expression, indicating that FGF9 does not work by inhibiting for FGF9 in inhibiting meiotic entry in cultured mouse gonads (Barrios et al., 2010). Indeed, when we added FGF9 to cultured XX urogenital ridges (UGRs) at 11.5 dpc, Stra8 expression was significantly lower after 24 hr than in control XX samples (Figure 2C).
the expression of Stra8 per se, but by making germ cells less responsive to RA (Figures 2E and 2F, columns 2 and 1).

We tested also whether the augmented Stra8 expression observed in ketoconazole-treated cultures could be overcome by addition of exogenous FGF9. In XY cultures, additional FGF9 did not diminish Stra8 expression in the presence of ketoconazole, although there was some diminution in XX cultures (Figures 2E and 2F, columns 5 and 7). Presumably the different responses in the two sexes reflect the fact that XY cultures already contain endogenous FGF9 and, hence, may show no additional response when exogenous FGF9 is added.
FGF9 Regulates Sex-Specific Germ Cell Fate

FGF9 Impedes the Uprogulation of Stra8 by RA In Vivo

Our in vitro observations involving cultured genital ridges and purified germ cells suggested that FGF9 acts directly on germ cells to antagonize entry into meiosis. To investigate the role of FGF9 in this system in vivo, we next set out to analyze the effects of genetic ablation of Fgf9 in mice.

Because Stra8 expression was reduced in XY Cyp26b1 null gonads relative to XX Cyp26b1 null counterparts at 12.5 dpc despite the presence of RA in both (Figure 1A), we first wanted to test the hypothesis that additional ablation of FGF9 activity in these gonads might rescue Stra8 expression to wild-type levels. However, XY Fgf9 null mice show male-to-female sex reversal (Colvin et al., 2001a; Kim et al., 2006; Schmahl et al., 2004), and therefore germ cells are exposed to ovary-specific signals, precluding analysis of the direct role of FGF9 on germ cell development. We reasoned that loss of one copy of Fgf9 from Cyp26b1 null gonads might partially rescue the reduced Stra8 levels observed in those gonads, without affecting somatic development of the gonads.

We first confirmed that Fgf9 expression is reduced in Fgf9−/− testes, relative to wild-type testes, at 12.5 dpc (Figure 4A). We then crossed Cyp26b1−/− and Fgf9−/− animals to produce embryos of a range of genotypes and analyzed Stra8 expression in the gonads at 12.5 dpc. XY Cyp26b1+/−;Fgf9−/− germ cells expressed significantly more Stra8 at 12.5 dpc than did XY Cyp26b1−/−;Fgf9−/− germ cells (Figure 4B). In the same samples, Sox9 expression was not decreased in the XY Cyp26b1−/−;Fgf9−/− gonads, indicating that the absence of one copy of Fgf9 had not adversely affected testicular somatic development (Figure 4C). This result confirms that, in vivo, FGF9 acts to prevent germ cells from upregulating Stra8 and that this effect is independent of any sex-reversal of the gonadal soma.

In the course of these experiments, we re-examined the germ cell phenotype of Fgf9 null mice. Surprisingly, we found that in homozygous XY Fgf9-null sex-reversed ovaries, germ cell numbers were similar to those in wild-type XX ovaries at 12.5 dpc, as assessed by alkaline phosphatase staining (Figure S4A). These findings differ from a previous analysis of Fgf9 null ovaries (DiNapoli et al., 2006). The reason for the different phenotype is not clear, but may be related to differences in substrain or breeding regime between the two studies. As expected given the location of these germ cells in an ovarian somatic environment, germ cells in XY Fgf9 null ovaries were similar to XX germ cells of wild-type ovaries at 15.5 dpc for a range of markers of meiosis (Stra8, Sycp3, Dmc1, and Spo11 mRNA; γH2AX and

Figure 3. FGF9 Acts Directly on Germ Cells to Attenuate Stra8 Expression

(A) Germ cells were isolated from XY and XX pools of sexed gonads at 11.5 dpc and each population was cultured for 18 hr with FGF9 (25 ng/ml), RA (0.01 μM), or both. qRT-PCR analysis of Stra8 expression is shown. Bars represent mean and 1 SEM, n = 4 independent experiments. Mvh was used as the normalization control. The graph shows results from XY samples (left) and XX samples (right).

(B) 11.5 dpc gonads were dissociated and germ cell and somatic cell populations were purified by FACS sorting. Whole-embryo sample 11.5 dpc is included as a control. Fgfr1IIc, Fgfr2IIb, and Fgfr2IIc isoforms are expressed by germ cells (arrows).

(C) Fgfr2 (green) is observed concentrated at or near the cell surface (red, E-cadherin-positive) of germ cells in a 12.5 dpc testis.

Scale bar, 10 μm. See also Figure S3.

(FACS) at 10.5 dpc, just before colonization of the genital ridges (Takeuchi et al., 2005). The predominant FGF9 isoforms expressed by germ cells at that time point were Fgfr1-IIlc and Fgfr2-IIib. We analyzed Fgfr gene expression in FACS-sorted germ cells using 11.5 dpc gonads as the starting material (Figure 3B). Like 10.5 dpc germ cells, 11.5 dpc germ cells expressed Fgfr1-IIlc; FGF9-IIlc is a possible but weak receptor for FGF9 (Ornitz et al., 1996; Takeuchi et al., 2005). However, 10.5 dpc and 11.5 dpc germ cells differed in Fgfr2 isoform expression. Fgfr2-IIlb was the only form expressed by 10.5 dpc germ cells (Takeuchi et al., 2005), whereas 11.5 dpc germ cells also expressed Fgfr2-IIlc (Figure 4). Fgfr2-IIlb and -IIlc are alternatively spliced transcripts of the gene Fgfr2; they encode FGFR2 proteins that differ in the third loop of the Ig-like extracellular ligand-binding domain. Unlike FGFR2-IIlb, FGFR2-IIlc is a high affinity receptor for FGF9 (Eswarakumar et al., 2005; Ornitz et al., 1996). Our data suggest that germ cells undergo FGFR2 isoform switching which makes them particularly competent to respond to FGF9 after they enter the gonad.

We next examined the subcellular localization of FGFR2 in germ cells. At 12.5 dpc, the cell adhesion molecule E-cadherin is highly expressed on the surface of XY germ cells in a testicular environment. Antibodies recognizing FGFR2 were detected with a peripheral pattern of punctate staining, at and near the germ cell surface, showing some overlap with E-cadherin and some staining subjacent to the surface membrane (Figure 3C).
In the through meiosis, expression of pluripotency-associated genes germ cells express meiotic marker genes and indeed progress our analysis of FGF9 Actively Promotes Male Germ Cell Fate was carried out for both copies of when one copy of knockout and Cyp26b1 Fgf9 KO Wt het KO Wt het KO Wt het KO Cyp26b1 KO Fgf9 KO WT WT WT het het het het het het het XY XY XY XY XY XY XX XX Expn. rel. to Tbp *** Expn. rel. to Mvh ** Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tbp *** Expn. rel. to Tbp ns XY XY XY XX XY XX Expn. rel. to Tp ** Fgf9 ** Fgf9 *** K 15 dpc when RA was blocked with below-baseline expression levels for pluripotency (Oct4, Sox2) and male fate markers (Dnmt3L, Nanos2, P15). These results suggest that germ cell sexual fate is determined by the antagonist actions of two signaling factors, RA and FGF9, with RA pushing germ cells toward oogenesis and FGF9 pushing germ cells toward a male fate (Figure 6).

**DISCUSSION**

Here, we present evidence that mouse germ cell sexual fate is determined by two different signaling factors, FGF9 and RA. This work significantly extends earlier findings concerning the dominant role of RA in the induction of meiosis and the function of CYP26B1 as a male-specific meiosis-inhibiting factor. We show that FGF9 makes germ cells less responsive to RA, possibly because it forces germ cells to maintain expression of pluripotency genes and to upregulate male germ cell fate genes, and that both RA and FGF9 act directly on germ cells rather than by signaling through somatic cells. These results demonstrate that FGF9 acts as a diffusible meiosis-inhibiting substance.

The model suggested by our data is presented in Figure 6. In the ovary, where RA levels are high and FGF9 levels low, germ cells upregulate Stra8, downregulate pluripotency markers, and enter meiosis. In the developing testis, where RA is degraded by CYP26B1 and FGF9 levels are high, germ cells do not upregulate Stra8 and instead maintain expression of pluripotency markers and upregulate male germ cell fate marker genes Nanos2, Dnmt3L, and P15. Although some elements of this model have been proposed recently (Barrios et al., 2010), we greatly extend the evidence offered in that study. In particular, we base our conclusions on in vivo as well as in vitro studies and, importantly, we demonstrate a direct effect of FGF9 on germ cell sexual fate determination without the confounding effect of somatic cell influences.
Antagonism between FGFs and RA is a recurring theme in development, in each instance associated with cell lineage decisions (for review, see Diez del Corral and Storey, 2004; Niederreither and Dolle, 2008). For example, in the elongating embryo axis, FGF8 acts to maintain “stemness” in the caudal region while RA stimulates differentiation in the ventral domain (Diez del Corral et al., 2003). FGF and RA also act antagonistically during limb bud development (Mercader et al., 2000). Our study provides another example of this phenomenon: RA triggers germ cells to enter meiosis, thereby committing to a female fate, while FGF9 acts to maintain pluripotency in germ cells and to push them toward a male fate.

The finding that FGF9 plays a key role in regulating germ cell sexual fate helps clarify a number of previous observations relating to the behavior of germ cells resulting from a variety of genetic and pharmacological perturbations. In Cyp26b1 null germ cells have been removed by apoptosis, suggesting that there is something abnormal about their development (MacLean et al., 2007). Before birth, however, all of these mice, XY germ cells in a testis are exposed to RA and so express Stra8 and enter and progress through meiosis (Bowles et al., 2006; MacLean et al., 2007). The testis while FGF9 levels are high. In the ovary, CYP26B1 is highly expressed in the testis (blue) but is downregulated in the ovary (pink). Since CYP26B1 degrades endogenous RA, RA levels are low in the testis while FGF9 levels are high. In the ovary, RA is not degraded and FGF9 levels are low. RA and FGF9 both act directly on germ cells (one white cell shown) to upregulate Stra8 (RA) or to prevent its upregulation (FGF9). Stra8 expression in gonadal germ cells is essential for entry into meiosis, by an unknown mechanism. FGF9 acts directly on germ cells to antagonize Stra8 expression, maintain expression of pluripotency markers, Oct4 and Sox2, and to induce male germ cell fate markers, Nanos2, Dnmt3L, and P15.
Our findings also help to explain observations made in Nanos2 null mice (Suzuki and Saga, 2008). When Nanos2 is deleted, XY germ cells die by apoptosis. However, if apoptotic death is rescued by deletion of the Bax1 gene (which encodes a proapoptotic protein), some XY germ cells do not commit to the male program of development but instead enter meiosis, albeit about 2 days later than their XX counterparts. Presumably expression of both Fgf9 and Cyp26b1 is normal in Nanos2 null XY gonads, so the XY germ cells do not enter meiosis on schedule, i.e., with Stra8 expression beginning at 12.5 dpc as is the case for XX germ cells in a wild-type background. It seems likely that, in the absence of Nanos2, male fate is not “locked in” and, therefore, XY germ cells remain vulnerable to the influence of RA as late as 14.5–15.5 dpc, after CYP26B1 levels start to fall (Suzuki and Saga, 2008). In the wild-type situation, Nanos2 expression appears to be regulated by both FGF9 (this study; Barrios et al., 2010) and RA (this study; Barrios et al., 2010; Suzuki and Saga, 2008). It is possible that apparent repression of Nanos2 expression by RA (Barrios et al., 2010; Suzuki and Saga, 2008) reflects mutually exclusive expression of Stra8 and Nanos2 in germ cells.

Our findings likely also explain the observation that treatment of XY gonads in culture at 11.5 dpc with Brefeldin A, a potent inhibitor of secretion, causes some germ cells at one end of the gonads to embark on meiosis (Best et al., 2008). If FGF9 secretion by pre-Sertoli cells were blocked at 11.5 dpc, XY germ cells might be sensitive to RA even at concentrations that are not normally sufficient to induceStra8 expression in the testis. We would predict that any meiotic germ cells would be located at the anterior end of the gonad where RA is likely to be present at highest levels at 11.5 dpc (Bowles et al., 2006). Interestingly, during normal development, a small cohort of meiotic germ cells is observed transiently at the anterior end of the XY gonad near the mesonephros/gonad junction at 14.5 dpc: these cells are rapidly removed by apoptosis (McLaren, 1984; Yao et al., 2003).

In XX Wnt4 null gonads, germ cells begin to enter meiosis but then degenerate (Yao et al., 2004). WNT4 and FGF9 are believed to act antagonistically to orchestrate somatic sex determination and, in support of this, FGF9 protein is readily detectable in XX Wnt4−/− gonads, but not in XX Wnt4+/− littermates at 12.5 dpc (Kim et al., 2006). Hence, in a XX Wnt4 null environment, we would predict high RA levels but also high levels of FGF9. As in XY Cyp26b1 null gonads, the high RA levels likely are responsible for initiating meiosis. Based on our findings, we would predict that continued exposure to FGF9 would cause the germ cells to retain expression of pluripotency markers, a factor that may lead to their removal by apoptosis. Thus, it is likely that the germ cell phenotype observed in Wnt4 null embryos reflects an abnormal FGF9/RA balance in the gonad.

A model whereby both RA and FGF9 are involved in germ cell fate determination is compatible with previous ideas concerning the timing of meiotic susceptibility and of commitment to oogonic or spermatogenetic fates. It has been suggested that the “window of opportunity” for XY germ cells to be triggered to enter meiosis closes by about 12.5 dpc and that some XY germ cells are already committed by 11.5 dpc (Adams and McLaren, 2002). In contrast, XX germ cells are reportedly not committed until 12.5–13.5 dpc (Adams and McLaren, 2002). This suggests that XY germ cells are positively instructed by a male-specific factor to commit to spermatogenesis rather than committing to spermatogenesis because they have not encountered RA and therefore have not entered meiosis. Our results demonstrate that FGF9 expression is male specific by 11.5 dpc, early enough to actively commit germ cells to the spermatogenetic fate, thereby closing the window of opportunity to adopt a meiotic fate. Although Nanos2 is clearly involved in commitment to the spermatogenetic lineage (Suzuki and Saga, 2008), it does not appear to be expressed in XY germ cells until about 13.5 dpc (Tsuda et al., 2003). XY germ cells at 11.5 and 12.5 dpc may be refractory to meiosis because FGF9 maintains expression of genes such as Oct4 and Sox2. However, since we have shown that germ cells can enter meiosis without downregulating pluripotency genes, perhaps the most likely explanation is that FGF9 induces as yet unidentified intrinsic factors in XY germ cells and that these prevent meiotic susceptibility until NANOS2 takes over.

We provide in vitro and in vivo evidence that FGF9 acts directly on germ cells to effect maintenance of pluripotency marker expression. Such retained expression is a hallmark of early male germ cell development. We show, further, that FGF9 pushes germ cells toward a male fate, as marked by upregulated expression of a gene that appears to be key for male fate, Nanos2 (encoding an RNA-binding protein), as well as later markers, Dmnt3L (encoding a de novo methylase enzyme) and P15 (Cdkn2b, encoding an inhibitor of CDK4) in isolated germ cell culture. Although it was recently demonstrated that exogously added FGF9 is able to induce expression of Nanos2 in whole and dissociated organ culture (Barrios et al., 2010), we add substantially to that work by showing that the effect of FGF9 on germ cells is independent of any influence on somatic cells. Because each of these marker genes is more highly expressed in FGF9-treated than in control culture, it appears that FGF9 actually pushes germ cells toward a male fate, rather than merely permitting their survival in the absence of RA. Because of the complication of the role of FGF9 in somatic development of the gonad, ultimate proof that FGF9 instructs male germ cell fate in vivo will require an efficient and specific deletion of FGFR2 in fetal germ cells.

If FGF9 and RA each act as key active determinants of germ cell sexual fate, as we propose, then a significant mystery remains. In XY Cyp26b1 null gonads, germ cells robustly expressed Stra8 and entered meiosis by 13.5 dpc, despite the fact that FGF9 was presumably present at normal levels (Bowles et al., 2006; MacLean et al., 2007). This result appears to indicate, therefore, that RA is the dominant force with respect to germ cell fate determination and that FGF9 must play only a supporting role. We do not believe that such an interpretation is correct. Instead, we postulate that, in the Cyp26b1 null model, RA has an advantage over FGF9 because RA is present earlier than is normal, even compared with the wild-type XX gonad. Therefore, XY germ cells in the Cyp26b1 null robustly enter meiosis by 13.5 dpc, even in the presence of FGF9. Evidence that this hypothesis is correct comes from our earlier observation that germ cells in XX Cyp26b1 null gonads begin to express Stra8 earlier than those in wild-type XX gonads (Bowles et al., 2006).

In summary, the present study adds a new layer of understanding to the problem of germ cell sex determination by...
revealing that FGF9 plays an important role in vivo. We propose that while RA acts to push germ cells toward an oogenic fate, FGF9 acts to oppose their entry into meiosis and to push them toward a spermatogenic fate. Since RA is more abundant in the developing ovary and FGF9 is more abundant in the developing testis, the model we propose allows for reinforcement, and improves robustness, of the crucial decision of whether a germ cell commits to the oogenic or spermatogenic fate.

**EXPERIMENTAL PROCEDURES**

**Mice**

Cyp26b1-knockout mice (Yashiro et al., 2004) and Fgf9-knockout mice (Colvin et al., 2001b) were on the pure C57BL/6 background. X-linked green fluorescent protein (GFP) mice (Hadjantonakis et al., 1998) were on a random bred out-bred Swiss albino background (Quackenbush strain). Oct4×PE-eGFP studs (OG2 line) were on a CD1 background (Szabo et al., 2002). Details of embryo collection and dissection are given in the Supplemental Experimental Procedures.

**Immunomagnetic Germ Cell Isolation**

Tissues were dissociated using Cell Dissociation Buffer (GIBCO) and germ cells were isolated using magnetic sorting (MACS, Miltenyi Biotech) as described (Pesce and De Felici, 1995). For details see Supplemental Experimental Procedures.

**FACS Germ Cell Purification and Analysis of FGFR Isoform Expression**

Oct4×PE-eGFP studs were mated with wild-type CD1 females and unsexed UGR tissue collected at 11.5 dpc. Germ cell and somatic cell populations were separated by fluorescence-activated cell sorting (FACS) using standard procedures. Details for FACS, preparation of DNA and cDNA, primer sequences and conditions for PCR reactions are given in the Supplemental Experimental Procedures.

**UGR and Germ Cell Culture**

Composition of media used for culture of UGRs and germ cells, culture conditions, and details of culture additives are given in the Supplemental Experimental Procedures.

**Quantitative RT-PCR**

Relative cDNA levels were analyzed by the comparative cycle time (Ct) method of quantitative RT-PCR (qRT-PCR) with reactions including Taqman PCR master mix (Applied Biosystems, ABI) and Taqman gene expression sets or SYBR master mix (Applied Biosystems, ABI) and standard PCR primers (Sigma Aldrich). Analysis details and primer sets used for qRT-PCR are documented in the Supplemental Experimental Procedures.

**Statistical Analysis**

Asterisks highlight the pertinent comparisons and indicate level of statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001; ns = not significant). For details of analyses, see Supplemental Experimental Procedures.

**Immunohistochemistry**

Analyses were carried out on fixed, paraffin-embedded 5 μm sections using standard methods. For details see Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at doi:10.1016/j.devcel.2010.08.010.

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**REFERENCES**


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