Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination

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Targeted mutagenesis of *Fgf9* in mice causes male-to-female sex reversal. Among the four FGF receptors, FGFR2 showed two highly specific patterns based on antibody staining, suggesting that it might be the receptor-mediating FGF9 signaling in the gonad. FGFR2 was detected at the plasma membrane in proliferating coelomic epithelial cells and in the nucleus in Sertoli progenitor cells. This expression pattern suggested that *Fgfr2* might play more than one role in testis development. To test the hypothesis that *Fgfr2* is required for male sex determination, we crossed mice carrying a floxed allele of *Fgfr2* with two different *Cre* lines to induce a temporal or cell-specific deletion of this receptor. Results show that deletion of *Fgfr2* in embryonic gonads phenocopies deletion of *Fgf9* and leads to male-to-female sex reversal. Using these two *Cre* lines, we provide the first genetic evidence that *Fgfr2* plays distinct roles in proliferation and Sertoli cell differentiation during testis development.

Fgfr2 | growth factor signaling | organogenesis | Sertoli cells | testis determination

The differentiation of the gonad is directed toward the testis pathway when the Y chromosome gene, *Sry*, is expressed in somatic progenitor cells (1–3). Precursor cells expressing *Sry* up-regulate the expression of a related gene, *SRY-box containing gene 9* (*Sox9*), and initiate differentiation as Sertoli cells (4, 5). The differentiation of Sertoli cells is the pivotal event essential for testis differentiation and male sex determination. As the first testis-specific cell type, Sertoli cells coordinate early testicular morphogenesis, including testis cord formation, mesonephric cell migration, and testis-specific vascularization. Most evidence suggests that Sertoli cells induce the differentiation of other testis cell types, such as peritubular myoid cells and Leydig cells (6, 7). The male fate is established in somatic precursor cells by collective functions of transcription factors and signaling molecules. Targeted deletion of the extracellular signaling factor (*Fgf9*) results in disrupted Sertoli cell differentiation and male-to-female sex reversal, indicating that *Fgf9* is required for testis fate determination (8).

Fgfs regulate a broad range of cellular activities, including proliferation, survival, migration, and differentiation in many organs during embryonic development. *In vivo* and *in vitro* studies suggested that *Fgf9* plays several direct or indirect roles in testis development and male sex determination (8–14). However, it has been difficult to tease these roles apart.

FGF9 is required for XY-specific proliferation of cells in the coelomic epithelium, which is the earliest cellular process known to occur as XX and XY gonad development begins to diverge downstream of *Sry.* Cell proliferation increases the Sertoli cell population and is an obligate event for testis formation based on blocking assays (15). These data, combined with the finding that a threshold number of XY cells is required to initiate testis development in XX ↔ XY chimeric gonads (16), have led to the hypothesis that FGF9 is required for proliferation to establish numbers of Sertoli cells sufficient to block the competing female pathway and to establish testis development. However, there was no clear evidence for a direct role of FGF9 in Sertoli precursor proliferation. Furthermore, because proliferation and differentiation of Sertoli cells are so closely intertwined, it has not been clear whether loss of proliferation and reduced Sertoli cell numbers could be solely responsible for failure of the testis pathway in *Fgf9* mutants or whether *Fgf9* has a distinct function within Sertoli cells to maintain *Sox9* during differentiation. One way to dissect the potential roles of FGF9 is to genetically address the function of Fgf receptors in the gonad.

In mammals, Fgfs bind to four high-affinity receptor tyrosine kinases, FGFR1–FGFR4, and to heparan sulfate proteoglycans in the extracellular matrix (17, 18). The expression patterns of Fgf ligands and Fgf receptors are spatiotemporally distinctive but often overlapping, and ligand–recepter interactions are possible in various combinations, complicating the understanding of their *in vivo* functions. Previous immunocytochemical analysis of Fgf receptors in mouse embryonic gonads indicated that all four receptors (FGFR1–FGFR4) are expressed in XX and XY gonads during early development (10). Among the four receptors, FGFR2 showed plasma membrane localization in coelomic epithelial cells, consistent with a role of *Fgf9* in mediating proliferation of these cells. In contrast, it showed a nuclear localization specifically in Sertoli progenitor cells within the interior of the gonad. This expression pattern made FGFR2 a good candidate for the receptor that mediates the activity of *Fgf9* in Sertoli progenitors, and suggested that it might play a role at multiple steps in the testis pathway. However, the unexpected nuclear localization of this tyrosine kinase receptor led to skepticism about the fidelity of the polyclonal antibody. To sidestep this problem we chose to take a genetic approach to test the role of *Fgfr2* in testis formation.

Fgfr2 -null mice die before gonad formation (19). To overcome the embryonic lethality, we generated conditional inactivation of *Fgfr2* by crossing mice carrying a *Fgfr2* allele flanked with *loxP sites* (*Fgfr2*^{flox}) (20) with two different transgenic *Cre* lines that direct either a temporal or a cell-specific deletion of this receptor. Phenotypic and molecular marker analysis in *Fgfr2* deletion mutant gonads uncovered genetic evidence that *Fgfr2*

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Abbreviations: dpc, days post coitum; SCP, synaptonemal complex protein.

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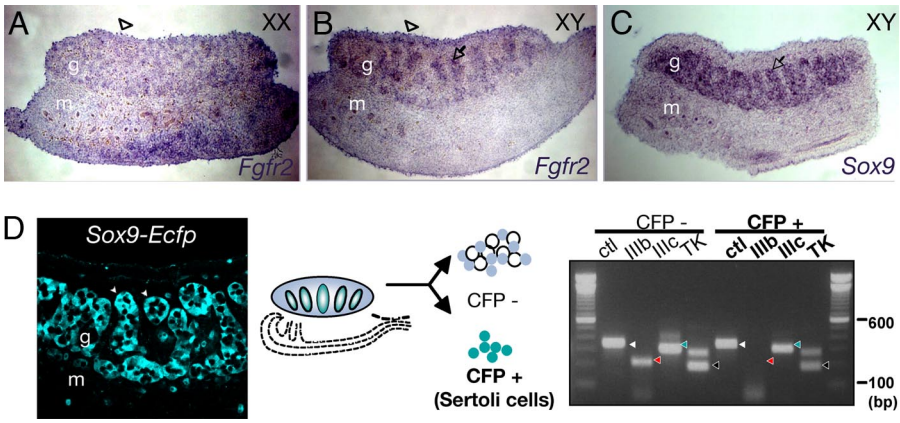

Fig. 1. Expression of *Fgfr2* transcripts in embryonic gonads. (*A–C*) mRNA *in situ* hybridization of *Fgfr2* and *Sox9* on frozen sections of XX and XY gonads at 12.5 dpc. *Fgfr2* antisense riboprobe reveals *Fgfr2* expression in the coelomic domain (arrowhead) of XX and XY gonads and inside the testis cords (arrow), but not in the interstitium, of XY gonads. *Sox9* is a comparative control for expression in Sertoli cells inside testis cords. (*D*) RT-PCR analysis of splicing isoforms of *Fgfr2* in fluorescence activation-sorted Sertoli cells. A confocal image of *Sox9-Ecfp* XY gonad shows Sertoli cells marked with EGFP (arrowheads). EGFP+ (Sertoli) cells express mainly *Fgfr2-IIIc*; whereas EGFP− cells express both isoforms. PCR products are *Hprt* (white arrowhead), *Fgfr2-IIIb* (red arrowhead), *Fgfr2-IIIc* (green arrowhead), and the tyrosine kinase domain of *Fgfr2* (black arrowhead).

plays an essential role during testis determination. The finding that deletion of *Fgfr2* phenocopies *Fgf9* knockout gonads strongly suggests that *Fgfr2* is the receptor for FGF9 in the gonad. Furthermore, these results, generated using two complementary *Cre* lines, support the hypothesis that *Fgfr2* plays distinct roles in the proliferation of progenitor cells and Sertoli cell differentiation during testis development.

Results

The Expression of *Fgfr2* in Gonads and Isolated Sertoli Cells. Because of concerns about the fidelity of the FGFR2 antibody, we set out to confirm the expression pattern of *Fgfr2* at the mRNA level. Using mRNA *in situ* hybridization on sections of gonads at 12.5 days post coitum (dpc), *Fgfr2* transcripts were detected in the coelomic domain of XX and XY gonads and in testis cords in a pattern similar to that of *Sox9* (Fig. 1 *A–C*). This finding supports our previous results using an antibody against the tyrosine kinase domain of FGFR2, in which the receptor protein was detected in coelomic epithelial cells and Sertoli precursor cells (10).

Using RT-PCR analysis with two different primer sets specific to the sequences within the transmembrane domain and the intracellular tyrosine kinase domain of *Fgfr2*, transcripts were detected in both XX and XY gonads (data not shown), consistent with our *in situ* hybridization data. To validate the expression of *Fgfr2* in Sertoli cells, we took advantage of transgenic mice expressing a Sertoli cell reporter, *Sox9-Ecfp*. ECFP-positive Sertoli cells were isolated by fluorescence-activated cell sorting, and RT-PCR of *Fgfr2* was performed using primers that distinguish the expression of the two major receptor isoform variants, *Fgfr2-IIIb* and *-IIIc*. The results showed that *Fgfr2-IIIc* was detectable in the isolated cells, whereas *Fgfr2-IIIb* expression was beneath the detection limit. This finding indicates that Sertoli cells express *Fgfr2* and that *Fgfr2-IIIc*, to which FGF9 binds with high affinity in *in vitro* assays (21), is the major isoform of *Fgfr2* in Sertoli cells (Fig. 1*D*).

Conditional Deletion of *Fgfr2* in Gonads Between 10.5 and 11.5 dpc Results in Disruption of Testis Morphogenesis. Because deletion of one isoform of *Fgfr2* can lead to compensation by the other isoform (refs. 22 and 23), we chose to analyze a null mutation of *Fgfr2.* To avoid the early embryonic lethality associated with deletion of *Fgfr2*, we obtained mice carrying a floxed allele ($Fgfr2^{flox/+}$) to generate a conditional mutation of *Fgfr2.* CRE recombinase-mediated excision of the floxed allele deletes a region including the ligand-binding domain and transmembrane domain of *Fgfr2* and creates a functional null allele (20). We conditionally deleted *Fgfr2* by using a heat-shock-inducible *Cre*, *Hs-Cre* (24), which mediates a stage-specific deletion of the receptor.

To test the efficiency of the heat-shock-activated CRE, male *Hs-Cre*/+ mice were crossed to female *LacZ*/*Egfp* (*Z*/*EG*) transgenic mice in which CRE-mediated *loxP* excision activates *Egfp* expression. Pregnant females were treated by heat shock at 10.5 dpc, and embryos were dissected at 11.5 dpc to examine the gonad for EGFP expression. Greater than 90% of cells in the genital ridge were positive for EGFP at the time of dissection (Fig. 2 *A* and *B*), indicating that excision by *Hs-Cre* is highly effective throughout the gonads. Male mice heterozygous for both *Hs-Cre* and $Fgfr2^{flox}$ (*Hs-Cre*/+;$Fgfr2^{flox/+}$) were crossed with $Fgfr2^{flox/+}$ female mice for timed matings. A heat shock was administered to pregnant females at 10.5 dpc, such that loss of *Fgfr2* would affect gonads after the initial formation of the genital ridge. After the heat-shock treatment, embryos were allowed to develop for a short period, until dissection at 12.5–13.5 dpc. Conditional mutants at 13.5 dpc were normal in body size (Fig. 2*C*), indicating that significant deletion of *Fgfr2* before heat-shock treatment was unlikely to have occurred during earlier stages, when the gene is essential for the survival and growth of embryos. However, in mutant embryos at 12.5–13.5 dpc, all XY mutant gonads failed to grow to normal sizes (Fig. 2 *E* and *F*), and limb defects (typically, a short limb with abnormal digits) were often detected (Fig. 2*C* and data not shown). Affected XY gonads were indistinguishable from normal XX gonads at the level of stereomicroscopy (data not shown). Heat-shock treatment alone does not impair the growth of gonads, because littermates that were wild type for either *Hs-Cre* or $Fgfr^{flox}$ appeared phenotypically normal after the treatment (data not shown). These results indicate that the deletion of *Fgfr2* affected growth of the limb and the XY gonad during a temporal window during which cell proliferation increases. The small XY *Hs-Cre*;$Fgfr2^{flox/flox}$ gonads showed no sign of testis structures such as testis cords and coelomic vasculature, which are normally discernable by 12.5 dpc. Immunostaining at 13.5 dpc for laminin, a protein localized to the perimeter of testis cords, and PECAM, a marker for endothelial cells and germ cells, verified that the mutant XY gonads fail to form testis cords or the typical testicular coelomic vessel (Fig. 2 *D–F*).

Because Sertoli cells are critical for organizing testis cord

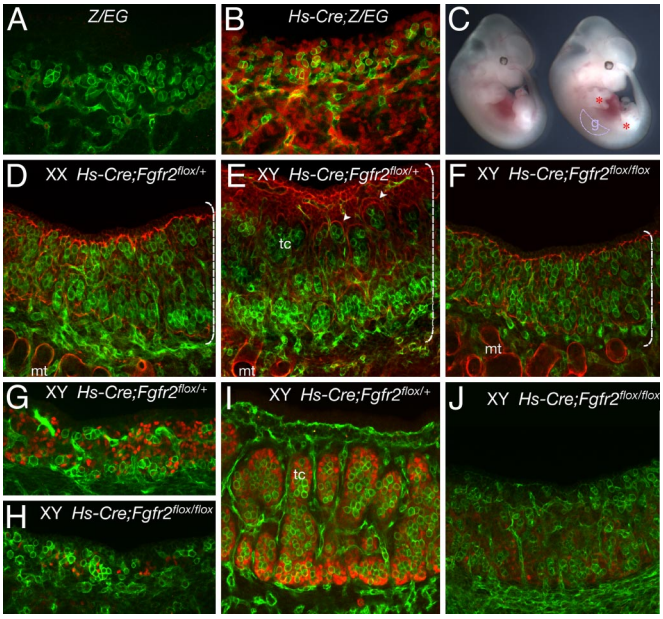

Fig. 2. Sex reversal caused by stage-specific conditional inactivation of *Fgfr2* by *Hs-Cre*. (*A* and *B*) *Hs-Cre* efficiency in gonads was accessed in *Z/EG* reporter mice. Confocal scanning microscopy shows that EGFP reporter (red) is expressed in most cells throughout the gonad and mesonephros after heat shock, but expression is absent in *Z/EG* gonads in the absence of *Hs-Cre*, indicating time-specific global recombination in the genital ridge. PECAM (green) is a marker for germ cells and endothelial cells. (*C*) Stereomicroscopy image of control (left) and *Hs-Cre/+;Fgfr2*$^{flox/flox}$ (right) embryos 48 h after heat-shock treatment. Body shape and size is grossly normal after the treatment. However, inactivation of *Fgfr2* results in defective growth in limbs and gonads. Asterisks indicate mutant limbs in heat-shock-treated animals. g, gonad, outlined. (*D–F*) Immunohistohemistry and confocal scanning microscopy of gonads from the heat-shock-treated embryos. Immunostaining with laminin (red) and PECAM (green) reveals that *Hs-Cre/+;Fgfr2*$^{flox/flox}$ XY gonads lack testis cords (tc), which normally form by this stage in littermate controls (arrowheads in *E*). Brackets highlight relative size of control and mutant XY gonads. mt, mesonephric tubules. (*G–J*) Control and mutant gonads immunostained for SOX9 at 11.5 dpc (*G* and *H*) or 12.5 dpc (*I* and *J*). SOX9 (red) is present in the mutant gonads at 11.5 dpc, albeit in fewer cells. By 12.5 dpc, SOX9 expression is almost absent in the mutant XY gonads.

structures, we investigated whether Sertoli cells differentiate in these mutant XY gonads by monitoring SOX9 expression between 11.5 and 12.5 dpc. SOX9 was present both in the littermate control XY gonads and in the mutant XY gonads at 11.5 dpc, but the number of SOX9-expressing cells in the mutants was reduced, often to only a few positive cells (Fig. 2 *G* and *H*). SOX9 expression was detectable in only a few cells by 12.5 dpc (Fig. 2 *I* and *J*), indicating that Sertoli differentiation was blocked. Overall, the conditional deletion of *Fgfr2* by *Hs-Cre* at the critical stages of sex determination recapitulated the phenotype of *Fgf9* null-mutant gonads: XY-specific gonad growth was blocked and testis differentiation was disrupted downstream of the initiation of SOX9 expression.

The Conditional Deletion of *Fgfr2* in Pre-Sertoli Cells Rescues Gonad Growth but Blocks Sertoli Cell Differentiation. Our previous study suggested a model in which *Fgf9* is required for maintenance of SOX9 and the stable commitment of precursor cells to the Sertoli cell fate (13). However, it did not distinguish between a role of *Fgf9* in the proliferation/expansion of Sertoli precursors or in the differentiation/maintenance of Sertoli precursors. The model predicts that if *Fgfr2* were the receptor mediating *Fgf9* activity in Sertoli precursor cells, the deletion of *Fgfr2* specifically in these cells would disrupt testis determination. To generate an *Fgfr2* deletion specific to somatic progenitor cells, we used a different *Cre* transgenic line, *Sf1-Cre*, which express a *Cre* transgene driven by the regulatory sequences of *steroidogenic factor 1* (SF1) (also called *Nr5a1*) (25). Endogenous SF1 expression is detected at high levels at 11.0–11.5 dpc in coelomic epithelial cells that proliferate and give rise to somatic progenitors within the gonad and in Sertoli precursors at the time when testis cords form (26). To determine which cells express the transgenic Sf1-Cre in gonads at bipotential stages, we crossed *Z/EG* females to *Sf1-Cre* males and examined EGFP reporter expression. In *Sf1-Cre/+;Z/EG* gonads at 11.5 dpc, EGFP was detected in a subset of somatic cells within the gonad and only a few coelomic epithelial cells (four of four gonad pairs examined) (Fig. 3*A*). *Sf1-Cre/+;Fgfr2*$^{flox/+}$ males were mated with *Fgfr2*$^{flox/flox}$ females, directing *Fgfr2* deletion to a subset of Sertoli precursor cells. In *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ embryos, early gonad formation occurred normally. Mutant XY gonads were only slightly smaller than those of littermate controls (compare Fig. 3*F* with Fig. 3*G* versus the size difference in Fig. 2*E* and *F* with *Hs-Cre*). Consistent with this observation, immunostaining for phosphorylated histone H3 (pHH3), a mitotic cell marker, revealed little difference in proliferation in the coelomic domain of *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ XY gonads and *Sf1-Cre/+;Fgfr2*$^{flox/+}$ littermates (Fig. 3 *D* and *E*), in contrast to the difference between wild-type XY and XX at 11.5 dpc (Fig. 3 *B* and *C* and ref. 26). However, *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ XY gonads showed aberrant testis cord formation based on laminin immunostaining (Fig. 3 *F* and *G*): Little detectable laminin deposition occurred, although cells aggregated as if cord formation were initiated.

Unlike *Hs-Cre/+;Fgfr2*$^{flox/flox}$ mutant gonads, in which SOX9 expression was reduced at this stage (Fig. 2 *G* and *H*), SOX9 was expressed at near-normal levels at 11.5 dpc in *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ gonads (Fig. 3 *H* and *I*), suggesting that a significant population of pre-Sertoli cells is initially established, likely through the rescue of early proliferation by using *Sf1-Cre* as opposed to *Hs-Cre* to trigger deletion of *Fgfr2*. However, SOX9 is dramatically decreased by 12.5 dpc (Fig. 3 *J* and *K*). These results indicate that the maintenance of the Sertoli cell differentiation pathway is impaired, owing to the deletion of *Fgfr2* in pre-Sertoli cells in the mutant gonads. Earlier work (13) predicted that the loss of *Fgfr2* would cause the disruption of the *Sox9/Fgf9* feed-forward loop and a failure to repress the ovary pathway. Accordingly, in *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ XY gonads, *Wnt4,* an ovary-specific marker that is normally down-regulated in XY gonads by 12.5 dpc, was derepressed and expressed at variable levels (Fig. 3 *L–N*). This finding suggests that the competing female pathway was activated in the mutant gonads.

Ovotestis Formation in *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ XY Gonads. By 15.5 dpc, *Sf1-Cre/+;Fgfr2*$^{flox/flox}$ XY gonads resolved into an ovotestis structure evident at the stereomicroscopy level, in which the adrenal is also seen to be reduced or absent (Fig. 4 *A–H*). On the basis of histological analysis, testis cords were present in the central regions, albeit rather disrupted (Fig. 4 *E* and *G*) relative to those of XY littermate control (Fig. 4 *D* and *F*); however, they were absent from anterior (and often posterior) regions of the gonad where meiotic figures characteristic of ovarian germ cells were histologically evident in germ cell nuclei (Fig. 4*H*). SOX9 was detected only in domains where there was some evidence of cord-like organization, but not in the anterior region of the gonad (Fig. 4*K*). Instead, the meiotic marker synaptonemal complex protein 3 (SCP3) was detected in the anterior domains of the mutant XY gonads, implying that these germ cells may be under the influence of the ovarian somatic environment, as described in recent studies (27, 28). Interestingly, there was a region of overlap in which SOX9 and the meiotic marker SCP3 were simultaneously detected (Fig. 4 *K* and *K*″). This finding raises questions about the role of FGF signaling in establishing the block to meiosis that is characteristic of the male pathway.

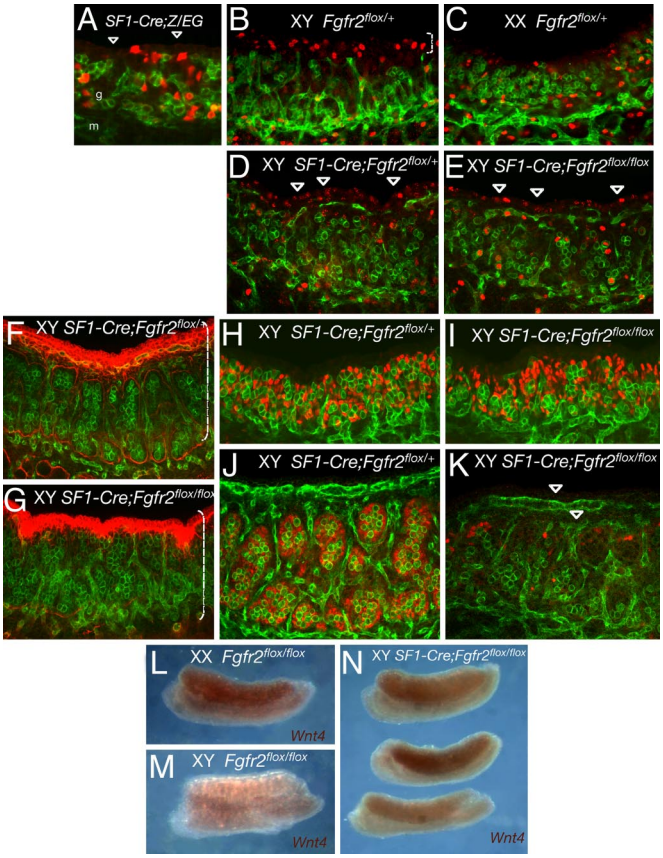

Fig. 3. Abnormal testis development caused by conditional deletion of *Fgfr2* by *SF1-Cre* in somatic progenitor cells. PECAM1 is green throughout. (*A*) Confocal scanning microscopy of a *SF1-Cre/+;Z/EG* gonad at 11.5 dpc shows a typical example of *SF1-Cre*-mediated activation of the Z/EG reporter (red) in a subset of gonadal somatic progenitor cells. Arrowheads indicate coelomic domain where few cells show recombination (compare with Fig. 2*B*). g, gonad; m, mesonephros. (*B–E*) Immunostaining and confocal scanning microscopy of a mitotic marker pHH3 (red) in controls and *SF1-Cre;Fgfr2*$^{flox/flox}$ gonads at 11.5 dpc. Somatic cells in coelomic domain show increased proliferation specific to XY gonads (bracket in *B*). (*D and E*) Proliferation in the coelomic domain (arrowheads) is only slightly decreased in XY *SF1-Cre;Fgfr2*$^{flox/flox}$ gonads relative to the normal levels in XY littermates. (*F–K*) There is little difference in gonad size between controls and mutant XY gonads at 12.5 dpc (brackets in *F* and *G*). (*G*) However, immunostaining of laminin (red) reveals aberrant testis cord formation in the mutant gonad. (*I* and *K*) SOX9 expression (red) appears normal in the mutant gonads at 11.5 dpc (*I*), but by 12.5 dpc it is severely reduced (*K*). PECAM reveals fragments of the testis vasculature (arrowheads in *K*). (*L–N*) Whole-mount *in situ* hybridization of *Wnt4* in control and mutant gonads at 12.5 dpc. (*L* and *M*) In control gonads, *Wnt4* expression is specific to XX gonads. (*N*) In XY *SF1-Cre/+;Fgfr2*$^{flox/flox}$ gonads, *Wnt4* is activated but variable between samples and across the gonad field.

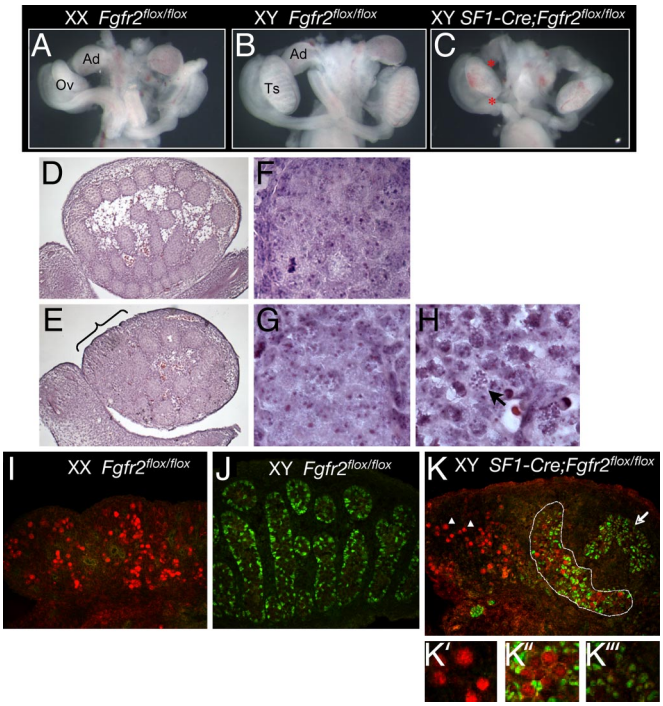

Fig. 4. Ovotestis formation in XY *SF1-Cre/+;Fgfr2*$^{flox/flox}$ gonads at 15.5 dpc. (*A–C*) Stereomicroscopy images of urogenital systems of controls and XY *SF1-Cre/+;Fgfr2*$^{flox/flox}$ mutant. In addition to gonadal defects, the adrenal glad is severely reduced or absent in these mutants. Ad, adrenal; Ov, ovary; Ts, testis; *, mutant adrenal and gonad. (*D–H*) Histology of wild-type XY and *SF1-Cre/+;Fgfr2*$^{flox/flox}$ XY gonads at 15.5 dpc. (*E–H*) Higher magnification views show testis cords in the wild-type XY gonad (*F*), disrupted cords from the central region in the mutant XY gonad (*G*), and ovarian region (*H*) located in the anterior of the mutant gonad (bracket in *E*). Arrow indicates the nucleus of a germ cell in meiosis. (*I–K*) Confocal scanning microscopy of sections of 15.5 dpc gonads immunostained with SOX9 (green) and SCP3 (red). (*I* and *J*) SCP3-positive germ cells are characteristic of wild-type XX gonads, but not XY gonads at this stage, whereas, SOX9 shows the reverse pattern. (*K′–K‴*) The ovotestis contains a region of SCP3-positive cells in the anterior domain (arrowhead in *K′*), a region where both markers are present (dashed line in *K″*), and a separated cluster of SOX9-positive cells where SCP3 is absent (arrow in *K‴*).

Taken together, findings in this study provide genetic evidence that *Fgfr2* is necessary for testis formation and male sex determination and support a model in which paracrine signals are crucial to the establishment of testis or ovarian patterning of early gonads.

Discussion

The similarities in the phenotypes of *Fgfr2* and *Fgf9* mutants suggest that FGFR2 is the receptor for FGF9 in the XY gonad. The generation of conditional deletions of *Fgfr2* allowed us to examine the role played by *Fgfr2* in gonads during sex determination. *Fgfr2* is essential for the wave of male-specific proliferation that establishes Sertoli progenitor cells in the XY gonad. We further demonstrated that *Fgfr2* is required in pre-Sertoli cells to direct Sertoli cell differentiation, separating this role for Fgf signaling from the early proliferation defect characteristic of *Fgf9* mutants.

The XY-specific cell proliferation is concentrated mostly at the surface of the gonad at 11.5 dpc (Fig. 3 *B* and *C* and ref. 26). This proliferation increase is not only responsible for the growth difference between XY and XX gonads by 12.5 dpc (15, 26), it is also critical for testis determination as cells dividing at the coelomic epithelium give rise to Sertoli and interstitial cells (29). Although it is evident that there is no size increase and no testicular morphogenesis in the *Hs-Cre;Fgfr2*$^{flox/flox}$ XY gonads (Fig. 2 *E* and *F*), *Sf1-Cre;Fgfr2*$^{flox/flox}$ XY gonads display near wild-type growth and improved testicular morphology (Fig. 3 *F* and *G*). Immunohistochemistry of a mitotic marker in *Sf1-Cre;Fgfr2*$^{flox/flox}$ XY gonads showed that proliferation of cells in the coelomic domain was close to normal levels, suggesting that the conditional deletion using *Sf1-Cre* did not affect these proliferating cells. Despite the substantial proliferation, *Sf1-Cre;Fgfr2*$^{flox/flox}$ XY gonads fail to maintain pre-Sertoli cells by 12.5 dpc (Fig. 3 *J* and *K*), suggesting that *Fgfr2* is required at a step subsequent to proliferation to direct the differentiation of Sertoli cells. The partial phenotype in the mutant XY gonads may be due to incomplete deletion of *Fgfr2*^{flox} alleles. The presence of ovarian regions restricted to the poles of the gonad is typical of other cases of ovotestis formation (30). The polar

DEVELOPMENTAL BIOLOGY

regions of the gonad may be more sensitive to loss of paracrine signals than the central region, where *Sry* is expressed at its highest levels (3, 31, 32).

Numbers of SOX9-positive cells in *HS-Cre* gonads are variable but usually reduced (Fig. 2*H*) compared with normal gonads or $Fgf9^{-/-}$ gonads. In normal development, the number of these precursors increases as a result of cell proliferation induced by feed-forward loops between Fgf9 and Sox9 (13, 26, 33). One possible explanation for the normal SOX9 expression in $Fgf9^{-/-}$ gonads at 11.5 dpc is that another Fgf can partially compensate for the loss of *Fgf9*. There are other Fgfs expressed at early stages in embryonic gonads (34–36). Perhaps in the absence of *Fgfr2*, cells are insensitive to an additional Fgf signal.

We previously reported the expression of FGFR2 in the plasma membrane of coelomic epithelial cells and in the nuclei of pre-Sertoli cells based on antibody staining patterns (10). Although this expression pattern implicated *Fgfr2* in testis determination, the data were inconclusive because (*i*) all four FGFRs were detected in somatic progenitors of XY gonads and (*ii*) the specificity of the polyclonal antibody reagent detecting the unusual localization of FGFR2 in the nuclei of pre-Sertoli cells could not be conclusively verified. Our genetic approach demonstrates that *Fgfr2* is required for proliferation of coelomic epithelial cells and in pre-Sertoli cells to establish the Sertoli cell fate, strongly suggesting that differential intracellular localization of the receptor has distinct functional relevance.

We show that *Fgfr2-IIIc* is expressed in isolated pre-Sertoli cells, whereas both *Fgfr2-IIIb* and *Fgfr2-IIIc* are expressed in other gonadal cells, including coelomic epithelial cells. It is interesting to speculate whether the regulation of alternative splicing in somatic progenitor cells is related to a switch from the proliferation of progenitors to the differentiation of Sertoli cells. Eswarakumar *et al.* (37) reported that homozygous $Fgfr2\text{-}IIIc^{-/-}$ mice were fertile, which suggests that Sertoli cells in the mutant gonads were functional. Homozygous $Fgfr2\text{-}IIIb^{-/-}$ mice are perinatal lethal (38), thus the fertility of the mutant animals cannot be assessed. We examined the gonads in $Fgfr2\text{-}IIIb^{-/-}$ embryos at 12.5–14.5 dpc and found no evidence of the disruption of Sertoli cell differentiation (data not shown). A disruption of one isoform of *Fgfr2* led to the compensatory expression of another alternative splicoform in other studies (22, 23). Thus, it is not clear how alternative isoform variants of *Fgfr2* underlie the regulation of Sertoli cell fate determination. This possibility should be investigated in a new system in which both alternative splicing and nuclear localization of FGFR2 can be visualized. If this regulation can account for a mechanism leading to a transition between proliferation and differentiation, it will have important implications, particularly in epithelial-to-mesenchymal transition during normal organogenesis and tumorigenesis.

Our study suggests that mutations in FGFR2 may account for an unassigned subset of human patients who show ambiguous sexual development. We hope our findings will motivate molecular analysis of FGF signaling in human syndromes associated with congenital malformations, including sexual development defects.

Methods

Mice. The *Sox9-Ecfp* transgenic mice were generated in the Duke University Medical Center Transgenic Facility by using a construct provided by R.S. and R.L.-B. (unpublished manuscript). *Cre* transgenic lines *Hs-Cre6* and *Sf1-Cre*, in which *Hsp70–1* promoter and a regulatory region of *Sf1* (also called *Nr5a1*) respectively drive expression of *Cre* recombinase, are described in refs. 24 and 25. The $Fgfr2^{flox}$ line was originally generated by Yu *et al.* (20). In the mating scheme in this study, the stage-specific inactivation of *Fgfr2* was achieved by crossing $Fgfr2^{flox/flox}$ females to F_1 generation *Hs-Cre*/+;$Fgfr2^{flox/+}$ males. Pregnant females were heat-shocked at 10.5–11.0 dpc to delete *Fgfr2* conditionally at bipotential stages in embryonic gonads. For heat shock treatment, pregnant mice were placed in a prewarmed container inside a 42° C oven for 12 min. The cell lineage-specific inactivation of *Fgfr2* was generated by crossing male *Sf1-Cre*/+;$Fgfr2^{flox/+}$ to $Fgfr2^{flox/flox}$ females. Mutant animals were bred on a mixed C57BL/6;129 genetic background. Genotyping for the floxed *Fgfr2* allele and the *Hs-Cre* and *SF1-Cre* transgenes was determined by PCR using specific primers described in refs. 20, 24, and 25.

***In Situ* Hybridization and RT-PCR.** mRNA *in situ* hybridization was performed on frozen sections (*Fgfr2*) or on whole-mount preparations (*Wnt4*) of gonads by using a standard protocol. The plasmid template for riboprobes of *Fgfr2* and *Wnt4* were gifts from David Ornitz (Washington University, St. Louis, MO) (39) and Andy McMahon (Harvard University, Cambridge, MA) (40). The urogenital ridge was isolated from timed *Sox9-Ecfp* transgenic embryos. Gonads were separated from the mesonephros and treated with collagenase (0.025%; Sigma, St. Louis, MO) to dissociate cells, and Sertoli cells were isolated by fluorescence-activated cell sorting. Total RNA was prepared either from embryonic gonad tissues (dissected free of the mesonephros) or isolated Sertoli cells by using TRIzol reagent as instructed by the manufacturer (GIBCO/BRL, Carlsbad, CA), and quantified using a standard spectrophotometer method (Bio-Rad Laboratories, Hercules, CA). The total RNA sample was amplified with the SuperScript One-Step RT-PCR system (Invitrogen, Carlsbad, CA) using specific primer sets. The RT-PCR program was one cycle of 45° C for 15 min; followed by one cycle of 94° C for 2 min; and 32 cycles of 94° C for 30 sec, 50° C for 30 sec, and 72°C for 1 min. The primer sequences were 5′CGCCTGTGAGAGAGAAGGAGATCACG3′ and 5′AACAACGCGTCTGTCCTCAACAGC3′ to detect the tyrosine kinase domain of *Fgfr2*; 5′CCCATCCTCCAAGCTGGACTGCCT 3′ and 5′CAGAGCCAGCACTTCTGCATTG 3′ for *Fgfr2-IIIB*; 5′CCGCCGGTGTTAACACCAC3′ and 5′TGTTACCTGTCTCCGCAG 3′ for *Fgfr2-IIIC*; 5′CCTGCTGGATTACATTAAAGCACTG3′ and 5′GTCAAGGGCATATCCAACAACAAAC3′ for *Hprt*. Reaction products were resolved on a 3% agarose gel alongside a 100-bp DNA ladder.

Immunohistochemistry and Histology. Whole-mount embryonic gonads were immunostained with antibodies directed against laminin (1:250; a gift of H. Erickson, Duke University Medical Center), SOX9 (1:1,000; a gift of F. Poulat, Institut de Genetique Humaine, Montpelier, France), phospho-histone H3 (1:250; Cell Signaling Technology, Danvers, MA), SCP3 (1:400; Novagen, San Diego, CA) and PECAM (1:250; BD BioScience, San Jose, CA). Double immunohistochemistry was detected by Cy3- and Cy5-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), and imaged using confocal scanning microscopy (LSM510; Zeiss, Thornwood, NY). For histology, embryos were fixed in 4% paraformaldehyde in PBS, saturated through a sucrose series, and embedded in optimal cutting temperature (OCT; Sakura Rinetek, Torrence, CA). Frozen samples were sectioned at 8-μm thickness and stained with hematoxylin and eosin.

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