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Fgf9 and Wnt4 Act as Antagonistic Signals to Regulate Mammalian Sex Determination

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The genes encoding members of the wingless-related MMTV integration site (WNT) and fibroblast growth factor (FGF) families coordinate growth, morphogenesis, and differentiation in many fields of cells during development. In the mouse, Fgf9 and Wnt4 are expressed in gonads of both sexes prior to sex determination. Loss of Fgf9 leads to XY sex reversal, whereas loss of Wnt4 results in partial testis development in XX gonads. However, the relationship between these signals and the male sex-determining gene, Sry, was unknown. We show through gain- and loss-of-function experiments that fibroblast growth factor 9 (FGF9) and WNT4 act as opposing signals to regulate sex determination. In mice, Sry normally initiates a feed-forward loop between Sox9 and Fgf9, which up-regulates Fgf9 and represses Wnt4 to establish the testis pathway. Surprisingly, loss of Wnt4 in XX gonads is sufficient to up-regulate Fgf9 and Sox9 in the absence of Sry. These data suggest that the fate of the gonad is controlled by antagonism between Fgf9 and Wnt4. The role of the male sex-determining switch—Sry—in the case of mammals—is to tip the balance between these underlying patterning signals. In principle, sex determination in other vertebrates may operate through any switch that introduces an imbalance between these two signaling pathways.

Introduction

The development of sexually dimorphic reproductive organs is a common feature among animal species. The testis and ovary represent two divergent pathways of development from the bipotential embryonic gonad. The switch that initiates divergent development of the gonad is highly diverse among species; however, the underlying mechanisms that lead to the establishment of ovary or testis pathways are likely to be conserved. In all species, the embryonic gonad is made up of a mixed population of germ cells and somatic cells. This tissue is remarkable in that all of its cells are believed to be bipotential, and can differentiate into ovarian or testicular lineages [1,2]. Consistent with the idea that cells in this primordium are poised between two developmental pathways, some of the genes that are involved in establishing sexual dimorphism, including Dax1 (dosage-sensitive sex reversal-congenital adrenal hypoplasia critical region on the X chromosome protein 1), Sox9 (Sry-like HMG box 9), Fgf9 (fibroblast growth factor 9), and Wnt4 (wingless-related MMTV integration site 4), are initially expressed in similar patterns in XX and XY gonads [3–8]. The conventional view of mammalian sex determination is that the basic pathway of organ development is ovarian, and that the testis-determining gene operates by diverting this program toward testis development by simultaneously influencing the fate of the key supporting cell lineage and initiating a male-specific morphogenetic program. All of the experimental evidence suggests that these two processes are closely interwoven. For example, both proliferation [9] and migration of cells to target testis cord formation [10,11] appear to be closely integrated with Sertoli cell differentiation.

Sry, a Y chromosome-linked gene, is the primary sex-determining gene in mammals [12–14]. In the absence of Sry expression—in XX embryos, or in XY embryos carrying a deletion of Sry—cells in the gonad follow an ovarian differentiation pathway. Genetic evidence from chimeric mice [15], and expression studies using reporter transgenes [2,16], indicate that Sry expression is required only in precursors of the somatic supporting cell lineage. Expression of Sry in these bipotential cells leads to their differentiation as testis-specific Sertoli cells rather than as follicle cells, the parallel cell type of the ovary [2]. It is believed that the Sertoli cell is the first cell type to differentiate in the gonad [17]. There is substantial evidence that a critical threshold number of Sertoli cells is required to establish testis differentiation [9,15,18–20]. In cases where this threshold is not reached, ovary differentiation ensues. Once Sry expression begins, expression patterns of other genes in the gonad begin to diverge. The first gene downstream of Sry known to show male-specific up-regulation in Sertoli cell precursors is a related gene

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Abbreviations: dpc, day post coitum; EGF, enhanced green fluorescent protein; FGF, fibroblast growth factor; PECAM, platelet/endothelial cell adhesion molecule; SOX, SRY-like HMG box; SRY, sex-determining region of the Y; WNT, wingless-related MMTV integration site

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expressed in many tissues in the developing embryo, Sox9. Disruption of Sox9 expression in the XY gonad causes male-to-female sex reversal [21,22], whereas increasing the dose of Sox9 in the XX gonad leads to testis development [23–25]. These studies indicate that Sox9 plays a central role in sex determination. Unlike Sry, which is specific to mammals, expression of Sox9 is known to be conserved in the gonad of many species. In mammals, Sox9 is up-regulated immediately after Sry expression initiates. Experiments tracing Sry-expressing cells using a stable reporter demonstrated that once testis differentiation is established, all Sertoli cells that express Sox9 are descendants of cells that have expressed Sry [16], suggesting that activation of Sox9 is a cell-autonomous effect of Sry. However, mutations in several signaling pathways including Fgf9 and Igf1r/Irr/Ir (insulin-like growth factor 1 receptor/insulin receptor-related receptor/insulin receptor) resulted in loss of Sox9 expression and partial or complete sex reversal [26,27], suggesting that extracellular signaling pathways play a significant role during primary sex determination.

Mice homozygous for a null mutation in Fgf9 display male-to-female sex reversal caused by disruption of all testis-specific cellular events, including cell proliferation, mesonephric cell migration, testis cord formation, and the differentiation of Sertoli cells [26,27]. Fgf9, like many of the founding signals in the gonads, is initially expressed in gonads of both sexes, but becomes male-specific after Sry is expressed. In a reciprocal manner, expression of Wnt4, which is also initially common to gonads of both sexes, becomes female-specific [8]. XX gonads with a null mutation in Wnt4 display some obvious aspects of testicular differentiation [28]. Based on the theory that Sertoli cells initiate all downstream founding signals in the gonads, is initially expressed in gonads [16], suggesting that activation of Sox9 is a cell-autonomous effect of Sry. However, mutations in several signaling pathways including Fgf9 and Igf1r/Irr/Ir (insulin-like growth factor 1 receptor/insulin receptor-related receptor/insulin receptor) resulted in loss of Sox9 expression and partial or complete sex reversal [26,27], suggesting that extracellular signaling pathways play a significant role during primary sex determination.

To investigate these findings, we examined the genetic relationship of Sry, Sox9, Fgf9, and Wnt4 in the regulatory network that governs the gonadal field. We show that the loss of Fgf9 in homozygous mutant XY gonads does not affect the expression of SRY or the initial up-regulation of SOX9; however, SOX9 expression is not maintained in the Fgf9+/− mutant gonads, and testis differentiation is aborted. We also demonstrate that FGF9 represses the ovary-promoting gene, Wnt4. We hypothesize that FGF9 functions in a feed-forward loop to expand Sertoli precursor cells, which secrete FGF9, to a critical threshold number sufficient to suppress Wnt4. This directly or indirectly stabilizes SOX9 expression and secures the male fate of the gonad. WNT4 seems to oppose the male pathway by repressing expression of SOX9 and FGF9. Surprisingly, both male pathway genes are transiently activated in Wnt4+/− XX gonads in the absence of the Y-linked gene Sry. Based on this genetic and in vitro data, we suggest that the plasticity of the bipotential gonad is controlled by mutually antagonistic signals between FGF9 and WNT4 in the gonadal field. These signals coordinate sexually dimorphic patterns of growth, morphogenesis, and cellular differentiation.

Figure 1. Stage- and Cell-Specific Expression of FGF9 in Embryonic Gonads
(A–F) Detection of FGF9 protein (red) at different stages of gonad development. FGF9 is up-regulated in XY gonads at 11.5 (B), 12.5 (D), and 13.5 dpc (F) while it is down-regulated in XX after 11.5 dpc (A, C, and E). No signal was detected in XY Fgf9+/− gonads (unpublished data).
(G–J) Serial sections of wild-type XX and compound heterozygous KI/C0/W/Wv XY gonads stained for alkaline phosphatase (purple; G and I) and FGF9 (red; H and J). Testis cords are formed in the absence of germ cells in XY KI/C0/W/Wv mutant gonads at 12.5 dpc (arrowhead in J). Expression of FGF9 is present in the mutant gonads where Sertoli cells are the only remaining cell type in the cords (J). Semitransparent dotted line indicates the boundary between gonad and mesonephroi. PECAM (green) marks germ cells and vascular endothelial cells (C–F, H, and J). The scale bars represent 25 μm.

Results
Early Bipotential Expression of FGF9 Resolves to an XY-Specific Pattern by 12.5 dpc
Using an antibody specific to FGF9, we examined expression during normal gonad development. FGF9 protein was distributed throughout the 11.5 dpc gonad in both sexes (Figure 1A and 1B). However, by 12.5 dpc, FGF9 was detected only in XY gonads in two domains: in cells near the surface of gonads and in cells located within testis cords. This sex-specific expression pattern was maintained in gonads at 13.5 dpc (Figure 1C–1F). FGF9 expression within testis cords was localized to Sertoli cells and excluded from germ cells based on the germ cell membrane marker platelet/endothelial cell adhesion molecule (PECAM) (Figure 1D and 1F). To confirm this result, we examined agametic gonads from KI/C0/W/Wv compound heterozygous embryos, which develop testis cords containing Sertoli cells only. FGF9 was detectable at normal levels in testis cords in XY KI/C0/W/Wv gonads, where alkaline phosphatase staining verified the absence of germ cells (Figure 1G–1J), indicating that FGF9 is expressed by Sertoli cells, and that its expression is not dependent on the...
presence of germ cells. In summary, FGF9 expression was present in both XX and XY gonads at bipotential stages, and became restricted to XY gonads as testis differentiation proceeded.

Sry Expression Is Normal in Homozygous Null Fgf9 XY Gonads

The early expression of Fgf9 in bipotential gonads raised the question of whether Fgf9 is an upstream regulator of Sry. To investigate this possibility, we mated Fgf9+/− mice with a transgenic reporter line that carries an enhanced green fluorescent protein (EGFP) transgene driven by the Sry promoter Sry-EGFP. At early stages, this transgene represents the pattern of endogenous Sry expression [2]. The expression of the EGFP reporter was detected in Fgf9+/− XY gonads comparable to Fgf9+/+ littermate controls (Figure 2A and 2B), suggesting that transcriptional regulation of Sry is independent of Fgf9. Sex reversal is caused not only by the loss of normal levels of Sry expression [30,31], but also by mutations disrupting SRY import into the nucleus [32]. The transgene, Sry-EGFP, does not reflect the intracellular distribution of the SRY protein. To investigate this aspect of SRY regulation, we bred Fgf9+/− mice with another Sry reporter mouse line carrying a Myc-tagged Sry transgene, SryMyc, which recapitulates the endogenous intracellular SRY expression pattern [16]. Using an antibody against c-MYC, the expression and nuclear localization of SRYMyc in SryMyc; Fgf9+/− gonads was indistinguishable from littermate controls (Figure 2C and 2D). These data using two different Sry transgenic reporter lines provide evidence that Sry expression is not dependent on Fgf9. Therefore, Fgf9 signaling must act in parallel and/or downstream of Sry to regulate testis development.

FGF9 Can Up-Regulate SOX9 Expression

In our previous study we did not observe SOX9 expression at 12.5 dpc in Fgf9−/− XY gonads that fail to develop into normal testes [26]. However, the loss of SOX9 expression at 12.5 dpc could be a consequence of the loss of Sertoli differentiation rather than a reflection of the genetic interaction between FGF9 and SOX9. Normally, Sox9 is weakly expressed in wild-type genital ridges of both XX and XY embryos at 10.5 dpc and, after the onset of Sry expression, is up-regulated in XY gonads [5,33,16]. As results indicated that Fgf9 functioned downstream of, or in parallel with Sry, we investigated whether Fgf9 was involved in the up-regulation of Sox9 expression. Primary cell culture and gonad culture systems were used to assess Sox9 activation by exogenous FGF9. For in vitro cell culture, cells were isolated from 11.5 dpc gonads free of mesonephroi, and cultured on extracellular matrix-coated coverslips with or without addition of purified FGF9 in culture media. After culture for 24 h, Sox9 expression was monitored by an antibody specific to Sox9 in XX cells and control XY cells. Exogenous FGF9 increased cell number in XX and XY cell cultures compared with cells in a duplicate culture without FGF9 treatment (unpublished data), and caused the up-regulation of Sox9 in XX cells (Figure 2E–2H). Up-regulation of Sox9 had not previously been seen in whole XX gonads cultured with exogenous FGF9 [9]. To explain the difference between experimental results from dissociated XX gonadal cells and XX gonads, we reasoned that the local concentrations of FGF9 might not be high enough to override blocking signals in the intact XX gonad, or that active FGF9 was not efficiently localized or presented in the extracellular matrix of the XX gonad. To test the local effect of FGF9, we modified the XX gonad culture by stably immobilizing FGF9, or BSA as a control, on beads (Figure 2I–2K). Under these conditions, Sox9 expression was up-regulated locally in cells near the surface of the XX gonad in contact with the FGF9 bead (Figure 2K). Taken together, these in vitro data demonstrate that ectopic FGF9 signaling can induce SOX9 expression in XX gonadal cells, suggesting a positive interaction between Fgf9 and Sox9.
To investigate whether Fgf9 is essential for the up-regulation of Sox9 in vivo, we assessed SOX9 expression in loss-of-function Fgf9+/− and Fgf9−/− XY gonads at 11.5 dpc (D) compared with heterozygous littermate controls (A). However, SOX9 is detected in fewer cells in mutant gonads at 12.0 dpc (B and E), and is lost by 12.5 dpc (C and F).

Figure 3. Interdependent Relationship between Fgf9 and Sox9

(A–F) Immunostaining of SOX9 (red) in Fgf9+/− and Fgf9−/− XY gonads shows that Fgf9 is required for maintenance of SOX9. The up-regulation of SOX9 in Sertoli precursor cells appears normal in Fgf9+/− gonads at 11.5 dpc (D) compared with heterozygous littermate controls (A). However, SOX9 is detected in fewer cells in mutant gonads at 12.0 dpc (B and E), and is lost by 12.5 dpc (C and F).

(G–J) mRNA whole-mount in situ hybridization for Sry and Fgf9 in Sox9lox/− and Sox9−/− XY gonads shows that Sox9 is required for Fgf9 expression. Sry expression is detected in both Sox9lox/− and Sox9−/− gonads at 11.5 dpc (G and H), whereas Fgf9 expression is markedly decreased or absent in Sox9−/− gonads at 11.5 dpc (I and J).

(K–O) Comparison of cell proliferation in Sox9−/− versus Sox9lox/− gonads at 11.5 dpc using immunostaining for phosphorylated histone H3. XY-specific proliferation at the gonad surface (K) is reduced in the absence of Sox9 (L). Bar graph (O) shows quantitation of proliferation obtained by counting positive cells in the cortical region of each gonad (right brace) and normalizing to the number obtained from XY Sox9lox/− gonads. n = 30, with five sections of each gonad and three pairs of gonads for each genotype. PECAM, green (A–F and K–N). The scale bars represent 25 μm. DOI: 10.1371/journal.pbio.0040187.g003

Fgf9 Is Required for Maintaining SOX9 Expression in XY Gonads

To investigate whether Fgf9 is essential for the up-regulation of Sox9 in vivo, we assessed SOX9 expression in loss-of-function Fgf9+/− and Fgf9−/− XY gonads at 11.5–12.5 dpc (Figure 3A–3F). In wild-type and heterozygous mutant XY gonads at 11.5 dpc, SOX9 is detected in a small number of cells in the gonad (Figure 3A). Over the next 6 h of development, nuclear SOX9 accumulated rapidly in cells toward the cortex and the anterior and posterior poles of the gonad, replicating patterns previously reported for both Sry and Sox9 expression [2,16,34–36]. This unique pattern was also observed in Fgf9−/− XY gonads (Figure 3D and 3E). Somatic cells within Fgf9−/− gonads were positive for SOX9 at 11.5 dpc, the earliest stages examined, demonstrating that initial expression and up-regulation of SOX9 were not disrupted in Fgf9+/− mutant XY gonads prior to 12.0 dpc. Notably, in Fgf9+/− XY gonads, SOX9 was no longer detectable by 12.5 dpc, and Sertoli precursor cells never began to organize into normal testis cord structures (Figure 3F). These data indicate that although Fgf9 is not required for the up-regulation of Sox9 in vivo, it is indispensable to maintain Sox9 expression in Sertoli precursor cells.

Sox9 Is Required for Fgf9 Up-Regulation in XY Gonads

We hypothesized that if the linear relationship among the three genes were Sry → Fgf9 → Sox9, expression of Fgf9 would be normal in XY gonads in the absence of Sox9. Alternatively, if the relationship were Sry → Sox9 → Fgf9, expression of Fgf9 should be reduced or absent in XY gonads in the absence of Sox9. We examined Fgf9 expression in Sox9 homozygous mutant (Sox9−/−) XY gonads generated by crossing mice...
homozygous for a conditional null (flox) allele of Sox9 (Sox9flox/flox) with mice carrying germline-specific Cre transgenes, Prm1-Cre in male and Zp3-Cre in female [22,37]. The Sox9 null mutant embryos die after 11.5 dpc because of cardiovascular defects [37]. Chaboissier et al. [22] successfully cultured 11.5 dpc Sox9null mutant gonads in vitro and detected male and female markers after 2–3 d of culture, suggesting that Sox9 mutant gonads are viable and developmentally competent at 11.5 dpc—the time point at which we collected samples to perform mRNA in situ hybridization (Figure 3G–3J). The expression of Fgf9 was significantly decreased or absent in XY Sox9D/D gonads at 11.5 dpc (Figure 3J), while Sry expression was similar to wild-type (Figure 3G and 3H), as previously reported [20], suggesting that Fgf9 expression in wild-type XY gonads is dependent on the expression of Sox9. These findings also indicate that expression of Sry is not sufficient to regulate Fgf9 in the absence of Sox9. Therefore, we conclude that Sox9 is essential for Fgf9 expression, and Fgf9, in return, maintains Sox9 expression, generating a positive feed-forward loop between these two genes in XY gonads.

Like Fgf9 Mutant Gonads, Sox9A/Δ XY Gonads Show Defects in Cell Proliferation

We previously reported that XY-specific cell proliferation is defective in Fgf9−/− XY gonads [7]. Because Sox9 acts as a positive regulator of Fgf9 expression, we questioned whether cell proliferation in XY gonads was also compromised by the loss of Sox9. We examined proliferation in Sox9A/Δ gonads at 11.5 dpc using a mitotic cell marker, phosphorylated histone H3. Proliferating cells were more abundant and concentrated in a domain near the surface of wild-type XY gonads, and this XY-specific cell proliferation was evident in XY Sox9flox/+ littermate controls (Figure 3K). However, in Sox9A/Δ XY gonads proliferation was reduced and similar to XX gonads (Figure 3L–3O). This result supports the idea that there is a mutual interdependence between Sox9 and Fgf9 generating a positive feed-forward loop, and that both genes are required for the expansion of somatic cells, including Sertoli cell precursors, in XY gonads.

The Male Pathway Is Aborted in Fgf9−/− Sertoli Precursors

Based on the fact that Sox9 is initially expressed in Fgf9−/− gonads, we investigated whether other genes in the male pathway are activated. We examined two markers for Sertoli cell differentiation, anti-Müllerian hormone (Amh) [38] and Desert hedgehog (Dhh) [39] in Fgf9−/− XY gonads using whole-mount in situ hybridization. Dhh, which is expressed in XY wild-type and heterozygous gonads beginning at 11.5 dpc, was absent from Fgf9−/− XY gonads, although mesonephric expression was still detected (Figure 4A and 4B). Amh, which is a direct transcriptional target of SOX9 activated after 11.5 dpc in wild-type XY gonads, was expressed at levels similar to the XX controls (Figure 4C and 4D). This result further supports the idea that Sox9 and Fgf9 are essential for the expansion of somatic cells, including Sertoli cell precursors, in XY gonads.
dpc [38,40], was detected at reduced levels in Fgf9−/− XY gonads at 12.5 dpc (Figure 4C–4F). The residual level of Ambh suggested that the transient expression of SOX9 in Fgf9−/− gonads at 11.5 dpc was sufficient to activate Amh, a direct downstream target. However, the absence of Dhh indicated that not all Sertoli pathways are initiated.

The initial specification of Sertoli cell precursors was not affected by the loss of Fgf9, as evidenced by normal Sry and Sox9 expression in Fgf9−/− gonads at 11.5 dpc (Figures 2 and 3D). However, SOX9 expression in XY Fgf9−/− gonads rapidly disappeared (Figure 3E and 3F), and other Sertoli markers were absent or severely reduced (Figure 4A–4F). To investigate the possibility that this loss was due to cell death, we found that FGF9 was expressed in XY gonads (Figure 6A–6C). Using an antibody against FGF9, we found that FGF9 was expressed in Fgf9+/- XY gonads rapidly—tubules and ducts, another site of FGF9 expression (Figure 4G and 4H). These data suggested that the loss of SOX9 expression in Fgf9−/− XY gonads was not caused by cell death but by the disruption of FGF9/SOX9 feed-forward regulation.

To determine whether the aborting of the male pathway in Fgf9−/− Sertoli precursors was associated with the transition of supporting cells from male to female differentiation, we investigated expression of Wnt4, an ovary-promoting gene. At 12.5 dpc Wnt4 was up-regulated in XY Fgf9−/− but not in XY Fgf9+/- gonads (Figure 4I–4K). This result suggests that Fgf9 is necessary for the down-regulation of Wnt4 in differentiating XY gonads after bipotential stages.

**Fgf9 and Wnt4 Antagonize Each Other**

Our finding that high levels of Wnt4 persist in Fgf9−/− XY gonads implies a genetic antagonism specifically between Fgf9 and Wnt4, as both Sry and SOX9 are initially expressed in Fgf9−/− XY gonads at 11.5 dpc, yet this is not sufficient to down-regulate Wnt4. To test whether exogenous FGF9 could down-regulate expression of Wnt4, we cultured the XX gonads mesonephros complex with or without FGF9 protein, and examined Wnt4 expression by whole-mount in situ hybridization. Treatment of XX gonads with exogenous FGF9 suppressed the normal expression of Wnt4 (Figure 5A–5C), supporting the hypothesis that Fgf9, rather than Sry or Sox9, functions to down-regulate Wnt4 in wild-type XY gonads.

We reasoned that if FGF9 and Wnt4 do act as opposing signals, then reduction in the dose of Wnt4 might render the XX gonad more susceptible to the male-promoting effects of Fgf9. This result suggested that FGF9 is normally down-regulated by WNT4 in XX gonads.

**Figure 5. Mutual Antagonism between Fgf9 and Wnt4**

(A–C) Wnt4 whole-mount in situ hybridization on gonad cultures. Adding exogenous FGF9 in gonad cultures results in the down-regulation of Wnt4 expression in cultured XX gonads (C). Controls (A and B) were cultured without FGF9 peptide.

(D–G) Reduction in the dose of Wnt4 allows FGF9 to induce SOX9 in XX gonads. Immunostaining of SOX9 (red) shows that addition of FGF9 up-regulates SOX9 expression in heterozygous Wnt4+/- XX gonads (G), but not in Wnt4−/− XX gonads (F), PCAM, green. The scale bars represent 50 μm. DOI: 10.1371/journal.pbio.0040187.g005

Given our finding that FGF9, a positive regulator of Sox9, is derepressed in XX Wnt4+/- gonads, we asked whether expression of SOX9 might also occur in XX Wnt4−/− gonads (Figure 6D–6L). An antibody against SOX9 revealed that expression was initially up-regulated in Wnt4−/− XX gonads at 11.5 dpc (Figure 6F and 6I), although it was rapidly down-regulated by 12.0 dpc and absent at 12.5 dpc (Figure 6L and 6K). This finding was confirmed by mRNA in situ hybridization, which also detected Sox9 transcripts in 11.5 dpc Wnt4−/+ XX gonads (Figure 6I). Wnt4−/+ XX gonads do not increase in size comparable to normal XY gonads (Figure 6C, 6F, 6L, and 6L), and Sertoli cell differentiation and testis cord formation do not occur. Nevertheless, it is noteworthy that up-regulation of Sox9 occurs in this case in the absence of Sry, by eliminating the antagonistic effect of Wnt4 and up-regulating FGF9, supporting our hypothesis that sex determination occurs by tipping the balance between these two opposing signals.

**Discussion**

Many studies support the view that cells in the undifferentiated gonad are bipotential; the supporting cell precursor lineage can develop into follicle cells or Sertoli cells. In Fgf9−/− XY gonads, cells initially embark on the Sertoli pathway, but in the absence of Fgf9 can neither maintain Sox9 expression nor establish downstream male pathways. The loss of Sertoli cells in XY Fgf9−/− gonads is not due to cell death, but instead...
be recruited to the Sertoli lineage, indicating that non-cell-autonomous signaling mechanisms operate under these conditions [15]. Other more recent studies have suggested that paracrine signals could be involved in the establishment of Sertoli cells [43–46]. The current study reveals that ectopic FGF9 can induce SOX9 under conditions in which XX cells are dissociated (Figure 2F), when an FGF9-coated bead is directly applied to the XX gonad (Figure 2K), or when the dose of Wnt4 is reduced (Figure 5G). Whether FGF9 normally acts non-cell-autonomously in vivo to recruit XY cells to the Sertoli lineage by up-regulating SOX9 is not clear. We show that Sry can initially up-regulate SOX9 in the absence of Fgf9, suggesting that FGF9 is not necessary for this step. However, FGF9 may act to trigger cell proliferation, increasing the number of Sertoli precursors above a threshold needed to stabilize the male pathway, consistent with threshold requirements deduced from earlier studies using XX→XY chimeric gonads [15]. Since Sertoli cells produce FGF9, loss of proliferation of Sertoli precursors may result in a reduction of the overall level of FGF9, and/or other male paracrine signals, below a critical threshold level required to antagonize the influence of WNT4. This model is appealing, because it links cell proliferation, believed to be required for establishment of the male pathway [9], with Sertoli fate determination.

A recent study by Yoshioka et al. [47] showed that misexpression of Fgf9 in chick nephrogenous mesenchyme led to the expansion of gonadal marker gene expression, implicating Fgf9 in gonadal cell proliferation across species.

It has been suggested that SOX9 represses WNT4 based on misexpression studies [48]. Here we show that the addition of FGF9 protein to XX gonad explant cultures repressed the expression of Wnt4. Down-regulation of Wnt4 is unlikely to occur through SOX9, as SOX9 is not up-regulated in this situation [7]. Furthermore, although both SRY and SOX9 are initially expressed in Fgf9+/− XY gonads, Wnt4 is not down-regulated in the absence of Fgf9 (Figure 4K). These findings support the idea that FGF9 acts as the antagonist of Wnt4. Antagonism of WNT signals may be a multistep process involving both the transcriptional down-regulation of Wnt4 observed in this study and the destabilization of downstream Wnt intracellular pathways that antagonize SOX9 expression, as shown in chondrocyte differentiation [49], or that compete for intracellular signal transducers as has been reported in other systems [50,51]. Future work will address these possibilities.

In support of the idea that Wnt4 antagonizes the male pathway, we found that the loss of Wnt4 caused the up-regulation of both SOX9 and FGF9 in XX gonads where Sry is absent. It appears that the male pathway can be initiated by disrupting the balance between Wnt4 and Fgf9, a finding that has strong implications for other vertebrate sex-determination systems in which Sry is not the sex determining factor. However, up-regulation of Sox9 is not sufficient to establish testis development in this mutant, as occurs in Odsex and other gain-of-function mutants where Sox9 is misexpressed in the XX gonad [24,25]. In those two misexpression cases, Sox9 expression may have been artificially sustained by exogenous regulatory sequences that bypass the fine dosage balance in this signaling network.

In Wnt4 mutants, SOX9 expression is not maintained. In light of the observation that the Wnt4+/− XX gonad does not increase significantly in size (Figure 6), it is possible that the
FGF9/Sox9-expressing population did not reach a critical threshold. Alternatively (or in addition), another male-specific factor normally dependent on Sry may be required to sustain Sox9 expression, possibly FGF-binding proteins in the extracellular matrix or FGF receptors. It is equally plausible that there are other female-specific factors that antagonize the establishment of Sox9 expression. It has been observed that several other Wnts are expressed in the XX gonad [52], and these or other factors may partially compensate for the loss of Wnt4.

These findings suggest that Wnt4 signaling normally acts as a repressor of the male pathway by interfering with the up-regulation of Sox9 expression. One report of a duplication of the region of human Chromosome 1, which includes Wnt4, led to an intersex phenotype [53]. However, the report constitutes only circumstantial evidence. Such a role is not supported by efforts to misexpress Wnt4 in XY gonads, which have led to very mild phenotypes with no evidence for defects in Sertoli cell differentiation [54]. It is possible that Wnt4 protein did not function as an active signal in these transgenic mice, either because it was not expressed in the right cells, at the right time, or at the right level. Consistent with our data and the partially sex-reversed phenotype of Wnt4+/- XX mutants, other Wnts or additional female factors may be required.

The switch that controls sex determination is biologically diverse. Sry is not present in nonmammalian systems; however, antagonistic signaling between FGFs and WNTs may be the conserved mechanism that balances the gonad between testicular and ovarian fates in vertebrates. In theory, any genetic or environmental switch may tip the balance toward the male pathway. Based on our findings we propose that cells in the mammalian gonad are balanced between two competing cell fates by counterbalanced signaling pathways, Fgf9, expressed near the coelomic surface, and Wnt4, expressed near the mesonephric border (Figure 7). In mammalian XY gonads, the onset of Sry expression initiates the male pathway by up-regulating Sox9. Sox9 up-regulates Fgf9, which initiates a Sox9/Fgf9 feed-forward loop that accelerates commitment to the male pathway. In XX gonads or XY mutant gonads lacking Sry, Sox9, or Fgf9, the Sox9/Fgf9 feed-forward loop is not established, and Wnt4 gains control of the gonadal field. This results in the down-regulation of Sox9 and Fgf9, tilting the balance toward commitment to the female pathway. Further experiments will be required to define the molecular mechanism of Fgf9 and Wnt4 action. However, our in vivo and in vitro data strongly support the antagonistic relationship of these two signaling pathways in regulating expression of the testis-determining factor Sox9.

**Materials and Methods**

**Animals and genotyping.** The Fgf9 mutation was maintained on a C57BL/6J (B6) background that leads to sex reversal in 100% of XY Fgf9+/- offspring. Sry-EGFP mice, a kind gift from K. Albrecht and E. Eicher, were initially on a mixed B6/129 and were backcrossed to B6 for five generations. Offspring were then crossed to Fgf9+/- and intercrossed and backcrossed to B6 in alternating generations. All XY Fgf9+/- offspring showed complete sex reversal. Sry+/- mice were maintained on a CBA background, and Wnt4 on a mixed 129/SVJ background. Mutant embryos were sexed by PCR using Y chromosome-specific primers and were genotyped as described [2,16,26,55]. Mice homozygous for the Sox9 deletion were generated using a germline-specific gene deletion system as described [20].

**In situ hybridization and immunocytochemistry.** In situ hybridization was performed on paraformaldehyde-fixed/OCT embedded cryosections, as described [56]. Whole-mount in situ hybridization was performed as previously described [57]. Probes used for in situ hybridization were: Amh [58], Dhh [20], Wnt4 [8], and Fgf9 [59]. Digoxigenin-labeled probes were prepared according to the Boehringer-Mannheim-Roche protocol.

Antibodies used in whole-mount immunocytochemistry were: mouse monoclonal anti-N-MYC (Cell Signaling Technology, Beverly, Massachusetts, United States; 1:100), rabbit anti-caspase-3 fragment (BD Bioscience, San Diego, California, United States; 1:500), rabbit anti-phosphorylated histone H3 (Cell Signaling; 1:250). Antibody binding was detected using fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania, United States).
were incubated in 50 μg/ml FGF9 for 2 h, and washed five times in culture medium.

Supporting Information
Figure S1. Confirmation that Transcription of Sox9 Is Up-Regulated in Wnt4−/− XX Gonads
Whole-mount in situ hybridization for Sox9 detected Sox9 expression in 11.5 dpc Wnt4−/− XY controls and Wnt4−/− XX gonads. Found at DOI: 10.1371/journal.pbio.0040187.sg001 (425 KB PPT).

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Reference.