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FGF9 promotes survival of germ cells in the fetal testis

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In addition to its role in somatic cell development in the testis, our data have revealed a role for *Fgf9* in XY germ cell survival. In *Fgf9*-null mice, germ cells in the XY gonad decline in numbers after 11.5 days post coitum (dpc), while germ cell numbers in XX gonads are unaffected. We present evidence that germ cells resident in the XY gonad become dependent on FGF9 signaling between 10.5 dpc and 11.5 dpc, and that FGF9 directly promotes XY gonocyte survival after 11.5 dpc, independently from Sertoli cell differentiation. Furthermore, XY *Fgf9*-null gonads undergo true male-to-female sex reversal as they initiate but fail to maintain the male pathway and subsequently express markers of ovarian differentiation (*Fst* and *Bmp2*). By 14.5 dpc, these gonads contain germ cells that enter meiosis synchronously with ovarian gonocytes. FGF9 is necessary for 11.5 dpc XY gonocyte survival and is the earliest reported factor with a sex-specific role in regulating germ cell survival.

KEY WORDS: *Fgf9*, Fetal testis, Gonad, Germ cell

INTRODUCTION

The mammalian gonad provides a unique model system because it is the only organ primordium that forms and then chooses between two fates, ovary (female) or testis (male). This choice is regulated by the Y-linked gene *Sry*; mammals with *Sry* develop as males, while ones without *Sry* develop as females (Lovell-Badge and Robertson, 1990; Koopman et al., 1991; Gubbay et al., 1992). Although *Sry* expression is initiated at 10.5 days post coitum (dpc), the gonad is still highly plastic at 11.5 dpc in mice and is capable of ovarian or testicular differentiation (Tilmann and Capel, 1999). By 12.0 dpc, gonads that expressed *Sry* have initiated the male pathway and are morphologically distinct from female gonads. Although no direct downstream targets of *Sry* have been definitively identified, upregulation of a number of genes, including *Sry*-like HMG-box protein 9 (*Sox9*) (Wright et al., 1995; da Silva et al., 1996), desert hedgehog (*Dhh*) (Bitgood et al., 1996; Yao et al., 2002), platelet derived growth factor receptor α (*Pdgfra*) (Brennan et al., 2003), anti-Müllerian hormone (*Amh*) (Behringer et al., 1994) and fibroblast growth factor 9 (*Fgf9*) (Colvin et al., 2001), occurs in somatic cells of the XY gonad. Interestingly, expression of either *Sry* or *Sox9* in gonadal somatic cells is sufficient to induce the differentiation of Sertoli cells (Koopman et al., 1991; Bishop et al., 2000; Vidal et al., 2001). Sertoli cells are a male-specific somatic cell type that drive testis development and organize male morphology by sequestering germ cells inside testis cords (reviewed by McLaren, 1991; Brennan and Capel, 2004).

In mice, primordial germ cells (PGCs) are specified at the base of the allantois around 7.5 dpc (Chiquoine, 1954; Mintz and Russell, 1957; Ginsburg et al., 1990; Tam and Zhou, 1996; Saitou et al., 2002). From there, they divide, migrate through the gut mesentery and enter the forming gonad between 10.0 and 11.5 dpc (Ginsburg et al., 1990; Anderson et al., 2000). In the early stages of their development, mouse XX and XY germ cells behave in an identical manner with respect to their formation, proliferation and migration (reviewed in Wylie, 1999; McLaren, 2003; Molyneaux et al., 2004). Once in the gonad, PGCs are termed gonocytes and become the precursors of oogonia in the adult ovary or spermatogonia in the adult testis. Moreover, when gonocytes find themselves in a gonad of the alternate sex, they conform to the sex of the gonad (Adams and McLaren, 2002). Gonocytes number ~3000 at 11.5 dpc (Tam and Snow, 1981) and continue to divide mitotically until 13.5 dpc when they enter meiosis in ovaries or mitotic arrest in testes (McLaren, 2000; Adams and McLaren, 2002) (reviewed by McLaren, 2003). Gonocyte mitotic arrest in testes is known to be dependent upon masculinizing interactions between germ cells and somatic cells in XY gonads (reviewed by McLaren, 2003). Gonocyte entry into meiosis in ovaries has been considered to be cell-autonomous, although it should be noted that recent evidence suggests that this process may be influenced by somatic cues (Menke et al., 2003; Koubova et al., 2006).

As there are no apparent differences in the rate of proliferation between XX and XY gonocytes between 10.5 and 13.5 dpc (reviewed by McLaren, 2003; Schmahl and Capel, 2003), it had been assumed that gonocyte survival and proliferation were under similar regulatory control at early stages. However, the mouse *Vasa* homolog (*Mvh*; *Ddx4* – Mouse Genome Informatics) was recently shown to differentially affect gonocyte proliferation in XY gonads between 11.5 and 12.5 dpc (Tanaka et al., 2000). This indicates that although no phenotypic difference has been detected in germ cells at these stages, the underlying pathways regulating them are different.

Fibroblast growth factors (FGFs) are known to play a role in the proliferation and survival of many cell types (reviewed by Ornitz and Itoh, 2001). *Fgf9*-null mutant mice die shortly after birth owing to defects in lung formation, but XY embryos also present with phenotypic male-to-female sex-reversal (Colvin et al., 2001). Although, *Fgf9* is expressed in XX and XY gonads at 11.5 dpc, it becomes XY specific by 12.5 dpc and is expressed throughout testis cords (Schmahl et al., 2004). Testis differentiation is disrupted in *Fgf9* mutants as the result of a failure of Sertoli precursors to proliferate and differentiate into Sertoli cells (Schmahl et al., 2004).

Here, we show that, although *Sry* is initially expressed, testis differentiation is aborted in XY *Fgf9* gonads, and the majority of germ cells (97%) are lost through cell death by 12.5 dpc. We also demonstrate that, XY $Fgf9^{-/-}$ gonads begin to express ovarian markers by 12.5 dpc and contain meiotic gonocytes at 14.5 dpc. Our data indicate that FGF9 acts independently of its role in Sertoli cell differentiation to promote germ cell survival in XY, but not XX, gonads.

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MATERIALS AND METHODS

Staging and genotyping embryonic gonads

For timed matings, noon on the day following mating was scored as 0.5 days post coitum (0.5 dpc). Sex of gonads prior to visible differentiation (before 12.5 dpc) was determined by staining of Barr bodies as previously described (Palmer and Burgoyne, 1991). For more precise staging, the number of tail somites (ts) between the hind limb and the tip of the tail were counted at the time of dissection (Hacker et al., 1995). Using this method, 10.5 dpc corresponds to 8 ts, 11.5 dpc to 18 ts and 12.5 dpc to 30 ts. Genotypes were determined by PCR as previously described for *Fgf9* (Colvin et al., 2001), *Oct4:GFP* (Anderson et al., 1999) and XX*Sry*^{Myc} (Sekido et al., 2004). Mice carrying the *Fgf9* deletion are maintained on the C57BL/6 J background as described by Schmahl et al. (Schmahl et al., 2004). *Oct4:GFP* mice are maintained on the C57BL/6 J background. XX*Sry*^{Myc}; *Fgf9* mice are maintained on a mixed DBA; C57BL/6 J background. On this mixed background, XX*Sry*^{Myc}; *Fgf9*$^{-/-}$ mice fail to form testis cords.

Alkaline phosphatase staining and germ cell counting

The alkaline phosphatase staining procedure was modified from Lawson et al. (Lawson et al., 1999). Briefly, embryos or genital ridges were dissected and fixed in 4% paraformaldehyde for 2 hours at 4°C, rinsed in PBS and then placed in 70% ethanol for over 2 hours with rocking at room temperature. Samples were then stained using α-naphthyl phosphate/Fast Red TR (Sigma F-8764) (Ginsburg et al., 1990) and cleared in 70% glycerol. Squash preps of embryos were made and total germ cells were counted using a cell counter. For later stages, samples were sliced into several pieces which were then made into squash preps. The number of germ cells per genotype was compared using the Student's *t*-test.

Organ and gonocyte culture

Organ cultures were performed as previously described (Martineau et al., 1997). Briefly, genital ridges were cultured at 37°C with 5% CO_2/95% air on a 1.5% agar block in Dulbecco's Minimal Eagle Medium (DMEM), supplemented with 10% fetal calf serum (BioWhittaker, Lot # 013987) and 50 μg/ml ampicillin. Human recombinant FGF9 (25 ng/ml, R&D Systems) was added to the culture medium as designated. An equivalent volume of carrier (PBS) was added to control organ cultures. The number of germ cells and Sertoli cells was compared using the Student's *t*-test. Gonocytes were purified for culture using a protocol modified from MacKay et al. (MacKay et al., 1999) with the supernatant taken in lieu of the pellet. Briefly, gonads from *Oct4:GFP* (Anderson et al., 2000) animals separated from mesonephroi were washed in Ca^{2+}- and Mg^{2+}-free Hanks Buffer without Phenol Red. XX and XY gonads were pooled separately (between 2 and 16 each per litter), then enzymatically disrupted using 0.025% collagenase and 0.025% trypsin at 37°C for 5 minutes. Gonads were washed twice in Ca^{2+}- and Mg^{2+}-free Hanks Buffer without Phenol Red and mechanically dispersed using a pipette. This solution was centrifuged for 1 minute at 100 ***g***. The supernatant was removed and re-spun at 100 ***g*** three more times. The final spin at 1000 ***g*** for 3 minutes pelleted the germ cells. Supernatant was removed and germ cells were resuspended in the desired volume of DMEM with or without FGF2, FGF7, FGF9 or FGF16 (233-FB, 251-KG, 273-F9 and 1212-FG respectively; R&D Systems). Two gonad equivalents of germ cells were used for each timepoint. Gonocytes were placed into culture within 1 hour of genital ridge dissection. Roughly 20-30% of preparations were at least 95% pure gonocytes. Purity was confirmed by GFP expression examined using a fluorescent microscope. The WST-1 reagent (Roche) was used per instructions as a metabolic indicator. Conversion of WST-1 was measured using an ELISA plate reader (BioRad). Briefly, WST-1 is added to culture wells to a final concentration of 10% WST-1. Wells are then incubated for 2 hours at 37°C and subsequently measured for absorbance. WST-1 conversion of the samples was compared using ANOVA.

Reverse transcriptase PCR (RT-PCR) and quantitative PCR (QPCR)

Gonads were separated from mesonephroi and RNA was isolated from the gonads alone using RNeasy (Qiagen). Reverse transcription reactions were performed using the IScript cDNA Synthesis Kit (BioRad). The BioRad MyiQ iCycler was used per manufacturer's instructions. The iCycler software was used for QPCR data analysis. Primers for *Sry* were AGTTCCATGACCACCACCAC and ATGGAACTGCTGCTTCTGCT; primers for hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) were TGGACTGATTATGGACAGGACTGAA and TCCAGCAGGTCAGCAAAGAACT. *Sry* expression was normalized to *Hprt1* and compared using the Student's *t*-test.

Immunofluorescence and in situ hybridization

Gonad/mesonephroi complexes were dissected or removed from culture and fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS, and blocked in PBS with 5% BSA, 1% heat-inactivated goat serum and 0.1% Triton X-100 for 1 hour at room temperature before staining with antibodies. For immunohistochemistry, antibodies against PECAM (Pharmingen; 1:500 dilution), E-Cadherin (Zymed; 1:200), SOX9 (the kind gift of Francis Poulat, 1:1000), γH2AX (the kind gift of William Bonner, 1:800) or laminin (the kind gift of Harold Erikson, 1:500) were added to the blocking solution and were incubated rocking at 4°C overnight. Samples were rinsed three times for 30 minutes in PBT (PBS and 0.1% Triton X-100) with 5% BSA and 0.1% heat-inactivated goat serum, and incubated overnight at 4°C in blocking solution with Cy2-, Cy3- or Cy5-conjugated secondary antibodies (1:500; Jackson Laboratories). Samples were washed three times for 30 minutes in PBT and mounted in DABCO for confocal microscopy with a Zeiss LSM 410 laser-scanning confocal microscope. LysoTracker Red DND-99 (Molecular Probes) was used to measure cell death per instructions. Briefly, gonads were dissected and placed into 1 ml DMEM with 5 μl of 1 mM LysoTracker Red DND-99 for 30 minutes. They were then washed four times in PBS for 30 minutes per wash and then fixed and processed for immunohistochemistry. For LysoTracker and γH2AX, the total number of positive and/or negative cells per gonad was counted via optical sectioning

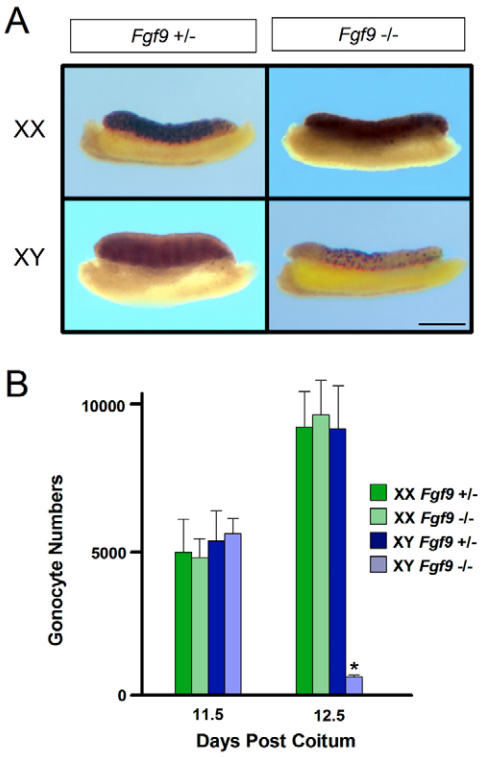

Fig. 1. Analysis of germ cell loss in *Fgf9*$^{-/-}$ gonads. (**A**) Alkaline phosphatase staining for germ cells in *Fgf9* mutant gonads at 12.5 dpc. XY *Fgf9*$^{-/-}$ gonads show a severe reduction in germ cell numbers relative to siblings. (**B**) Quantification of germ cell loss. Germ cell numbers are similar in mutants and controls from 7.5 to 11.5 dpc (11.5 dpc shown). At 12.5 dpc XY *Fgf9*$^{-/-}$ gonads contain significantly fewer germ cells than XY *Fgf9*$^{+/-}$, XX *Fgf9*$^{+/-}$ or XX *Fgf9*$^{-/-}$ (n=14 for each timepoint; P<0.01). Scale bar: 250 μm.

and compared using the Student's *t*-test. Whole-mount in situ hybridization was performed as previously described using NBT/BCIP (Wilkinson and Nieto, 1993) with probes for *Bmp2* and *Fst* (Yao et al., 2004).

RESULTS

Fgf9$^{-/-}$ gonads display an XY specific germ cell loss

Based on a preliminary examination of XY *Fgf9*-null gonads, which contained reduced germ cell numbers by 12.5 dpc, we performed alkaline phosphatase staining to assay for the presence or absence of germ cells at early stages of development. Tissue non-specific alkaline phosphatase (TNAP) is expressed by migratory (PGCs) and post-migratory germ cells (gonocytes) (Chiquone, 1954; Lawson and Hage, 1994). At 12.5 dpc, gonocyte numbers appeared qualitatively normal in controls and in the XX *Fgf9*$^{-/-}$ gonads; however, germ cell numbers were greatly reduced in the XY *Fgf9*$^{-/-}$ mutants (Fig. 1A). We then quantified this loss by counting total gonocyte numbers in the *Fgf9* mutants from stages 7.5 to 12.5 dpc (Fig. 1B, stages 11.5-12.5 dpc shown). Germ cell numbers were similar to expected values (Tam and Snow, 1981; Lawson and Hage, 1994; McLaren, 2000) in mutants and controls through 11.5 dpc. However, by 12.5 dpc gonocyte numbers were significantly reduced in XY *Fgf9*-null mutants compared with controls and 11.5 dpc XX null mutants (P<0.001). The number of gonocytes per embryo declined from a maximum at 11.5 dpc and reached a plateau at 389±31.3 after 12.5 dpc (Fig. 1B). We then examined later stages and observed no further decrease in germ cell numbers through 16.5 dpc (data not shown).

XY *Fgf9*$^{-/-}$ germ cells display elevated levels of cell death

Although gonocyte proliferation is reduced in XY *Fgf9*$^{-/-}$ gonads (Schmahl et al., 2004), cell counts indicated that numbers of gonocytes actually declined between 11.5 dpc and 12.5 dpc. This indicated that the gonocyte phenotype cannot be due solely to a defect in proliferation. Using LysoTracker Red DND-99 (Molecular Probes), which detects increased cellular pH associated with cell death, we investigated whether the XY *Fgf9*-null gonads had increased levels of cell death at 11.5, 12.0 and 12.5 dpc (Fig. 2). Cells in XX and XY *Fgf9*$^{+/-}$ and XX *Fgf9*$^{-/-}$ gonads have a very low basal level of cell death between 11.5 and 12.5 dpc (Fig. 2A-I). XY *Fgf9*$^{-/-}$ gonads show greater numbers of dying germ cells than XY controls at these three stages (Fig. 2J-L). The maximum level of germ cell loss is seen in XY *Fgf9*$^{-/-}$ gonads at 12.0 dpc (Fig. 2K) and returns to basal levels after 12.5 dpc (data not shown). We detected no somatic cell death in these gonads between 11.5 dpc and 12.5 dpc.

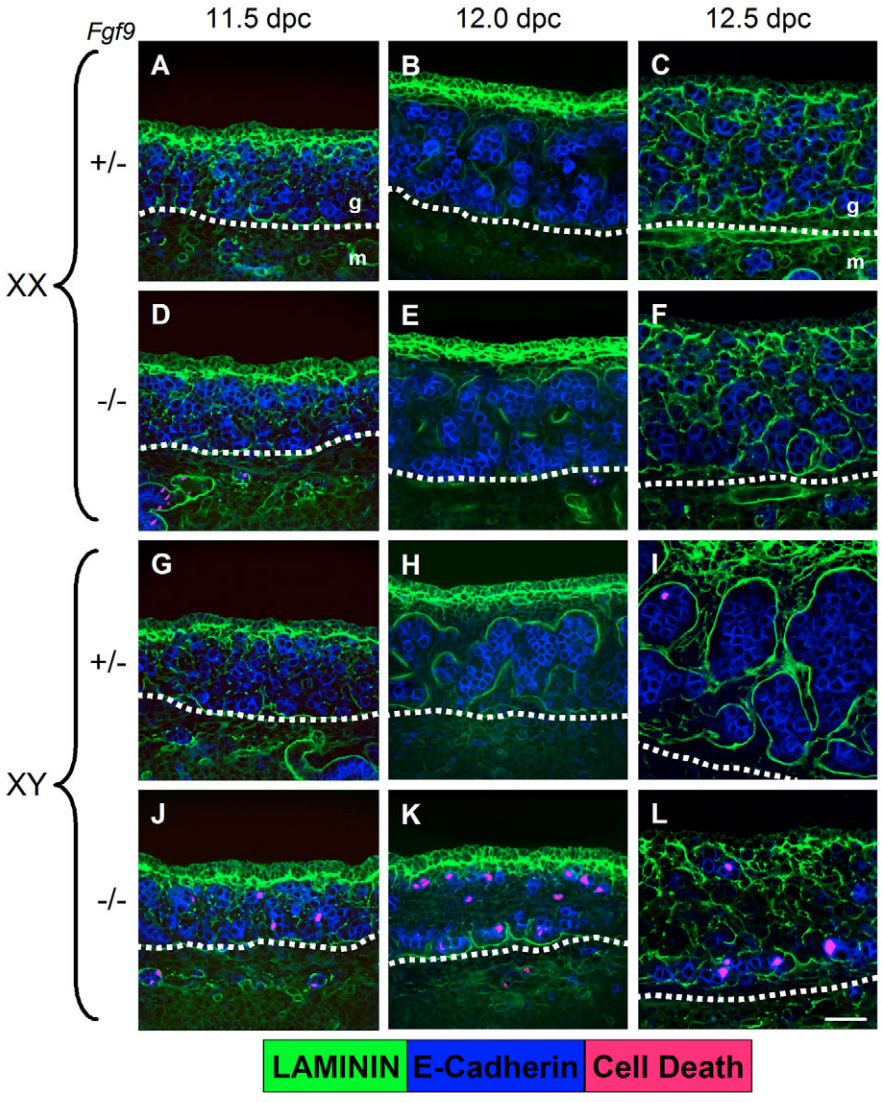

Fig. 2. XY *Fgf9*$^{-/-}$ gonads show elevated levels of germ cell death between 11.5 and 12.5 dpc. Germ cells are stained for E-Cadherin (blue), whereas laminin (green) labels the basal lamina of testis cords. Cell death is labeled by LysoTracker Red DND-99 (red). XX and XY *Fgf9*$^{+/-}$ gonads show low basal levels of cell death at both 11.5 dpc (**A**,**G**), 12.0 dpc (**B**,**H**) and 12.5 dpc (**C**,**I**). XX *Fgf9*$^{-/-}$ gonads contain similar low levels at 11.5 dpc (**D**), 12.0 dpc (**E**) and 12.5 dpc (**F**). XY *Fgf9*$^{-/-}$ gonads show significantly increased levels of dying germ cells at stages 11.5 to 12.5 dpc (**J-L**; P<0.05. g, gonad; m, mesonephros. Broken line indicates boundary of gonad and mesonephros. Figures are representative of three independent experiments of n≥3 each. Scale bar: 50 μm.

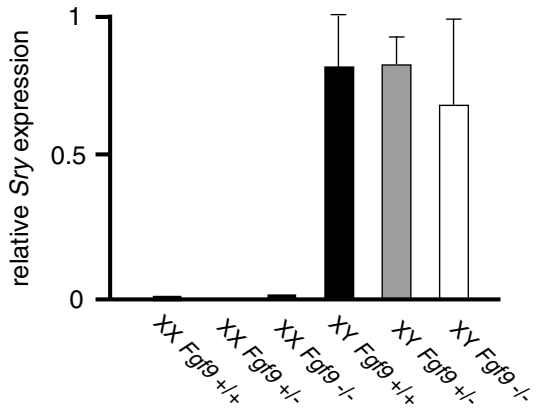

Fig. 3. XY *Fgf9*$^{-/-}$ gonads initiate male development. Quantitative PCR (QPCR) of *Sry* in 11.5 dpc XX and XY *Fgf9*$^{+/-}$ and$^{-/-}$ gonads. XY *Fgf9*$^{-/-}$ gonads express *Sry* at a similar level to XY *Fgf9*$^{+/-}$ gonads. *n*=4 for XX *Fgf9*$^{+/+}$, $^{+/-}$ and $^{-/-}$. *n*=4 for XY *Fgf9*$^{+/+}$ and $^{+/-}$. *n*=3 for XY *Fgf9*$^{-/-}$.

XY *Fgf9*$^{-/-}$ gonads express markers of ovarian development

Because XY *Fgf9*$^{-/-}$ animals do not form testes, we asked whether male sex determination was initiated in their gonads. To examine this, we assayed for expression of *Sry* in 11.5 dpc XY *Fgf9*$^{-/-}$ gonads using reverse transcriptase PCR (RT-PCR). *Sry* is expressed in 11.5 dpc XY *Fgf9*$^{-/-}$ and $^{+/-}$ gonads and not in XX samples (data not shown). We then used quantitative PCR (QPCR) to determine if *Sry* is expressed at normal levels in XY *Fgf9*$^{-/-}$ gonads. We found no significant difference in *Sry* expression levels between XY *Fgf9*$^{-/-}$, $^{+/-}$ or $^{+/+}$ gonads (Fig. 3).

Although *Sry* is initially expressed in XY *Fgf9* nulls, testis development is disrupted by 12.0 dpc (Fig. 2K). Therefore, we hypothesized that XY *Fgf9*$^{-/-}$ gonads might express female markers suggesting full sex-reversal to the ovarian pathway. Bone morphogenetic protein 2 (*Bmp2*) and follistatin (*Fst*) are markers of ovarian differentiation that are specific to XX gonads at 12.5 dpc (Yao et al., 2003). In situ hybridization revealed normal expression of *Bmp2* and *Fst* in 12.5 dpc XX *Fgf9*$^{+/-}$ and $^{-/-}$ gonads (Fig. 4A-D). XY *Fgf9*$^{+/-}$ gonads express neither of these markers at 12.5 dpc (Fig. 4E,F). However, 12.5 dpc XY *Fgf9*$^{-/-}$ gonads express both ovarian markers (Fig. 4G,H), indistinguishable from XX genotypes.

Because at least part of the ovarian pathway is active by 12.5 dpc in XY *Fgf9* nulls, we asked if the surviving primordial germ cells entered meiosis as they would in a wild-type XX gonad. Phosphorylated γH2AX is a marker for germ cells in the leptotene stage of meiosis (Mahadevaiah et al., 2001) and is visible at 14.5 dpc in wild-type XX gonads (Yao et al., 2003). Immunostaining for γH2AX revealed meiotic germ cells in XX *Fgf9*$^{+/-}$ gonads but not in XY *Fgf9*$^{+/-}$ gonads at 14.5 dpc (Fig. 4I,J respectively) as predicted. In XY *Fgf9*$^{-/-}$ gonads at 14.5 dpc, 47% of the XY germ cells were positive for phosphorylated γH2AX (Fig. 4K), indicating that some of the surviving gonocytes respond to an ovarian signaling environment and are competent to enter meiosis. By in situ hybridization, XY *Fgf9*$^{-/-}$ gonads also express the meiotic markers *Stra8* and *Dmc1* (Menke et al., 2003) at 14.5 dpc (data not shown).

Time-dependant requirement for FGF9

In XY *Fgf9*$^{-/-}$ gonads, markers of Sertoli cell differentiation are not expressed at 12.5 dpc (Colvin et al., 2001; Schmahl et al., 2004). As Sertoli cells are known to be important for the development of germ

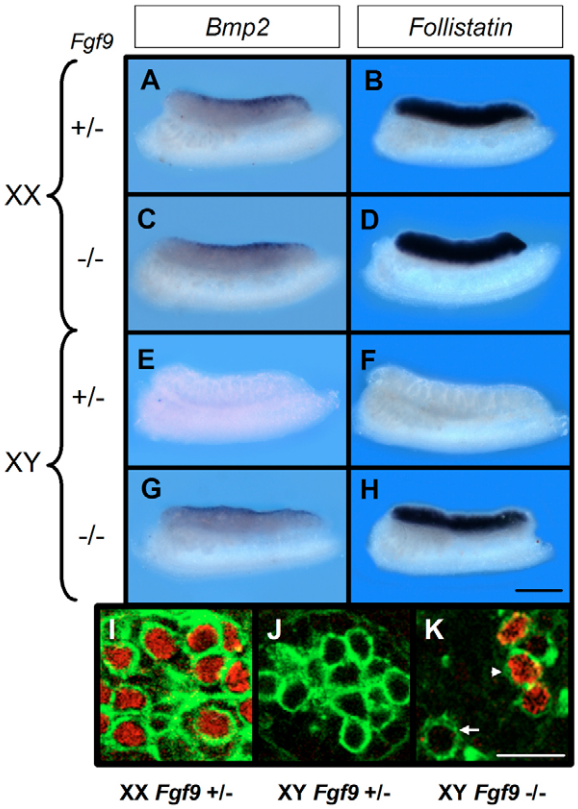

Fig. 4. 12.5 dpc XY *Fgf9* null gonads express markers of ovarian development. In situ hybridization using markers of ovarian fate *Bmp2* (A,C,E,G) and follistatin (B,D,F,H) on 12.5 dpc XX (A-D) and XY (E-H) gonads of either *Fgf9*$^{+/-}$ (A,B,E,F) or *Fgf9*$^{-/-}$ (C,D,G,H) genotypes. All XX gonads express *Bmp2* and follistatin normally (**A-D**). XY *Fgf9*$^{+/-}$ samples do not express either of these ovarian markers (**E**,**F**). At 12.5 dpc, XY *Fgf9*$^{-/-}$ gonads express both *Bmp2* and follistatin (**G**,**H**) in a pattern similar to XX gonads. Sections of 14.5 dpc XX and XY gonads containing germ cells immunostained for PECAM (green) and phosphorylated γH2AX (red) (**I-K**). Virtually all XX Fgf9$^{+/-}$ germ cells are positive for phosphorylated γH2AX staining, as expected (I), whereas XY Fgf9$^{+/-}$ germ cells are not (J). Forty-seven percent of the germ cells that survive to this stage in XY Fgf9$^{-/-}$ gonads are positive for phosphorylated γH2AX (arrowhead in K), but some are not (arrow in K). Figures representative of three independent experiments of *n*⩾5 (A-H) each and *n*=5 (I-K) each. Scale bar: 250 μm in A-H; 25 μm in I,K.

cells in the testis, we reasoned that the loss of germ cells in *Fgf9* mutants could be the indirect result of Sertoli cell loss. Preliminary experiments had shown that Sertoli cells can be partially rescued in *Fgf9* mutant XY gonads by explanting at 11.5 dpc and culturing for ~24 hours in DMEM and 10% FBS (discussed below). However, we found that even when Sertoli cells are rescued in early explant cultures, there is no rescue of the germ cells. This implies that *Fgf9*$^{-/-}$ Sertoli cells are not sufficient to promote gonocyte survival.

To determine whether FGF9 acts directly to support germ cells in the XY gonad, we cultured different stages of XY *Fgf9*$^{-/-}$ gonads in the presence or absence of exogenous FGF9. Uncultured 12.5 dpc XY *Fgf9*$^{-/-}$ gonads are gonocyte deficient, contain no Sertoli cells and display no male specific structures (Fig. 5A; compare with Fig. 5B). When *Fgf9*$^{-/-}$ gonads were explanted at 11.25 dpc and cultured, Sertoli cell rescue was robust, as indicated by SOX9 staining. Additionally, testis-specific vasculature was also present as were testis cord-like formations (arrow and arrowhead, respectively, in

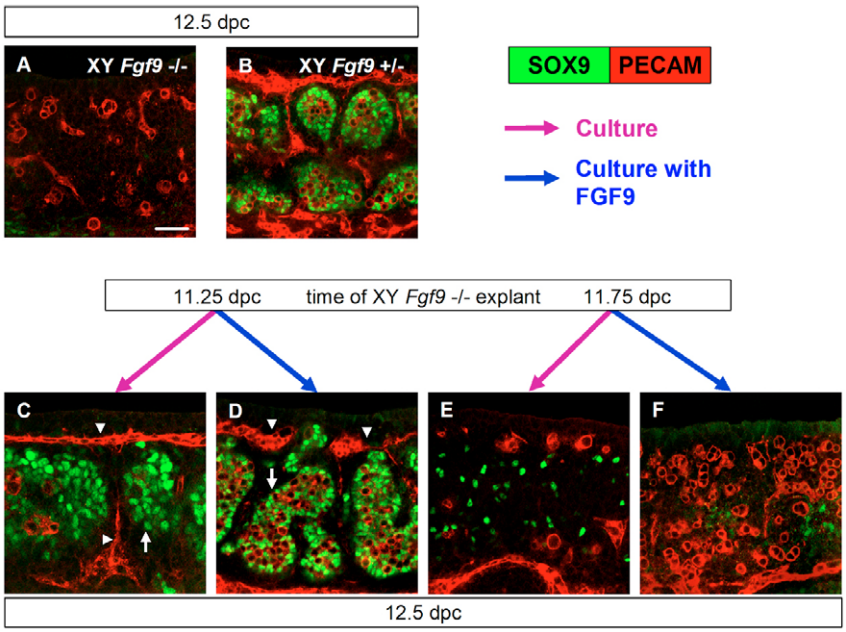

Fig. 5. Time-dependent exposure to culture medium with or without exogenous FGF9 reveals distinct effects of FGF9 on Sertoli and germ cells. Cultured gonads were labeled with antibodies against SOX9 to detect Sertoli cells (green) and PECAM to detect germ cells and vasculature (red). Blue and purple arrows indicate stages at which samples were placed into culture. (**A**) Uncultured XY *Fgf9*$^{-/-}$ control with few germ cells, no SOX9-positive cells and no male-specific vasculature. (**B**) *Fgf9*$^{+/-}$ 12.5 dpc XY gonad cultured without FGF9 for comparison. (**C**) XY *Fgf9*$^{-/-}$ gonad cultured without FGF9 from 11.25 to 12.5 dpc shows few germ cells and many SOX9-positive cells. Some male structures are rescued including testis cord-like organization (arrow) and male-specific vasculature (arrowheads); however, germ cell numbers are not rescued compared with *Fgf9*$^{+/-}$ (B). (**D**) When 25 ng/ml FGF9 is added to the culture medium of Fgf9$^{-/-}$ gonads explanted at 11.25 dpc and cultured to 12.5 dpc, germ cells are rescued as well as testis cord formation (arrow) and male-specific vasculature (arrowhead). (**E**) XY *Fgf9*$^{-/-}$ gonad cultured from 11.75 to 12.5 dpc shows few germ cells, few SOX9-positive cells and no male-specific vasculature or cord formation. (**F**) XY *Fgf9*$^{-/-}$ gonad cultured from 11.75 to 12.5 dpc in the presence of 25 ng/ml FGF9. Germ cell numbers are rescued but other features of testis development are not. SOX9-positive cells are reduced or absent. Figures representative of three independent experiments, n≥5 each. Scale bar: 50 μm.

Fig. 5C). However, despite the rescue of Sertoli cells in these gonads, there was still no rescue of germ cells (Fig. 5C). When XY *Fgf9*$^{-/-}$ gonads were explanted and cultured from 11.75 to 12.5 dpc, they did contain a few Sertoli cells; however, neither gonocytes nor testis morphology was rescued (Fig. 5E).

In *Fgf9*$^{-/-}$ XY gonads explanted at 11.25 dpc and cultured to 12.5 dpc in the presence of human recombinant FGF9 (25 ng/ml; R&D Systems) Sertoli cells, gonocytes, and testis morphology were all rescued (Fig. 5D; compare to 5B). By contrast, *Fgf9*$^{-/-}$ gonads explanted 12 hours later at 11.75 dpc, and cultured in the presence of FGF9 to 12.5 dpc showed no testis morphology and few, if any, Sertoli cells (Fig. 5F). Notably, however, gonocyte numbers were rescued. This effect was dose dependent as lower concentrations of FGF9 resulted in fewer gonocytes in the cultured mutant gonads (data not shown). There was no significant difference in Sertoli cell numbers in 11.75 dpc samples treated with or without FGF9. Germ cell numbers and gonad morphology were unchanged by any of the above culture procedures in XX *Fgf9*$^{+/+}$, $^{+/-}$ or $^{-/-}$ gonads (data not shown).

FGF9 promotes gonocyte survival in vitro at 11.5 dpc but not at 10.5 dpc

To examine whether FGF9 could act directly on germ cells in the absence of Sertoli or other somatic cells, we tested whether exogenous FGF9 could promote the survival of purified XY gonocytes in culture. Gonocytes from 10.5 dpc XX or XY gonads of *Oct4:GFP* transgenic embryos (Anderson et al., 2000) were removed and purified by differential centrifugation. In these animals, germ cells express green fluorescent protein (GFP). The purity of gonocyte preparations was determined by visualization of the proportion of GFP-expressing cells. Samples with less than 95% gonocytes were discarded. Survival of gonocytes was quantitatively determined by mitochondrial activity using the WST-1 reagent (Roche) and measured with an ELISA plate reader. 46% of 10.5 dpc XX and XY gonocytes cultured in only DMEM were still metabolically active after 18 hours in culture (Fig. 6A). After 36 hours in culture, the survival had dropped to under 15%. XX and XY gonocytes (10.5 dpc) had similar survival patterns when cultured in DMEM supplemented with FGF9 (25 ng/ml) (Fig. 6A). Comparable results were obtained from 11.5 dpc XX, XY and FGF9-treated XX gonocytes (Fig. 6B). However, 83% of 11.5 dpc XY gonocytes treated with FGF9 were still metabolically active after 18 hours, and ~43% after 36 hours (Fig. 6B). These results indicate that 11.5 dpc XY (but not XX) gonocyte survival significantly increased when treated with FGF9 (P<0.001).

It was possible that exogenous FGF9 was delaying or masking cell death in XY gonocyte cultures by inducing proliferation. To exclude this possibility, we assayed for the presence of phosphorylated Histone H3 (pHH3) as a marker of proliferation. pHH3 was never observed in FGF9-treated gonocytes (data not shown). Consistent with this finding, clonal expansion of gonocytes was never visually observed (data not shown).

Gonocyte requirement for FGF9 is non-cell autonomous

These data indicate that a transition to dependence on FGF9 occurs in XY gonocytes between 10.5 and 11.5 dpc. This could be a cell-autonomous effect of the Y-chromosome in gonocytes or it could be a dependence induced by the male somatic environment. To investigate this question, we explanted gonocytes from XX mice carrying an *Sry* transgene (XX*Sry*^{Myc}) (Sekido et al., 2004). Expression of the *Sry* transgene in Sertoli precursors leads to the development of a testis in XX*Sry*^{Myc} mice. However, the *Sry* transgene is not expressed in germ cells. Therefore, in these animals XX germ cells first encounter a male somatic environment when they enter a gonad expressing *Sry*. We hypothesized that XX gonocytes from *Sry*^{Myc} transgenic mice would respond to FGF9 in vitro after experiencing a male context in vivo. To investigate this possibility, we generated *Sry*^{Myc};*Oct4:GFP* and *Sry*^{Myc};*Fgf9*$^{-/-}$ animals. Importantly, the gonads of XX*Sry*^{Myc};*Fgf9*$^{-/-}$ lose

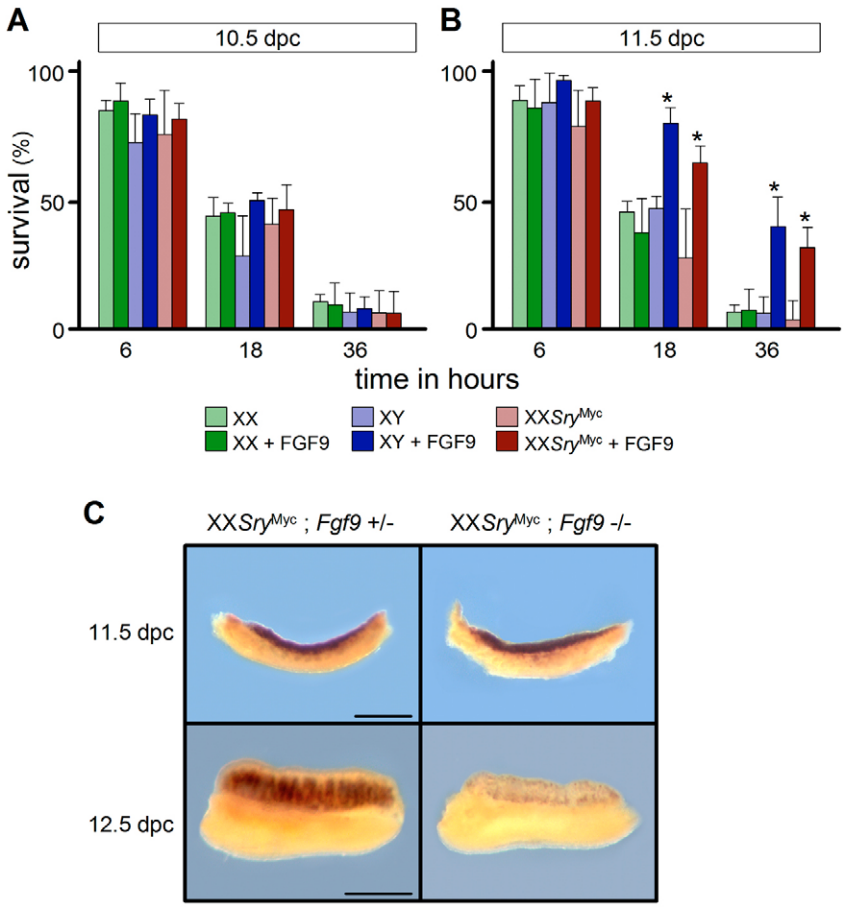

Fig. 6. Purified gonocyte culture with or without FGF9 demonstrates direct pro-survival effect of FGF9 on XY gonocytes. Gonocytes of 10.5 dpc (A) or 11.5 dpc (B) *Oct4:GFP* or XX*Sry*^{Myc};*Oct4:GFP* embryos were purified (>95%) and cultured in DMEM alone or in DMEM + FGF9 (25 ng/ml). (**A**) XX and XY gonocytes (10.5 dpc) decline rapidly in number regardless of treatment. (**B**) XX and XY gonocytes (11.5 dpc) in DMEM also decline rapidly as do XX gonocytes in DMEM + FGF9. However, XY gonocytes and XX*Sry*^{Myc} gonocytes in DMEM + FGF9 exhibit significantly improved survival after 18 and 36 hours in culture (n=10 for each time point; P<0.001). (**C**) Alkaline phosphatase staining of XX*Sry*^{Myc}; *Fgf9*$^{+/-}$ and XX*Sry*^{Myc}; *Fgf9*$^{-/-}$ gonads at 11.5 and 12.5 dpc. XX*Sry*^{Myc}; *Fgf9*$^{-/-}$ gonads at 12.5 dpc show reduced numbers of germ cells (n≥3 for each sample).

gonocytes between 11.5 dpc and 12.5 dpc similar to XY *Fgf9*$^{-/-}$ gonads (Fig. 6C; compare with Fig. 1A,B). When XX*Sry*^{Myc} gonocytes were purified at 10.5 dpc, they did not respond to exogenous FGF9 in culture similar to non-transgenic XX and XY samples at this stage (Fig. 6A). When purified at 11.5 dpc and cultured in the presence of FGF9, XX*Sry*^{Myc} gonocytes showed improved survival (P<0.001) at 18 and 36 hours comparable with FGF9-treated XY gonocytes (Fig. 5B). These findings are consistent with the hypothesis that a male somatic environment induces dependence of gonocytes on FGF9.

Gonocytes respond to members of the FGF9 subfamily

To test the specificity of the effect of FGF9 on XY gonocytes, we examined whether other FGFs could substitute for FGF9 in our gonocyte survival assays. We hypothesized that FGFs from different subfamilies and with different affinities for the four major FGF receptors may be able to rescue 11.5 XY gonocyte survival. FGF2/bFGF, a member of the FGF1 subfamily, is known to promote embryonic stem cell proliferation in culture and has recently been shown to do so in the absence of feeder cells or conditioned medium (Wang et al., 2005; Xu et al., 2005). FGF7/KGF, a founding member of the FGF7 subfamily, has been demonstrated to promote cell survival by inhibiting apoptosis (Hishikawa et al., 2004; Bao et al., 2005). We also tested FGF16, which is a member of the FGF9 subfamily (Itoh and Ornitz, 2004). Neither FGF2 nor FGF7 (25 ng/ml) altered 11.5 XX or XY gonocyte survival relative to controls (Fig. 7A,B). However, 25 ng/ml FGF16 was able to improve XY gonocyte survival after 36 hours (Fig. 7C). When the concentration of FGF16 was raised to 40 ng/ml, XY gonocyte survival was rescued to the same level as treatment with 25 ng/ml FGF9 (Fig. 7D; compare with Fig. 6B).

DISCUSSION

Our previous work characterized the effect of a null mutation in *Fgf9* on the proliferation and differentiation of Sertoli cells and the development of testis morphology (Colvin et al., 2001; Schmahl et al., 2004). In this study, we use in vitro cultures of whole gonads and purified gonocytes to show that FGF9 also acts directly to promote the survival of gonocytes from the testis, but not the ovary, after 11.5 dpc. Although XY *Fgf9*-null gonads initiate the male pathway, they fail to maintain testis development and lose most of their gonocytes between 11.5 and 12.5 dpc. Further analysis revealed that somatic cells within these gonads switch to the ovarian pathway, as indicated by in situ hybridization for the ovarian markers bone morphogenetic protein 2 (*Bmp2*) and follistatin (*Fst*). Concordantly, we observe that some of the surviving XY gonocytes enter meiosis, presumably in response to ovarian cues.

Timing is critical

These findings present an interesting paradox. We show that *Fgf9* is required for the survival of gonocytes in a testis, but not an ovarian environment. If the XY gonad is switching to the ovarian pathway in *Fgf9* mutants, why are XY gonocytes unable to survive there?

No differences have been shown to exist between early XX and XY germ cells (reviewed by McLaren, 2003). Furthermore, in several instances of complete male-to-female sex reversal, XY germ cells are known to be competent to develop as oocytes (Lovell-

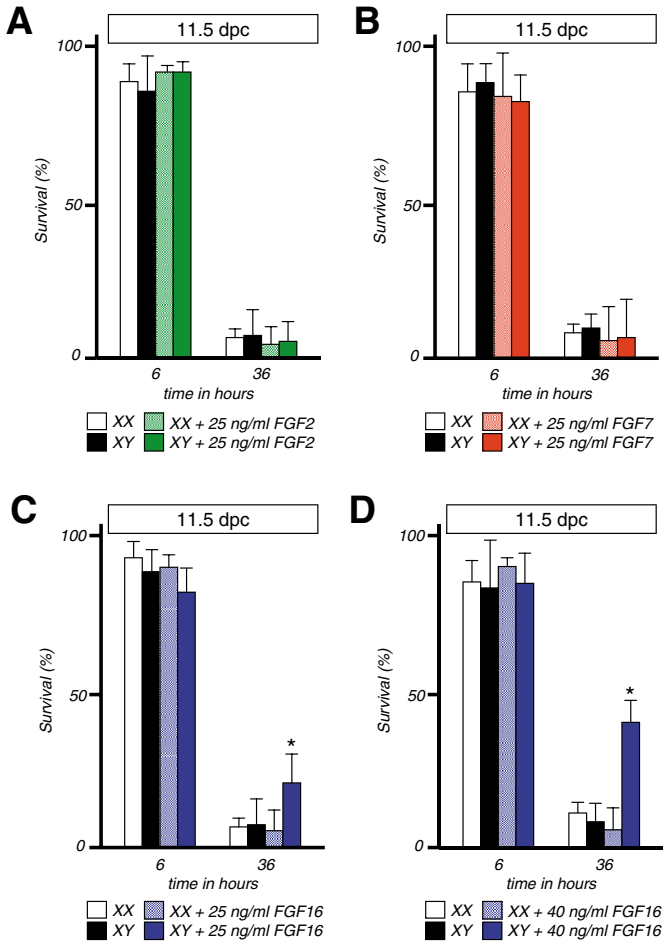

Fig. 7. FGF16, but not FGF2 or FGF7, promotes survival of 11.5 dpc XY gonocytes. Gonocytes of 11.5 dpc *Oct4:GFP* embryos were purified (>95%) and cultured in DMEM alone or in DMEM + 25 ng/ml FGF2 (**A**), + 25 ng/ml FGF7 (**B**), + 25 ng/ml FGF16 (**C**) or + 40 ng/ml FGF16 (**D**). FGF2 and FGF7 show no effect on gonocyte survival (A,B). However, XY gonocytes and gonocytes cultured in DMEM + 25 ng/ml FGF16 (C) or + 40 ng/ml FGF16 (D) exhibit significantly improved survival after 36 hours in culture (n=10 for each time point; P<0.01).

Badge and Robertson, 1990; Adams and McLaren, 2002). However, one formal possibility was that the heteromorphic sex chromosomes are responsible for a germ cell intrinsic differential response to FGF9 signals. To address this possibility, we investigated whether XX gonocytes from the testes of Sry^{Myc} transgenic embryos (Sekido et al., 2004) respond to FGF9 in vitro. In Sry^{Myc} transgenics, *Sry* expression is restricted to somatic cells of the gonad where it initiates normal testis development; therefore, XX gonocytes first encounter a male environment when they enter the testis. XX gonocytes isolated from the testes of these transgenics responded to FGF9 in vitro and showed survival rates similar to XY gonocytes. These data argue that dependence on FGF9 is conferred by the male somatic environment and not by the XY chromosomal constitution of the germ cells.

Other evidence also supports the idea that masculinizing factors in XY gonads influence gonocyte fate by 11.5 dpc. In germ cell transplantation experiments, Adams and McLaren found that a small number of XY gonocytes were committed to become prospermatogonia by 11.5 dpc (Adams and McLaren, 2002). Consistent with these data, our results imply that the putative masculinizing factors gradually confer dependence on FGF9 between 10.5 and 11.5 dpc such that soon after 11.5 dpc, most gonocytes in the wild-type XY gonad require FGF9 for their survival (Fig. 1A,B).

The male gonadal environment is initiated by expression of *Sry* in somatic cells between 10.5 and 12.5 dpc (Koopman et al., 1991; Hacker et al., 1995). In XY $Fgf9^{-/-}$ gonads, the male pathway is transiently initiated in somatic cells (Fig. 3). Other work in our laboratory has revealed that *Sox9* is also transiently expressed in $Fgf9^{-/-}$ gonads (Y. Kim, A. Kobayashi, R. Sekido, L.D., J. Brennan, M.-C. Chaboissier, F. Poulat, R. R. Behringer, R. Lovell-Badge and B.C., unpublished). We hypothesize that transient expression of *Sry* and *Sox9* is sufficient to initiate masculinization of germ cells in the $Fgf9^{-/-}$ XY gonad. In $Sox9^{-/-}$ and $Sox9^{-/-}$; $Sox8^{-/-}$ gonads, despite the fact that *Sry* is initially expressed, Sertoli cells fail to differentiate, testis development is aborted and markers of ovarian development are activated, as in the *Fgf9* mutants (Chaboissier et al., 2004). Interestingly, gonocyte numbers appear unaffected in these sex-reversed XY gonads. By contrast, in $Fgf9^{-/-}$ XY gonads, where both *Sry* and *Sox9* are initially expressed, more than 95% of germ cells are lost. These data suggest that the stage of development at which the male pathway is aborted is crucial to gonocyte survival, and imply that the crucial signal conferring dependence on FGF9 is downstream of *Sox9*. It is noteworthy that the majority of germ cell loss occurs in the central region of the gonad. This may be a result of the fact that testis differentiation is initiated in a center-to-pole pattern (Bullejos and Koopman, 2001; Albrecht and Eicher, 2001).

In $Fgf9^{-/-}$ gonads, some gonocytes escaped conversion to FGF9 dependence, survived and entered meiosis in a pattern similar to an XX gonocyte residing in an ovary. The number of such gonocytes may reflect the exact time at which the male pathway is aborted in XY $Fgf9^{-/-}$ gonads. It also seems likely that the establishment of ovarian differentiation seen by 12.5 dpc in the XY *Fgf9*-null mutants may be incomplete or delayed such that many gonocytes do not receive appropriate male or female survival signals at susceptible stages.

FGF9 as a survival factor in context

When 11.5 dpc $Fgf9^{-/-}$ gonads are cultured in the presence of FBS, Sertoli cells are rescued but gonocytes are not. We suspect that the fetal calf serum in the culture medium contains factors, perhaps FGFs, that are sufficient to rescue Sertoli cells when administered during the critical proliferative window (Fig. 5) (Schmahl et al., 2004). However, these factors are not sufficient to rescue gonocyte numbers. Our results suggest that gonocytes either require higher concentrations of the factors in FBS or that specific factors required by gonocytes are missing. Our experiments indicate that gonocytes are not responsive to all FGFs, but selectively to members of the FGF9 subfamily. Nevertheless, the addition of exogenous FGF9 to cultured gonads or purified cultured gonocytes in vitro clearly supports a direct role of FGF9 as a pro-survival factor for XY gonocytes (Figs 5, 6). FGF9 is the earliest example of a sex-specific germ cell survival factor.

A number of factors have been identified that support PGC survival in vitro, including *Kit*, *Kit* ligand (*Scf*; *Kitl* – Mouse Genome Informatics), LIF and another FGF, bFGF (Matsui et al., 1992) (reviewed by Wylie, 1999; McLaren, 2003). Null mutations in *Kit* or *Kitl* lead to a total loss of PGCs in XX and XY embryos by 9.5 dpc (reviewed by Besmer et al., 1993). There are no in vivo data demonstrating a requirement for LIF or bFGF in PGCs. Recently, it has been shown that the shared receptor for the LIF cytokine family (GP130) plays a role in XY PGC development. PGC-specific

ablation of GP130 led to reduced gonocyte numbers in 13.5 dpc XY animals (Molyneaux et al., 2003). However, the basis for this effect was unclear, and, interestingly, these GP130-null males are fertile. Pro-survival roles in other cell types have been assigned to FGFs, including FGF8 and FGF4 (Trumpp et al., 1999; Abu-Issa et al., 2002; Morini et al., 2000).

These findings make *Fgf9* one of a small but growing set of genes that display a sex-specific embryonic germ cell phenotype. Mouse *Vasa* homolog (*Mvh*) encodes an RNA helicase that plays a testis specific role in gonocyte development (Tanaka et al., 2000). In *Mvh* XY mutants, some gonocytes are mis-localized to the interstitial space between cords. Specifically, *Mvh* has been shown to act as a positive regulator of XY (but not XX) germ cell proliferation between 11.5 and 12.5 dpc, and was the first molecular evidence for dimorphic pathways regulating early gonocyte proliferation. Both the *Fgf9* and *Mvh*-null phenotypes point to the fundamental importance of germ cell/somatic cell interactions. FGF9 is the first secreted cell signaling molecule demonstrated to have a dimorphic effect on survival of germ cells in a male (but not a female) environment.

Future analysis will determine which FGF receptor (FGFR) is mediating the pro-survival effect. We have previously demonstrated the presence of FGFR1, FGFR2, FGFR3 and FGFR4 in the gonad (Schmahl et al., 2004). FGF9 can bind and activate the four major FGFRs, although it has greatest specificity for the FGFR1c, FGFR2c, FGFR3b,and FGFR3c isoforms (Ornitz et al., 1996). However, it is unlikely to function solely through FGFR3 or FGFR4, as mice with null mutations for either are at least partially fertile (Colvin et al., 1996; Deng et al., 1996; Weinstein et al., 1998). It has recently been shown that migratory germ cells express at least two FGFRs, FGFR1-IIIc and FGFR2-IIIb (Takeuchi et al., 2005). FGFR1-IIIc is thought to have high affinity for FGF9. Given that gonocytes do not express any of the FGF receptors sex specifically (Schmahl et al., 2004), how does FGF9 confer a sex specific effect? It is possible that a receptor is expressed at higher levels, or a differently spliced variant is present in the XY gonad. Alternatively, or in addition, there may be sex specific co-factors, e.g. extracellular heparin sulfate proteoglycans or intracellular signal transducers, in masculinized XY gonocytes that mediate the effects of FGF9. In addition, it is intriguing to speculate that there may be an ovarian specific pro-survival signal that has yet to be identified.

Finally, it would be informative to screen for differentially regulated genes in XY gonocytes treated with and without exogenous FGF9. Dissection of the pathways that regulate germ cell and somatic cell coordination would greatly aid our understanding of germ cell development.

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