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Testis development requires the repression of *Wnt4* by Fgf signaling

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Abstract

The bipotential gonad expresses genes associated with both the male and female pathways. Adoption of the male testicular fate is associated with the repression of many female genes including *Wnt4*. However, the importance of repression of *Wnt4* to the establishment of male development was not previously determined. Deletion of either *Fgf9* or *Fgfr2* in an XY gonad resulted in up-regulation of *Wnt4* and male-to-female sex reversal. We investigated whether the deletion if *Wnt4* could rescue sex reversal in *Fgf9* and *Fgfr2* mutants. XY *Fgf9*/*Wnt4* and *Fgfr2*/*Wnt4* double mutants developed testes with male somatic and germ cells present, suggesting that the primary role of Fgf signaling is the repression of female-promoting genes. Thus, the decision to adopt the male fate is based not only on whether male genes, such as *Sox9*, are expressed, but also on the active repression of female genes, such as *Wnt4*. Because loss of *Wnt4* results in the up-regulation of *Fgf9*, we also tested the possibility that derepression of *Fgf9* was responsible for the aspects of male development observed in XX *Wnt4* mutants. However, we found that the relationship between these two signaling factors is not symmetric: loss of *Fgf*9 in XX *Wnt4*$^{-/-}$ gonads does not rescue their partial female-to-male sex-reversal.

Keywords

Sex determination; Bipotential; Testis; Ovary; *Fgf9*; *Wnt4*

Introduction

The mammalian gonad forms initially as a bipotential primordium that is capable of developing into either a testis or ovary. The fate of a population of somatic cells called the supporting cells controls sex determination by either giving rise to Sertoli cells of the testis or a population of granulosa cells in the ovary. Prior to sex determination, supporting cells in XX and XY gonads have an identical expression profile. They express genes later associated with both the male and female pathways, although they show a bias toward the female pathway at the undifferentiated stage (Jameson et al., 2012).

In mammals, the adoption of the male fate is determined by the expression of the sex-determining gene from the Y-chromosome, *Sry* (sex determining region of Chr Y) in supporting cell precursors. Expression of *Sry* up-regulates *Sox9* (*Sry*-box containing gene 9), and initiates Sertoli cell differentiation and testis development (Gubbay et al., 1990; Koopman et al., 1991; Sekido et al., 2004; Sekido and Lovell-Badge, 2008). *Sox9* is critical for testis development (Bishop et al., 2000; Chaboissier et al., 2004; Huang et al., 1999; Vidal et al., 2001; Wagner et al., 1994), but other genes are also required, such as *Fgf9* and

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its receptor *Fgfr2*. Deletion of *Fgf9* (fibroblast growth factor 9) or *Fgfr2* (fibroblast growth factor receptor 2) results in male-to-female sex reversal (Bagheri-Fam et al., 2008; Colvin et al., 2001; Kim et al., 2007).

A robust transcriptional repression program characterizes the commitment of the supporting cells to the Sertoli fate (Jameson et al., 2012). Many female-associated genes (including *Wnt4*, wingless-related MMTV integration site 4) are initially expressed in the sexually undifferentiated supporting cells, and are repressed during male development (Jameson et al., 2012; Nef et al., 2005; Vainio et al., 1999). Exogenous FGF9 can repress *Wnt4* expression in cultured gonads (Kim et al., 2006), suggesting it is a mediator of female gene repression. Consistent with this result, in the absence of *Fgf9*, ($Fgf9^{-/-}$), *Wnt4* was up-regulated in XY gonads by E12.5. However, $Fgf9^{-/-}$ gonads also showed loss of *Sox9* expression and full male-to-female sex reversal (Kim et al., 2006). Based on these findings, we proposed that *Fgf9* and *Sox9* act in a feed-forward loop to reinforce SOX9 expression and repress *Wnt4* (Kim et al., 2006). However, within this general model, the primary cause of sex reversal was not determined.

There is reciprocal evidence that *Wnt4* acts as an antagonist of the male pathway. Stabilization of the *Wnt4* downstream target β-catenin in XY gonads leads to silencing of SOX9 and the male pathway (Maatouk et al., 2008). XX gonads mutant for the Wnt signaling components *Wnt4, Rspo1* (R-spondin homolog), or *Ctnnb1* (β-catenin) show partial female-to-male sex reversal. *Fgf9* is expressed in XX *Wnt4* mutants, endothelial cells migrate into the XX gonad and form male-type vasculature, and XX cells express steroidogenic enzymes characteristic of the testis, suggesting that *Wnt4* is normally involved in blocking male development of the gonad (Chassot et al., 2008; Jeays-Ward et al., 2003; Kim et al., 2006; Liu et al., 2009; Manuylov et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999). Although constitutively active β-catenin can block male development of XY gonads, the importance of repression of the normal female program to Sertoli cell differentiation and testis development was not clear.

One open possibility was that *Fgf9* signaling is primarily required to block *Wnt4* and the female pathway rather than to activate *Sox9* and male development. To test this possibility, we generated XY mice mutant for both *Wnt4* and *Fgf9* or its receptor *Fgfr2*. If the primary role of *Fgf9* is to activate the male pathway, double mutants should be female. However, if the primary role of *Fgf9* is to repress *Wnt4* and the female pathway, these mice should be male.

In both sets of double mutants, the additional deletion of *Wnt4* rescued the XY *Fgf9* and *Fgfr2* male-to-female sex reversal phenotypes in somatic and germ cells. This indicates that the repression of *Wnt4* is required for male development. On the other hand, we found that the phenotype of the XX *Wnt4* mutants was not rescued by deleting *Fgf9*, indicating that the partial male sex reversal in XX gonads does not depend on the up-regulation of *Fgf9*. Thus, *Fgf* signaling is required to repress female genes during testis development, but it is not required to maintain *Sox9*, regulate male germ cell development, or activate male vascular development and steroidogenesis.

Materials and methods

Mice

All animals were maintained and experiments were conducted according to DUMC-IACUC and NIH guidelines, based on existing protocols. $Fgf9^{+/-}$ (Colvin et al., 2001) and $Wnt4^{+/-}$ (Stark et al., 1994) were separately maintained as heterozygotes on the C57BL/6 strain. For qRT-PCR experiments, $Fgf9^{-/-}$ embryos and their control littermates were generated by

intercrossing *Fgf9*$^{+/-}$ mice. For more precise staging, tail somites distal to the hindlimb were counted (Hacker et al., 1995). E11.5 embryos had 16–19 tail somites, E11.75 embryos had 20–23 tail somites, and E12.5 embryos had 27–28 tail somites. Gonads were dissected away from the mesonephros prior to RNA extraction. "Control" mice were *Fgf9*$^{+/-}$ or *Fgf9*$^{+/+}$.

Fgf9$^{+/-}$ and *Wnt4*$^{+/-}$ mice (on the C57BL/6 strain) were intercrossed to generate *Fgf9*$^{+/-}$; *Wnt4*$^{+/-}$ offspring, which were intercrossed to generate double mutant embryos. The genotypes and chromosomal sex of the embryos were determined as previously described (Colvin et al., 2001; Munger et al., 2009; Stark et al., 1994). Embryos were isolated between about E16.5 and E17.5. Brightfield images of the gonads were collected on a Leica MZ12 following the dissection. "Control" mice were heterozygous or homozygous wild type at the *Fgf9* and *Wnt4* loci.

A mixed background *Fgfr2*$^{flox/flox}$ line (Yu et al., 2003) was backcrossed two or more generations to C57BL/6, during the processes of crossing on the *Wnt4* null allele (Stark et al., 1994) and the *Sf1-Cre* allele (Bingham et al., 2006) that are maintained on C57BL/6. These mice were intercrossed to generate *Fgfr2*$^{flox/flox}$; *Wnt4*$^{+/-}$ mice, some of which also had *Sf1-Cre*, and the line was maintained by intercrossing to preserve homozygosity at the *Fgfr2* locus. Timed matings were established between fertile *Sf1-Cre; Fgfr2*$^{flox/flox}$; *Wnt4*$^{+/-}$ males (see Fig. 2 for explanation) and *Fgfr2*$^{flox/flox}$; *Wnt4*$^{+/-}$ females. Similar methods were also used to generate mice that carried *Flk1-Cre* (Motoike et al., 2003) and *Fgfr2*$^{flox/+}$. *Flk1-Cre; Fgfr2*$^{flox/+}$ males were crossed to *Fgfr2*$^{flox/flox}$ females. E0.5 was defined as noon on the day a mating plug was detected. Embryos were collected from pregnant females on the morning of day E13.5. The genotypes (Stark et al., 1994; Yu et al., 2003) and chromosomal sex (Munger et al., 2009) of the embryos were determined as previously described. We determined whether Cre was present using the primers 5′-CCAGGGCGC-GAGTTGATAGC-3′ and 5′-CTGCCACGACCAAGTGACAGC-3′. "Control" mice were all *Fgfr2*$^{flox/flox}$, negative for *Sf1-Cre*, and either *Wnt4*$^{+/-}$ or *Wnt4*$^{+/+}$.

qRT-PCR

Isolated gonads were frozen at −80 °C. RNA was extracted as previously described (Munger et al., 2009), treated with DNaseI, and converted to cDNA using the iScript cDNA synthesis kit (170–8891, Bio-Rad, Hercules, CA). Each cDNA sample was run in technical triplicate on a StepOnePlus Real-time PCR system (Applied Biosystems, Carlsbad, CA). The threshold and baseline were set manually. Generally, the average C_T value of the technical replicates was analyzed using the "normalized expression" method described previously (Simon, 2003). *Canx* was used for normalization (van den Bergen et al., 2009). Since all primer sets appeared to have greater than 95% efficiency (data not shown), we assumed all primers were perfectly efficient. If the difference in C_T values of technical triplicates exceeded 0.5, the outlier sample was excluded from the average. If no sample was an outlier, all replicates were retained so long as the difference in C_T values was less than 0.75. The list of primers is provided (Table S1), several of which have been previously published (Manuylov et al., 2008; Munger et al., 2009).

Generally, three individual gonad pairs were analyzed for each genotype at each stage (three biological replicates). The normalized expression values from the biological replicates were averaged to calculate mean normalized expression (MNE). A significant difference in normalized expression between genotypes was determined using a *T*-test. To reduce technical variability in expression at E11.5 and E11.75, we used qRT-PCR data only when the reactions for all of the biological replicates were set up at the same time. Four independent E11.75 XY *Fgf9*$^{-/-}$ samples were analyzed. For one sample, the *Foxl2* reading was eliminated because the C_T value was within 3 cycles of the negative control, but data from that sample for other genes was retained in the analysis.

Immunofluorescence

Gonads were immunostained as previously described (Cook et al., 2009). All E13.5 gonads were stained as whole mounts with the mesonephros attached. At/after E16.5, phenotypically female samples (including XY *Fgf9*$^{-/-}$ samples) were stained as whole mounts after removing mesonephric structures. The whole mount samples were either immunostained immediately, or processed through a methanol series and stored at −80 °C prior to rehydration and immunostaining (Barske and Capel, 2010). Phenotypically male samples from E16.5 and after were cryosectioned after removing the mesonephric structures. Since the male and female samples were processed separately at E16.5–E17.5, we included CD-1 (Charles River, Wilmington, MA) positive/negative controls in each batch of immunostained samples (i.e., E13.5 CD-1 male gonads were stained as whole mounts with the E16.5 female samples, and E16.5 CD-1 female gonads were cryosectioned and stained with the E16.5–E17.5 males) (data not shown). E12.5 CD-1 male gonads were also used as the positive control in Fig. S3 for the Ki67 immunostaining. For the DNMT3L immunostaining (Fig. 5F–J), cryosectioned XY *Fgf9*$^{-/-}$ samples and CD-1 female controls are shown because the antibody did not work in whole mount.

Primary antibodies were anti-3β-HSD (sc-30820, Santa Cruz Biotechnology, Santa Cruz, CA) used 1:75 in samples processed through methanol or KO607 (TransGenic Inc., Kobe, Japan) used at 1:250; anti-AMH (MIS, sc-6886, Santa Cruz Biotechnology) used at 1:500; anti-CDH1 (13–1900, Invitrogen, Camarillo, CA) initially reconstituted in 400 μl and used at 1:250; anti-FOXL2 (NB100-1277, Novus Biologicals, Littleton, CO) used at 1:250; anti-Ki67 (RM-9106-S, Neomarkers, Thermo Scientific, Waltham, MA) used at 1:500; anti-NRP1 (AF566, R&D Biosystems, Minneapolis, MN) used at 1:350; anti-PECAM1 (CD31, 553370, BD Pharmingen, San Diego, CA) used at 1:250; anti-SCP3 (ab15093, Abcam, Cambridge, MA) used at 1:500-1:1000; anti-SOX9 (AB5535, Millipore, Billerica, MA) used at 1:3000-1:5000, and anti-DNMT3L (kindly provided by Dr. Shoji Tajima, Osaka University, Japan) used at 1:125. Secondary antibodies used included Alexa 647- and 488-conjugated secondary antibodies (Molecular Probes, Grand Island, NY) used at 1:500, and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) used at 1:500. DAPI (Sigma-Aldrich, St. Louis, MO) was used to label nuclei. Samples were mounted in 2.5% DABCO (Sigma-Aldrich) in 90% glycerol. In some samples, Hoechst 333420 (Invitrogen, Eugene, OR, USA) was also used to lable DNA (Fig. 5F–J).

Samples were immunostained at the same time as opposite sex controls to ensure that all antibodies were working properly. Samples stained at the same time were also imaged at the same time on a Leica SP2 confocal microscope. Leica confocal software, copyright 1997–2004, by Leica Microsystems Heidelberg GmbH was used to generate maximum intensity projections of multiple images along the *Z* axis (Figs. 4–6, S2–S4). In some cases, multiple overlapping images were taken along the *Y* axis in a single *Z* plane, and these were assembled by eye to survey the entire gonad (Fig. 2, S1 and S5). A Zeiss 710 inverted confocal microscope was used to collect some images as well, and the Zeiss free offline software was used to generate maximum intensity projections (Fig. 5F–J).

Results

Gene expression in XY Fgf9 mutants

Previous work demonstrated that exogenous FGF9 could repress *Wnt4*, and that *Wnt4* expression was elevated in XY *Fgf9*$^{-/-}$ gonads by E12.5 (Kim et al., 2006). To better characterize the timing of gene expression changes, we used qRT-PCR at E11.5, E11.75, and E12.5 to determine the transcriptional regulation of *Sox9* and female genes relative to the loss of SOX9 protein at E12.5 (Kim et al., 2006). We expected some expression

variability in samples collected before E12.5 (Munger et al., 2009), but we were able to identify distinct trends in the data even when the differences were not yet significant.

At all time points, *Sox9* expression was significantly higher in XY *Fgf9*$^{-/-}$ gonads than in XX control gonads (Fig. 1A). At E11.5 and E11.75, *Sox9* was not significantly different between XY control and *Fgf9*$^{-/-}$ gonads, but it did appear to be consistently lower in XY *Fgf9* mutant gonads. This difference became significant at E12.5. Therefore, we could not exclude the possibility that *Fgf9* has some role in up-regulating *Sox9* expression. Nevertheless, *Sox9* expression was not reduced to the female expression level, even though SOX9 protein was absent at E12.5 (Kim et al., 2006). This finding suggested post-transcriptional regulation of SOX9 in the XY *Fgf9* mutants.

We examined the expression of three female associated genes in XY *Fgf9*$^{-/-}$ gonads: *Wnt4*, *Rspo1*, and *Foxl2* (Forkhead box L2) (Chassot et al., 2008; Schmidt et al., 2004; Tomizuka et al., 2008; Vainio et al., 1999) (Fig. 1B–D). No significant differences were observed between the expression of these genes in the XX control and XY *Fgf9*$^{-/-}$ gonads at any time point. The expression of these genes in XY *Fgf9*$^{-/-}$ and XX control gonads appeared different from XY control gonads by E11.75, although this difference generally was not significant until E12.5. Thus, even though SOX9 protein was present at this time (Kim et al., 2006), there was an early derepression of female genes in XY *Fgf9*$^{-/-}$ gonads. This demonstrated that *Fgf9* is required for the repression of multiple female associated genes early in gonad development in vivo.

The partial sex reversal observed in XY Fgfr2 mutants was rescued by deleting Wnt4

In previous work, we found that many female genes are actively repressed during male gonad development (Jameson et al., 2012). The finding that multiple female genes were derepressed early in XY *Fgf9*$^{-/-}$ gonads in vivo (Fig. 1) led us to hypothesize that the primary cause of the male-to-female sex reversal in both the *Fgf9* and *Fgfr2* XY mutants (Bagheri-Fam et al., 2008; Colvin et al., 2001; Kim et al., 2007) was the derepression of the Wnt pathway. If this were the case, we expected that the deletion of *Wnt4* should rescue the *Fgf9* and *Fgfr2* XY mutant phenotypes.

We tested this first in the *Fgfr2* mutants. We used a null allele of *Wnt4* (Stark et al., 1994), and a floxed allele of *Fgfr2* (Yu et al., 2003) conditionally deleted using the *Sf1-Cre* transgene (Bingham et al., 2006). These mice were maintained on a mixed strain background with a large C57BL/6 contribution, since the sex reversal phenotype of *Fgfr2* mutants is sensitive to strain background (Bagheri-Fam et al., 2008). Gonads were collected at E13.5 and immunostained for FOXL2 (a marker of the female supporting cell fate) and SOX9 (a marker of the male supporting cell fate) (Fig. 2A and B), and analyzed for male-specific vasculature and the development of testis cord structures enclosing germ cells (Brennan and Capel, 2004) using PECAM1 to label the germ cells and the vasculature. Deleting *Wnt4* alone did not disrupt general male development (Fig. 2C), although it was previously observed that the XY gonad was slightly smaller and had early defects in gene expression (Jeays-Ward et al., 2004).

XY *Sf1-Cre; Fgfr2*$^{flox/flox}$ gonads exhibited partial male-to-female sex reversal, consistent with previous observations (Bagheri-Fam et al., 2008; Kim et al., 2007) (Fig. 2D). They expressed substantially more FOXL2 and less SOX9 than XY controls, and testis cord structures were disrupted at the poles of the gonad. In XY *Sf1-Cre; Fgfr2*$^{flox/flox}$ gonads that were heterozygous or homozygous null for *Wnt4*, there was substantial rescue of the *Fgfr2* mutant phenotype (Fig. 2E and F). The rescue phenotype of XY *Sf1-Cre; Fgfr2*$^{flox/flox}$; *Wnt4*$^{+/-}$ mice was robust and resulted in fertile males that were used in breeding (Fig. 2E), indicating that not only somatic, but male germ cell development occurs normally in double

mutants. In the *Wnt4* null samples, SOX9 positive testis cords extended to the poles of the gonad, and few cells were positive for FOXL2 (Fig. 2F). The rescue of the *Fgfr2* mutant phenotype was consistent and clear, despite variability observed among control samples that could be due to the mixed strain, minor differences in stage, or natural variability among individuals at this early stage (Fig. S1). Thus, as hypothesized, the deletion of *Wnt4* rescued the male-to-female sex reversal in the XY *Fgfr2* mutants, consistent with the idea that the derepression of female genes plays a primary role in the sex reversal in XY *Fgfr2* mutants.

Male-to-female sex reversal caused by Fgf9 deletion was rescued by deleting Wnt4

We also tested whether the deletion of *Wnt4* could rescue the more severe complete male-to-female sex reversal in the XY *Fgf9* mutants on the C57BL/6 strain (Schmahl et al., 2004). We collected *Fgf9*/*Wnt4* double mutant samples around E16.5–E17.5 to avoid some of the variability observed at the earlier stage (Fig. S1) and to assay other roles for Fgf (such as an involvement in germ cell commitment to the male fate).

XX and XY gonads were visibly distinct at this stage, and the XY *Wnt4* mutant gonads showed clear testicular morphology (Fig. 3A–C). In contrast, the XY *Fgf9* mutants showed full male-to-female sex reversal (Fig. 3D), consistent with previous findings (Schmahl et al., 2004). Unlike the case for *Fgfr2* mutants, XY *Fgf9*$^{-/-}$ gonads heterozygous for *Wnt4* (*Wnt4*$^{+/-}$) did not show rescue and instead resembled the *Fgf9* mutant alone. However, in XY *Fgf9*$^{-/-}$; *Wnt4*$^{-/-}$ gonads, we observed a complete rescue of the *Fgf9*$^{-/-}$ sex reversal phenotype, with possibly a slight reduction in testis size (Fig. 3E). All seven XY *Fgf9*/*Wnt4* double mutant gonads collected were phenotypically male.

Somatic cell fate was properly adopted and cellular organization was normal in XY Fgf9/Wnt4 double mutant gonads

All somatic cell types examined were properly specified in XY *Fgf9*$^{-/-}$; *Wnt4*$^{-/-}$ gonads (Fig. 4). First, we checked for the presence of maturing Sertoli cells in testis cords, which were immunostained with antibodies against SOX9 and AMH. The XX controls lacked Sertoli cells labeled with SOX9 and AMH (Fig. 4A). Sertoli cells were present in XY controls and *Wnt4* mutants (Fig. 4B and C). XY *Fgf9* mutants resembled ovaries. Occasional SOX9-positive cells were observed in the XY *Fgf9* mutant gonads (which were rarely seen in XX controls), but they were not AMH positive as Sertoli cells normally are at this stage (Fig. 4D). In contrast, the XY *Fgf9*$^{-/-}$; *Wnt4*$^{-/-}$ gonads had testis cords with SOX9- and AMH-positive Sertoli cells similar to control XY gonads (Fig. 4E).

We also examined whether germ cells, labeled by CDH1 (E-cadherin), were clustered inside testis cords, and whether Leydig cells, expressing the steroidogenic enzyme 3β-HSD, were present outside of the testis cords (in the interstitium). The germ cells in the XX control were not clustered into testis cords and Leydig cells labeled with 3β-HSD were not present (Fig. 4F). In contrast, in XY controls and *Wnt4* mutants, germ cells were clustered inside testis cords and Leydig cells were found in the interstitium (Fig. 4G and H). XY *Fgf9* mutants resembled ovaries in that germ cells were not clustered into testis cords and Leydig cells were absent (Fig. 4I). However, deletion of *Wnt4* in an *Fgf9* mutant rescued both the organization of germ cells in cords and the development of Leydig cells (Fig. 4J). In 1 of 4 double mutant XY samples, there was reduced CDH1 staining (data not shown), but based on nuclear morphology, germ cells were still present in testis cords. Similarly, we observed a range of 3β-HSD staining intensity in XY *Wnt4* mutants and *Fgf9*/*Wnt4* double mutants (data not shown). Nevertheless, cells positive for 3β-HSD were observed in all samples of these genotypes examined. Thus, all aspects of somatic male development that we examined were rescued in XY *Fgf9*$^{-/-}$ mice by deletion of *Wnt4*. We observed no sex reversed regions in these double mutant gonads (Fig. S2).

Male germ cell fate was properly adopted in XY Fgf9/Wnt4 double mutant gonads

Fgf9 was reported to inhibit the entry of the germ cells into meiosis (Barrios et al., 2010; Bowles et al., 2010). However, *Fgf9* is not required to prevent the entry of the germ cells into meiosis in the absence of *Wnt4*. Nuclear SCP3 labels meiotic germ cells, which are present in XX controls and absent from both XY controls and XY *Wnt4* mutants (Fig. 5A–C). The germ cells in *Fgf9* mutants were in meiosis (Fig. 5D) (Bowles et al., 2010), although there was somewhat variable loss of germ cells in these mutants (Fig. S2). However, when both *Fgf9* and *Wnt4* were deleted, SCP3 was absent (Fig. 5E). Together, these results showed that both the somatic and germ cell sex reversal phenotype of the XY *Fgf9* mutants was rescued by deleting *Wnt4*.

Because the germ cells in the XY *Fgf9/Wnt4* double mutants were not meiotic, we investigated whether they were positive for a male germ cell marker at this stage. DNMT3L is a DNA methyltransferase implicated in genomic imprinting. The expression of DNMT3L is specific to male germ cells from E14.5 to E18.5, while in the female, expression begins only after birth (Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Sakai et al., 2004) DNMT3L was absent in E16.5 XX gonads but present in the XY controls (Fig. 5F and G) and in XY *Wnt4* single mutants (Fig. 5H). In sex reversed *Fgf9* mutant XY gonads (Fig. 5I), no DNMT3L positive germ cells were observed, while in the $Fgf9^{-/-}$; $Wnt4^{-/-}$ double mutants (Fig. 5J), germ cells were positive for DNMT3L staining, indicating they had adopted the male fate. We also found that the germ cells properly entered mitotic arrest in the XY $Fgf9^{-/-}$; $Wnt4^{-/-}$ double mutants (Fig. S3), further confirming that the germ cells have properly adopted the male fate in the *Fgf9*/*Wnt4* double mutant males.

The partial sex reversal phenotype of XX Wnt4 mutants is not rescued by deletion of Fgf9

Unlike XX controls, XX $Wnt4^{-/-}$ gonads develop features normally seen only in XY gonads: a coelomic vessel and steroidogenic cells (Jeays-Ward et al., 2003; Vainio et al., 1999) (Fig. 6A–D). Previous work exploring the antagonism between FGF9 and *Wnt4* signaling showed that FGF9 was elevated in XX *Wnt4* mutants (Kim et al., 2006). It has also been shown previously that FGF9 can induce migration of mesonephric endothelial cells into the XX gonad (Colvin et al., 2001). Thus, we hypothesized that elevation of FGF9 in XX $Wnt4^{-/-}$ gonads might be responsible for development of the testis-like characteristics in these mutants. To investigate this possibility, we examined XX $Fgf9^{-/-}$; $Wnt4^{-/-}$ gonads. In E16.5–E17.5 XX wild type gonads, neither the coelomic vessel nor steroidogenic cells are detected (Fig. 6A and B). However, in *Wnt4* mutants, we identified the coelomic vessel as well as ectopic steroidogenic cells (Fig. 6C and D). Neither the steroidogenic cells nor the ectopic vessel were apparent in the XX $Fgf9^{-/-}$ gonads (Fig. 6E and F). However, the XX $Fgf9^{-/-}$; $Wnt4^{-/-}$ mice developed a coelomic vessel and steroidogenic cells similar to the *Wnt4* mutants (Fig. 6G and H). The number of steroidogenic cells and the appearance of the ectopic vessel were variable in both XX $Wnt4^{-/-}$ and $Fgf9^{-/-}$; $Wnt4^{-/-}$ gonads (Fig. S4), but both were present in three gonads of each genotype analyzed. The presence of an ectopic vessel and steroidogenic cells in the absence of *Fgf9* indicates that Fgf signaling is not necessary for coelomic vessel formation or steroidogenesis in vivo. In further support of this conclusion, deleting *Fgfr2* in endothelial cells had no effect on coelomic vessel formation in XY gonads (Fig. S5). These results show that development of the male-like characteristics in XX $Wnt4^{-/-}$ gonads does not require *Fgf9*.

Discussion

Mammalian sex determination depends on the fate decision that occurs in the supporting cell lineage of the gonad. We recently demonstrated that both XX and XY supporting cells are

"primed" to adopt the male and female fate. During the bipotential stage, supporting cells isolated from XX and XY gonads have a common transcriptome characterized by the expression of transcripts associated with both the male and female fates, but genes associated with the female fate are over-represented (Jameson et al., 2012). This results in the repression of many female genes when these cells adopt the male fate. This is consistent with antagonism between the male and female programs reported by multiple laboratories (Bradford et al., 2009; Chassot et al., 2008; Kent et al., 1996; Kim et al., 2006; Morais da Silva et al., 1996; Munger et al., 2009; Swain et al., 1998; Vainio et al., 1999). However, the functional importance of repression of the female program to the adoption of the male fate had not been directly tested. Here, we provide evidence that the fate commitment of the XY supporting cells requires repression of the female-associated gene, *Wnt4*. This finding is in accord with predictions in the theoretical sex determination literature (Koopman, 1999; McElreavey et al., 1993; Sekido and Lovell-Badge, 2009).

In XY gonads, expression of the Y-linked gene *Sry* diverts the fate of the supporting cells toward Sertoli cell differentiation (Fig. 7). SRY acts directly to up-regulate *Sox9* transcription by binding to the TESCO element in the *Sox9* promoter (Sekido and Lovell-Badge, 2008). Once activated, SOX9 binds its own promoter to maintain expression (Sekido and Lovell-Badge, 2008). However, continued SOX9 expression is also critically dependent on the extracellular signaling molecule *Fgf9* and its receptor *Fgfr2.* In the absence of Fgf signaling in XY gonads, SOX9 expression is lost, *Wnt4* is elevated, and the gonad switches to the female fate (Kim et al., 2007, 2006). We proposed that FGF9 and SOX9 create a positive feed-forward loop to stabilize SOX9 and repress *Wnt4* (Kim et al., 2006). However, our present results suggest that the primary role of *Fgf9* is the repression of female genes (including *Wnt4*) that would otherwise antagonize male development (Fig. 7).

Wnt4 is initially expressed in both XX and XY gonads and positively reinforces its own expression (Liu et al., 2009; Maatouk et al., 2008; Nef et al., 2005; Vainio et al., 1999) (Fig. 7). However, its expression declines by E11.5 in XY gonads, soon after *Sry/Sox9* activation and coincident with a pulse of *Fgf9* expression (Nef et al., 2005; Vainio et al., 1999). In XY $Fgf9^{-/-}$ gonads, *Wnt4* and other female genes showed some derepression by E11.75, preceding SOX9 protein loss (although it was generally not statistically significant until E12.5) (Kim et al., 2006). *Wnt4* and its downstream effector β-catenin are known to repress SOX9 in the gonad (Chang et al., 2008; Kim et al., 2006; Maatouk et al., 2008). This, coupled with the ability of *Wnt4* deletion to rescue both the *Fgfr2* and *Fgf9* male-to-female sex reversal phenotypes, suggests that the primary function of Fgf signaling is to repress *Wnt4*.

We cannot exclude the possibility that FGF9 has some direct role in activating *Sox9* expression in addition to repressing female transcripts. There is some evidence suggesting that FGF9 can up-regulate *Sox9* independent of *Wnt4* (Hiramatsu et al., 2010), and we detected a small difference in *Sox9* expression in XY *Fgf9* mutant gonads at all time points (although it is not statistically significant before E12.5). Nonetheless, our results demonstrate that when *Wnt4* and other genes associated with the female pathway are downregualted, the level of SOX9 in the XY *Fgf9* mutants is sufficient to maintain male development.

Interestingly, *Sox9* transcript is not repressed to female levels in XY *Fgf9* mutant gonads at E12.5, despite the fact that the protein is absent (Kim et al., 2006). This finding is reminiscent of the role of Wnt signaling in chondrocytes where β-catenin represses SOX9 at the protein level through a physical interaction, leading to mutual protein degradation (Akiyama et al., 2004). Wnt signaling may also repress *Sox9* at the transcriptional level

based on recent data (Bernard et al., 2011), but our results suggest that transcriptional repression is unlikely to be its sole function.

Our data suggests that expression of female genes at endogenous female levels is sufficient to block testis development, indicating that active repression of the female program is required for male development. This is consistent with previous data illustrating the ability of over-expressed female genes to block male development. In humans a duplication containing *Wnt4* and *Rspo1* disrupted XY male development (Jordan et al., 2001; Tevosian and Manuylov, 2008). In the mouse, constitutive activation of β-catenin in XY gonads caused sex reversal (Chang et al., 2008; Maatouk et al., 2008), although the over-expression of *Wnt4* or *Rspo1* alone was not sufficient to induce male to female sex reversal in mice (Buscara et al., 2009; Jeays-Ward et al., 2003; Jordan et al., 2003). Over-expression of multiple female genes, as occurs in the XY *Fgf9* mutants, may be required to achieve the level of β-catenin signaling to repress male development in mice. This is consistent with our finding that deletion of only one of these factors (*Wnt4*) is sufficient to prevent the male-to-female sex reversal of XY *Fgf9* mutants.

The differences between the human and mouse may reflect the relative strength of the male and female programs in different species. In humans, mutations in *RSPO1* alone can result in female-to-male sex reversal in the absence of *SRY* (Parma et al., 2006). However, full male development does not occur in XX mouse gonads if any one of the *Wnt* pathway components (*Wnt4*, *Rspo1*, or β-catenin) is deleted (Chassot et al., 2008; Jeays-Ward et al., 2003; Liu et al., 2009; Manuylov et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999). Although SOX9 was detected transiently in XX gonads mutant for *Wnt4* alone and the female program was disrupted (Coveney et al., 2008; Kim et al., 2006; Vainio et al., 1999), the full male program was not established. This may indicate that the male program in the mouse needs more reinforcement (from SRY) to become established than the male program in humans.

There is an asymmetry in the relationship between *Fgf9* and *Wnt4*. Since the *Fgf9* phenotypes were the result of *Wnt4* derepression, we investigated whether the XX *Wnt4* mutant phenotypes were caused by derepression of *Fgf9*. We show that this is not the case: the XX *Wnt4* mutant phenotypes were not rescued by the deletion of *Fgf9*. Even though exogenous FGF9 can induce migration of mesonephric endothelial cells into the XX gonad (Colvin et al., 2001), endogenous FGF9 does not appear to have this function in vivo. It may be that exogenous FGF9 induces endothelial migration through the repression of *Wnt4*. The finding that *Fgf9* loss does not affect coelomic vessel formation in the *Wnt4* mutants is also consistent with previous work showing that *Wnt4* results in vessel formation by repressing inhibin beta B (Yao et al., 2006).

These results also bring into question the role of *Fgf9* in the development of other cell types in the XY gonad. Both Sertoli cells and Leydig cells appeared to develop normally in double mutants. *Fgf9* has been reported to promote male germ cell development and repress the female meiotic program (Barrios et al., 2010; Bowles et al., 2010). However, while *Fgf9* may reinforce the male germ cell fate, we show that *Fgf9* is not required to block meiosis or induce mitotic arrest in the context of normal male development.

The primary role of *Fgf9* appears to be coupling the activation of the male program to the repression of the female program, which would otherwise repress the male program. This double negative mechanism is a common feature of many developmental systems (Bracken et al., 2008; Davidson et al., 2002; Ferrell, 2002; Johnston et al., 2005; Revilla-i-Domingo et al., 2007). Previous work in the gonad characterized these antagonistic programs and the repression of the female program in the XY gonad (Bradford et al., 2009; Chassot et al.,

2008; Jameson et al., 2012; Kent et al., 1996; Kim et al., 2006; Morais da Silva et al., 1996; Munger et al., 2009; Swain et al., 1998; Vainio et al., 1999). Here, we show that the repression of the female program is required for male development.

Conclusion

Fgf9 is critical to a repression program in the XY gonad that blocks female gene expression that would otherwise antagonize male development. Using *Fgfr2*/*Wnt4* and *Fgf9*/*Wnt4* double mutant mice, we show that Fgf signaling promotes male sex determination by repressing the female-promoting genes. If the male program is unable to repress *Wnt4* due to loss of either *Fgf9* or *Fgfr2*, male development is aborted. Thus, the decision to adopt the male fate is based not only on whether male genes, such as *Sox9*, are expressed, but also on the repression of female genes, such as *Wnt4*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.06.009.

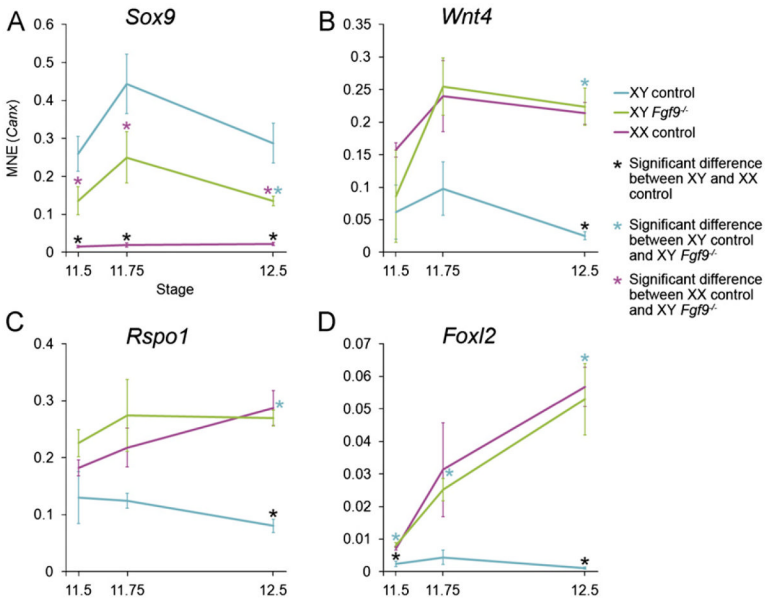

Fig. 1.

Wnt4, *Rspo1* and *Foxl2* transcript levels are indistinguishable from XX gonads, whereas *Sox9* levels are somewhat lower in XY *Fgf9*$^{-/-}$ gonads. ((A)–(D)) qRT-PCR data from XY control (cyan), XY *Fgf9*$^{-/-}$ (green), and XX control (magenta) gonads at E11.5, E11.75, and E12.5. n=3 or 4. Mean normalized expression (as described in the methods) is shown, using *Canx* as the normalizing gene. A significant difference (as determined by a *T*-test, p-value <0.05) between XX and XY samples is indicated by a black asterisk over the sex with lower expression. A significant difference between XY *Fgf9*$^{-/-}$ and XY control samples is indicated by a cyan asterisk, while a significant difference between the XY *Fgf9*$^{-/-}$ and XX control samples is indicated by a magenta asterisk. (A) Sox9 was expressed at an intermediate level at E12.5 in XY *Fgf9*$^{-/-}$ gonads when compared to both XX and XY controls, even though *Sox9* protein was lost by this stage (Kim et al., 2006). While there was no significant difference in expression of *Sox9* between XY control and *Fgf9*$^{-/-}$ gonads at E11.5 and E11.75, the average expression of *Sox9* in XY *Fgf9* mutants was lower than that in XY controls, indicating that *Fgf9* could play a role in up-regulating *Sox9* or fewer *Sox9*-positive cells may be present. ((B)–(D)) For female associated genes, no significant difference was observed at any time point between the XY *Fgf9*$^{-/-}$ and XX control gonads. While the differences between XX and XY control gonads were not always significant before E12.5, elevated expression of these female-associated genes was apparent in both the XX control and XY *Fgf9*$^{-/-}$ gonads when compared to the XY control gonads. Thus, loss of *Fgf9* resulted in the over-expression of multiple female genes.

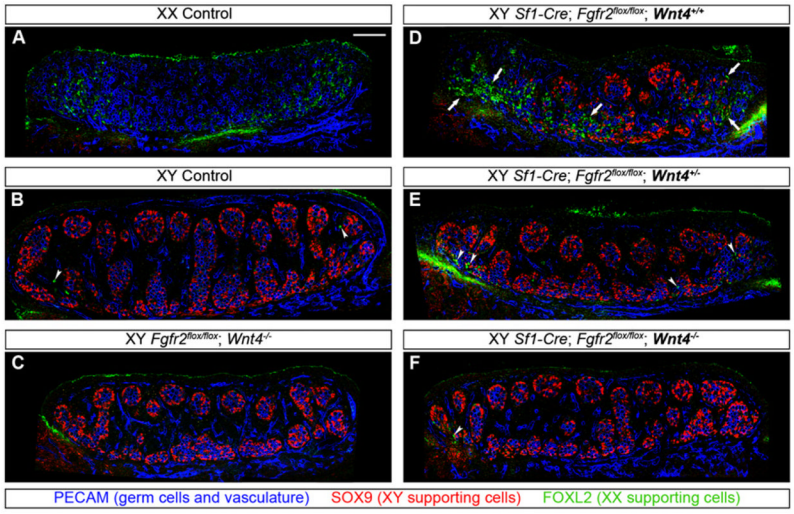

Fig. 2. Deleting Wnt4 rescued the partial sex reversal induced by loss of Fgfr2

Immunofluorescence of E13.5 gonads stained with PECAM1 (labeling germ cells and vasculature, blue), SOX9 (red), and FOXL2 (green). XX controls (A) had no testis cords and expressed FOXL2. In contrast, XY controls (B) and XY *Wnt4* mutants (C) had SOX9-positive Sertoli cells surrounding the germ cells in testis cords with rare FOXL2-positive cells (arrowheads). (D) XY mice with *Fgfr2* deleted in the supporting cells were partially sex reversed. Cells at the poles of the gonad (arrows) expressed FOXL2 and cords were absent, as in the female control. The center of the gonad was more testicular with SOX9-positive cells and testis cord structure. ((E) and (F)) Loss of one or two alleles of *Wnt4* substantially rescued the *Fgfr2* phenotype. Particularly in the XY *Sf1-Cre; Fgfr2*$^{flox/flox}$; *Wnt4*$^{-/-}$ samples (F), there were testis cords throughout the gonad with SOX9 and few FOXL2-positive cells (arrowheads). Images were captured using a 40X objective, assembled to span the entire gonad, and placed on a black background. Scale bar=100 μm. $n \geq 3$ of each genotype.

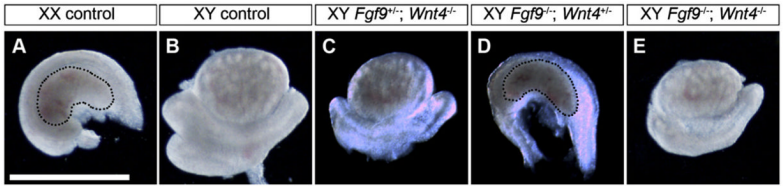

Fig. 3. Deleting Wnt4 rescued the sex reversal induced by loss of Fgf9, further supporting the Wnt4 repression model

Brightfield images of E16.5–E17.5 gonads with mesonephric-derived structures. Control XX ovaries (A) and XY testes (B) had distinct morphologies. Dotted lines surround the ovaries. (C) XY gonads carrying a deletion of *Wnt4* developed as testes, but (D) the deletion of *Fgf9* resulted in sex reversal and development of an ovary, even with the deletion of one copy of *Wnt4*. (E) Deleting both *Wnt4* and *Fgf9* rescued testis development. Scale bar=1 mm. $n \geq 3$ of each genotype.

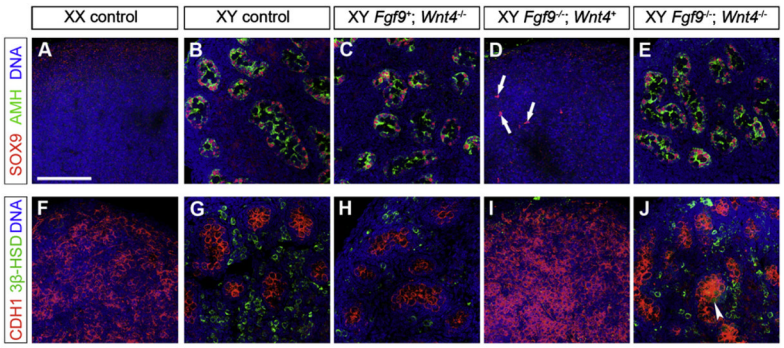

Fig. 4.

XY *Fgf9*/*Wnt4* double mutants express molecular markers typical of testis development consistent with a full rescue of sex reversal. Immunostaining of E16.5–E17.5 gonads. DNA is shown in blue. ((A)–(E)) Gonads labeled with the Sertoli-cell markers SOX9 (red) and AMH (green). Sertoli cells labeled with SOX9 and AMH were absent in XX controls (A), but present in XY controls (B) and XY *Wnt4* mutants (C). Similar to XX controls, sex reversed XY *Fgf9* mutants (D) lacked differentiating Sertoli cells, although a variable number of SOX9 positive cells were observed that did not express AMH (arrows). Sertoli cells were rescued by deleting *Wnt4* in XY *Fgf9* mutants (E). ((F)–(J)) Gonads labeled with the germ cell marker CDH1 (red) and the Leydig cell marker 3β-HSD (green). In the XX control (F), germ cells were present but not clustered into testis cords and Leydig cells were absent. In the XY control (G) and XY *Wnt4* mutant (H), germ cells were clustered into testis cords with Leydig cells positive for 3β-HSD outside of testis cords. Sex reversed XY *Fgf9* mutants (I) resembled XX controls, but deleting *Wnt4* in an XY *Fgf9* mutant (J) rescued Leydig cells and the presence of germ cells in testis cords. The arrowhead indicates occasional background staining with the 3β-HSD antibody in the testis cords. Thus, deleting *Wnt4* rescued the *Fgf9* phenotype in the XY samples based on the detection of molecular markers characteristic of the prominent testicular cell types. All images shown are maximum intensity projections of 12 images over approximately 12 μm using a 40X objective. For XY *Wnt4* ((C) and (H)) and *Fgf9* ((D) and (I)) single mutants, the other locus was either homozygous wild type or heterozygous (designated "*Fgf9*$^{+}$" and "*Wnt4*$^{+}$"). Scale bars=100 μm. $n \geq 3$ of each genotype.

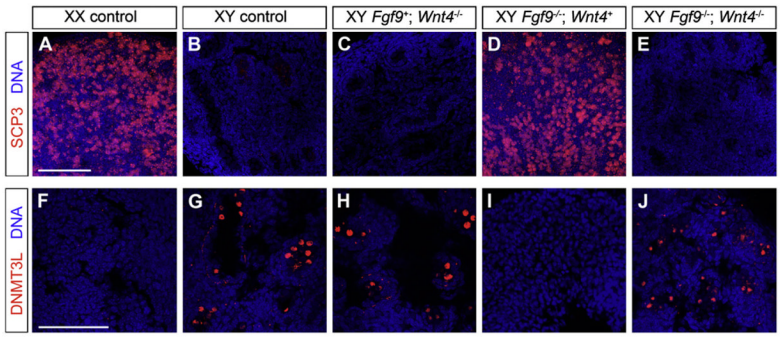

Fig. 5. Germ cells in the XY Fgf9/Wnt4 double mutants adopt the male fate

Immunostaining of E16.5–E17.5 gonads. DNA is shown in blue. ((A)–(E)) Gonads labeled with the meiosis marker SCP3 (red) that is nuclear in meiotic germ cells. Meiotic germ cells were present in XX controls (A), absent in XY controls (B) and XY *Wnt4* mutants (C), and present in sex reversed XY *Fgf9* mutants (D). The germ cells in the *Fgf9*/*Wnt4* double mutants were not in meiosis (E). Images are maximum intensity projections of 12 images over approximately 12 μm using a 40X objective. Scale bar=100 μm. n=3 of each genotype. ((F)–(J)) Gonads labeled with the male germ cell marker, DNMT3L (red). In E16.5 gonads, DNMT3L is absent in XX control gonads (F), but present in XY controls (G) and XY *Wnt4* single mutants (H). Germ cells in *Fgf9* mutants (I) do not express DNMT3L, consistent with their failure to enter the male pathway. However, DNMT3L expression is detected in *Fgf9*/*Wnt4* double mutants, suggesting rescue of the male pathway (J). Images are maximum intensity projections of 6 images over approximately 6 μm using a 40X objective. Scale bar=100 μm. n=3 of each genotype. For XY *Wnt4* ((C) and (H)) and *Fgf9* ((D) and (I)) single mutants, the other locus was either homozygous wild type or heterozygous (designated "*Fgf9*$^{+}$" and "*Wnt4*$^{+}$").

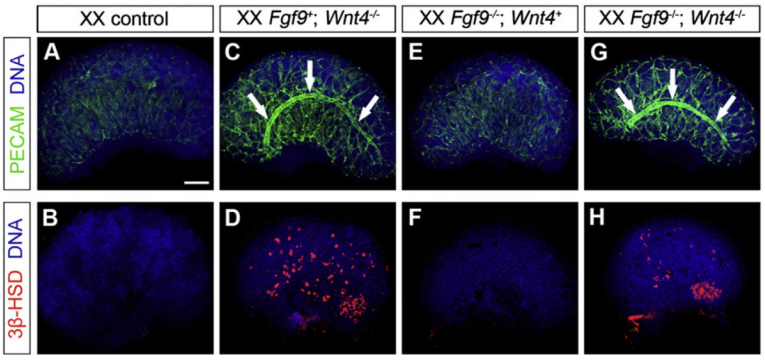

Fig. 6. Deleting Fgf9 did not rescue the phenotypes of XX Wnt4 mutants

DNA is shown in blue. ((A), (C), (E) and (G)) PECAM1 (green) labels both the endothelial cells and the germ cells, but with varying intensity in the germ cells at E16.5–E17.5. ((B), (D), (F) and (H)) E16.5–E17.5 gonads labeled with the steroidogenic marker 3β-HSD (red). Unlike XX control ovaries ((A) and (B)), XX *Wnt4* mutants developed an ectopic coelomic vessel ((C), arrows) and steroidogenic cells (D). ((E) and (F)) In the presence of one or more functional copies of *Wnt4*, *Fgf9* mutants did not develop this ectopic vessel or steroidogenic cells. However, both the ectopic vessel ((G), arrows) and steroidogenic cells (H) were present in the XX *Wnt4*/*Fgf9* double mutants. The images shown are maximum intensity projections of 5 images over approximately 10 μm ((A), (C), (E) and (G)) or 10 images over approximately 30 μm ((B), (D), (F) and (H)) taken using a 20X objective. For XX *Wnt4* ((C) and (D)) and *Fgf9* ((E) and (F)) single mutants, the other locus was homozygous wild type or heterozygous (designated "*Fgf9*$^{+}$" and "*Wnt4*$^{+}$"). Scale bar=100 μm. *n*=3 of each genotype.

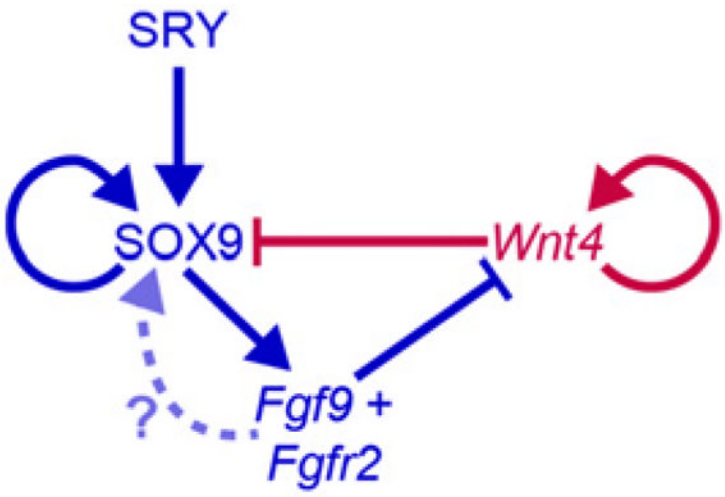

Fig. 7. Model of sex determination

A model of the genetic interactions during early gonad development. SRY up-regulates SOX9 in XY gonads, and SOX9 becomes self-regulating (Sekido and Lovell-Badge, 2008). SOX9 up-regulates *Fgf9* (Kim et al., 2006) to repress *Wnt4*, and possibly also reinforce its own expression. A failure to repress *Wnt4* (in *Fgf9* or *Fgfr2* mutants) results in repression of SOX9 by *Wnt4* signaling and loss of male development.