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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 (G_0/G_1 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 (Bowles 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 NANOS2 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 testis 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*, *Dnmt3L*, 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-Saldivia 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-Saldivia 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).

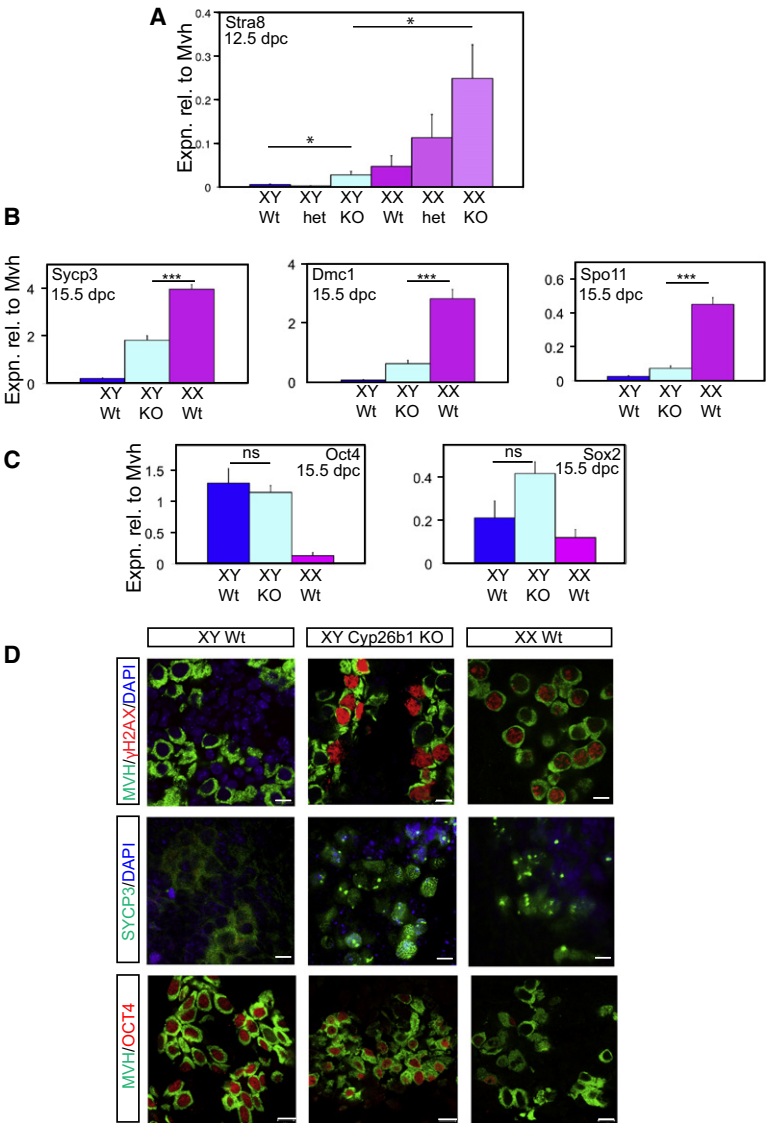

Figure 1. XY Germ Cells in the *Cyp26b1* Null Embryo Are Distinct from Both XY Germ Cells and XX Germ Cells

(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.

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

We hypothesized that FGF9 might have a role in influencing germ cell fate in the testis. *Fgf9* is initially expressed in gonads of both sexes (Kim et al., 2006), but by 11.5 dpc it is much more highly expressed in the developing testis than the ovary (Figure 2; Nef et al., 2005). A recent study has pointed to a role 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).

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 ketoconazole, 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 XX 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

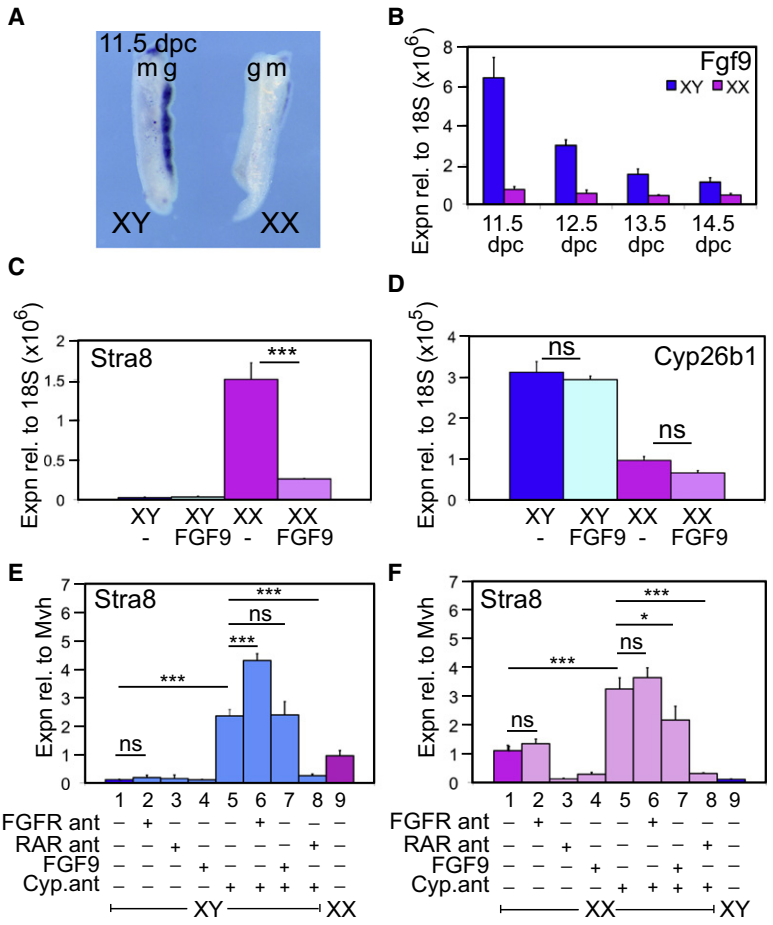

Figure 2. FGF9 Is Male-Specifically Expressed at 11.5 dpc and Affects Expression of *Stra8* in Gonadal Culture without Affecting *Cyp26b1* Expression

(A) Whole-mount in situ hybridization for *Fgf9* at 11.5 dpc in XY and XX UGR. m, mesonephros; g, gonad.

(B–D) (B) qRT-PCR analysis of *Fgf9* expression at various time points in XY and XX gonad samples. Bars represent mean + 1 SEM, n = 3 individual embryos. *18S rRNA* was used as the normalization control. UGRs (11.5 dpc) were cultured in the presence of 50 ng/ml of FGF9 for 24 hr and *Stra8* (C) and *Cyp26b1* (D) expression was analyzed by qRT-PCR. Bars represent mean + 1 SEM, n = 3 independent experiments. *18S RNA* was used as the normalization control.

(E and F) 11.5 dpc XY (E) or XX (F) UGRs were cultured for 18 hr with FGFR antagonist (FGFRant, SU5402, 5 μM), RAR antagonist (RARant, AGN193109, 5 μM), FGF9 (50 ng/ml), or ketoconazole (Cyp.ant, 10 μM) as indicated. qRT-PCR analysis of *Stra8* expression is shown (using *Mvh* as the normalization control).

Bars represent mean and 1 SEM, n = 4 or more independent experiments. See also Figure S2.

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. To address the issue of specificity of the CYP26B1 inhibitor ketoconazole, we tested the effect of treating in the presence of RAR antagonist (Figures 2E and 2F, columns 8 and 5). The effect of ketoconazole on *Stra8* expression was greatly diminished in the presence of RAR antagonist, suggesting that, as intended, the effect of ketoconazole in these cultures is to inhibit the degradation of RA. Overall, these data indicate that RA and FGF9 act in the gonad to oppositely influence expression of the meiotic gatekeeper gene, *Stra8*. Moreover, these results suggest that RA is instructive in the induction of meiosis and that the absence of FGF9 is permissive.

FGF9 Acts Directly on Germ Cells

Although FGF9 antagonized *Stra8* expression in organ culture (Figure 2C; Barrios et al., 2010), it remained possible that the effects might be mediated by somatic sex reversal, given the known role of FGF9 in maintaining *Sox9* expression (Kim et al., 2006). That is, administration of FGF9 to cultured XX gonads might upregulate *Sox9* expression, which might then cause Sertoli cell differentiation and result in a signaling cascade that inhibits germ cell meiosis. On the other hand, blockade of FGF signaling in cultured XY gonads might result in loss of *Sox9* expression and transdifferentiation of Sertoli cells, leading to a promeiotic signaling cascade. To assess the effects of FGF9 on germ cells in isolation, we purified them by immunomagnetic sorting (Pesce and De Felici, 1995) and established a serum-free germ cell culture system in order to exclude confounding effects of serum-borne RA and growth factors. Using this method, we were able to demonstrate that RA itself acts directly and dose-dependently on germ cells to induce *Stra8* expression (Figure S3).

We next tested the effect of FGF9 in isolated germ cell culture. We isolated separate XY and XX populations of germ cells from 11.5 dpc gonads and tested them in 18 hr serum-free culture. When germ cells of either population were treated with FGF9 (25 ng/ml), baseline levels of *Stra8* expression decreased after 18 hr, whereas treatment with RA (0.01 μM) strongly induced *Stra8* expression (Figure 3A). The level of *Stra8* expression was attenuated when FGF9 and RA were applied together. These experiments show that the effects of FGF9 in this system occur through direct action on germ cells, and are not indirect effects of changes to somatic cells of the gonad. Moreover, these studies demonstrate that 11.5 dpc germ cells respond to FGF9 and RA in the same manner, regardless of whether they are XX or XY or whether they have been exposed to a testicular or ovarian environment.

A corollary of this finding is that gonadal germ cells must express suitable FGF receptors (FGFRs). Previous studies have examined FGFR isoforms expressed by migratory germ cells that were purified by fluorescence-activated cell sorting

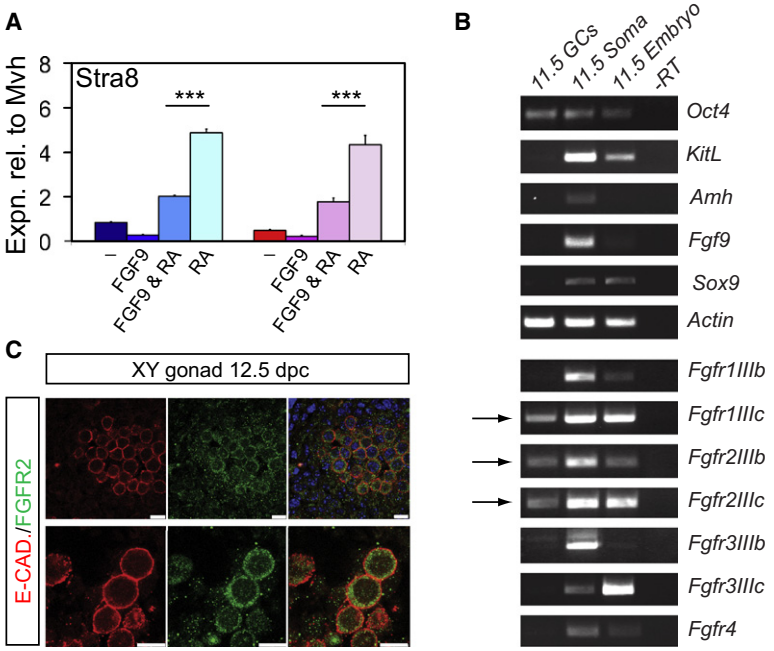

Figure 3. FGF9 Acts Directly on Germ Cells to Attenuate *Stra8* Expression

(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. *Fgfr1IIIc*, *Fgfr2IIIb*, and *Fgfr2IIIc* 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 FGFR isoform transcripts expressed by germ cells at that time point were *Fgfr1-IIIc* 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-IIIc*; FGFR1-IIIc 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-IIIb* was the only form expressed by 10.5 dpc germ cells (Takeuchi et al., 2005), whereas 11.5 dpc germ cells also expressed *Fgfr2-IIIc* (Figure 4). *Fgfr2-IIIb* and *-IIIc* 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-IIIb, FGFR2-IIIc is a high affinity receptor for FGF9 (Eswarakumar et al., 2005; Ornitz et al., 1996). Our data suggest that germ cells undergo FGFR2 isotype 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).

FGF9 Impedes the Upregulation 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*-knockout 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 mice demonstrating that 97% of germ cells in the XY null gonad undergo apoptosis between 11.5 and 12.5 dpc (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

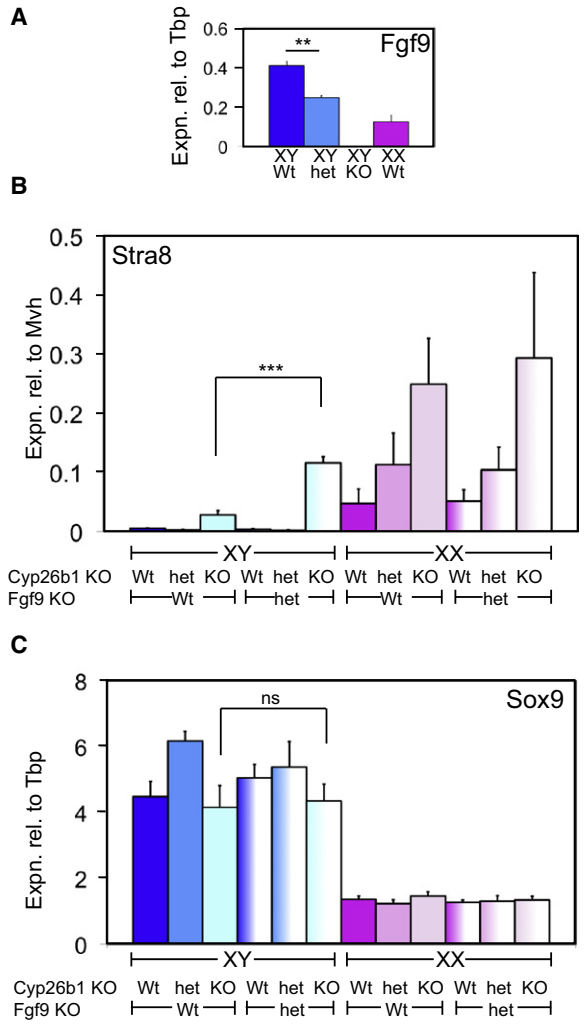

Figure 4. FGF9 Acts In Vivo to Attenuate *Stra8* Expression in the XY Gonad

UGRs were recovered at 12.5 dpc from various genotypes of *Cyp26b1*-knockout and *Cyp26b1*-knockout /*Fgf9*-knockout crosses and qRT-PCR was carried out for *Stra8* expression (using *Mvh* as the normalization control) and *Fgf9* and *Sox9* expression (using *Tbp* as the normalization control).

(A) In the *Fgf9* heterozygous XY gonads (XY het), *Fgf9* expression is significantly reduced compared with levels in the XY wild-type gonad (XY Wt).

(B) In the *Cyp26b1* null XY samples, *Stra8* expression is significantly higher when one copy of *Fgf9* is deleted (XY *Cyp26b1* KO;*Fgf9* het), than when both copies of *Fgf9* are intact (XY *Cyp26b1* KO; *Fgf9* Wt).

(C) There is no significant difference in *Sox9* expression when XY *Cyp26b1* KO;*Fgf9* het and XY *Cyp26b1* KO; *Fgf9* Wt samples are compared, indicating that *Fgf9* haploinsufficiency does not adversely affect male gonadal development at 12.5 dpc.

Bars represent mean + 1 SEM, n = 5, 8, 5, 7, 4, 4, 7, 5, 4, 5, 4, and 3 individual embryos, respectively.

SYCP3 protein), pluripotency (*Oct4*, *Sox2* mRNA; OCT4 protein), and male fate (*Nanos2*, *Dnmt3l*) (Figures S4B and S4C).

FGF9 Actively Promotes Male Germ Cell Fate

Our analysis of *Cyp26b1* null testes revealed that although XY germ cells express meiotic marker genes and indeed progress through meiosis, expression of pluripotency-associated genes is not downregulated (Figures 1C and 1D). Because retention of these markers is normally associated with the early male pathway of germ cell development, these findings raise the possibility that an additional role of FGF9 might be to actively promote male germ cell differentiation.

We tested whether FGF9 could induce expression of male cell fate marker genes when added to XX UGRs explanted at 11.5 dpc and cultured for 48 hr. FGF9 induced *Nanos2* and *Dnmt3L* expression in both XY and XX samples (Figure 5A). Conversely, when FGFRs were blocked in the XY gonad using a chemical antagonist (SU5402), *Nanos2* and *Dnmt3L* were downregulated (Figure 5B). These results are consistent with the hypothesis that FGF9 not only inhibits the initiation of germ cell meiosis but also that it actively pushes germ cells toward a spermatogenic fate.

To confirm that these effects result from direct action on germ cells and are not mediated by effects on somatic cells, we isolated XY germ cell populations at 12.5 dpc and cultured them in control media or in media containing FGF9 (25 ng/ml) or RA (0.01 μM) (Figure 5C). After 48 hr of culture, expression of *Oct4* and *Sox2* was upregulated in XY germ cells treated with FGF9. In addition, male fate markers *Nanos2*, *Dnmt3L*, and *P15* were upregulated consistently in the presence of FGF9. In contrast, treatment of 12.5 dpc XY germ cells with RA greatly induced *Stra8, Sycp3, and Dmc1* and was associated 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.

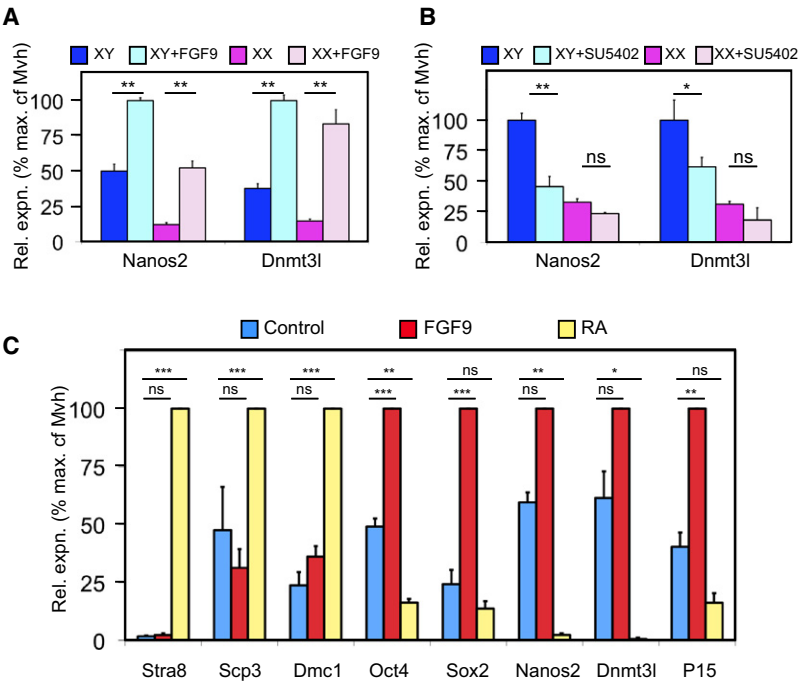

Figure 5. FGF9 Drives the Expression of Male Germ Cell Fate Markers in Gonad and Germ Cell Culture

UGRs (11.5 dpc) were cultured for 48 hr in the presence or absence of FGF9 (50 ng/ml, A) or FGFR antagonist (SU5402, 5 μM, B) and qRT-PCR was used to test expression of *Nanos2* and *Dnmt3l* (using *Mvh* as the normalization control). Bars represent mean + 1 SEM, n = 3 independent experiments. (C) XY germ cells were isolated from testes at 12.5 dpc and cultured for 48 hr in the presence of FGF9 (25 ng/ml) or RA (0.01 μM). qRT-PCR was used to test expression of meiosis markers (*Stra8*, *Sycp3*, *Dmc1*), pluripotency markers (*Oct4*, *Sox2*) and male germ cell fate markers (*Nanos2*, *Dnmt3l*, and *P15*). Bars represent mean + 1 SEM, n = 5 independent experiments. For each gene, expression is plotted relative to maximal expression for that gene (using *Mvh* as the normalization control). Statistical significance was calculated based on raw expression values from each independent experiment.

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 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). Before birth, however, all of these germ cells have been removed by apoptosis, suggesting that there is something abnormal about their development (MacLean et al., 2007). In this study, we find that XY germ cells in *Cyp26b1* null gonads do not downregulate *Oct4* and *Sox2*, as is normal for germ cells entering meiosis (Pesce et al., 1998). Our data suggest that FGF9 acts to maintain germ cell pluripotency genes in *Cyp26b* null gonads despite the fact that germ cells are in meiosis, a situation very much at odds with normal germ cell development. It may be that germ cells at the cross-roads of these mixed signals are frequently removed by apoptosis. The postulated "antagonism" between the testis and meiotic germ cells suggested by other studies (McLaren, 1984; Wai-sum and Baker, 1976; Vigier et al., 1987; Yao et al., 2003) may reflect a situation similar to that observed in the *Cyp26b1* null testis, with both meiotic and pluripotency markers being expressed simultaneously in germ cells and leading to their removal by apoptosis.

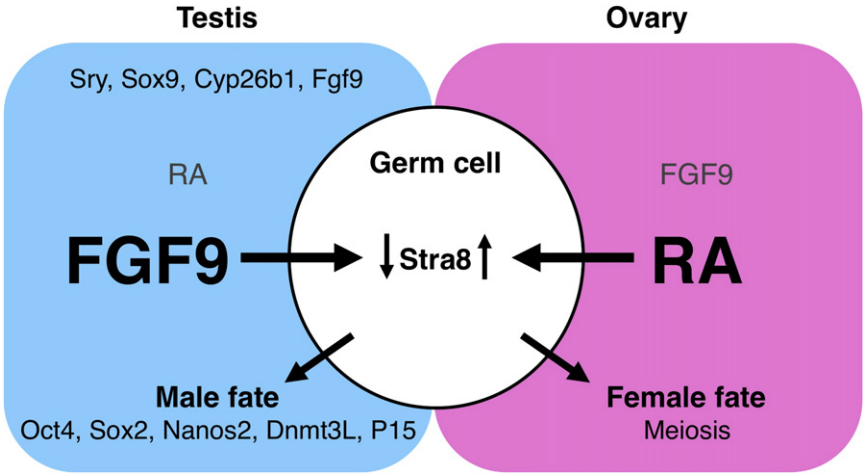

Figure 6. RA and FGF9 Act Antagonistically to Determine Germ Cell Fate

Germ cell sexual fate is determined by two signaling molecules produced by the somatic cells of the gonad, FGF9 and RA. *Cyp26b1* and *Fgf9* are highly expressed in the testis (blue) but are 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 induce *Stra8* 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 oogenic or spermatogenic 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 spermatogenic fate, thereby closing the window of opportunity to adopt a meiotic fate. Although *Nanos2* is clearly involved in commitment to the spermatogenic 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, *Dnmt3L* (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 exogenously 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). Oct4ΔPE: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

Oct4ΔPE: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 RNA 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 (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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