Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination

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Summary

Recently, we demonstrated that loss of Fgf9 results in a block of testis development and a male to female sex-reversed phenotype; however, the function of Fgf9 in sex determination was unknown. We now show that Fgf9 is necessary for two steps of testis development just downstream of the male sex-determining gene, Sry: (1) for the proliferation of a population of cells that give rise to Sertoli progenitors; and (2) for the nuclear localization of an FGF receptor (FGFR2) in Sertoli cell precursors. The nuclear localization of FGFR2 coincides with the initiation of Sry expression and the nuclear localization of SOX9 during the early differentiation of Sertoli cells and the determination of male fate.

Supplemental data available online

Key words: FGF, Sex determination, Sertoli cell, Sry, Gonad, Testes

Introduction

Sex determination provides an ideal system for examining how a binary fate choice (male or female) is integrated with processes that govern growth and morphogenesis during development. The early gonad is morphologically identical in XX and XY individuals, and the same genes and developmental processes appear to be essential to its formation in both sexes. However, between 10.5 and 12.5 days post coitum (dpc), the expression of a gene on the Y chromosome, Sry, initiates the developmental processes necessary to shape the XY gonad into a testis (Gubbay et al., 1990; Koopman et al., 1991). Subsequent to Sry expression, the development of the XY gonad diverges from the XX, and by 12.5 dpc drastic changes in gene expression, cell differentiation and organ morphology are observed in the XY gonad. By contrast, few changes are observed in the XX gonad during this period. Thus, Sry acts at the top of a hierarchical cascade of events that directs gonadal cells to form a testis.

The direct targets of Sry are not known. However, Sry expression is restricted to the lineage of a single cell type, the Sertoli cell (Albrecht and Eicher, 2001; Lovell-Badge et al., 2002). Sry acts through the Sertoli cell population to determine the sex of the gonad. Studies in XY↔XX chimeras indicate that if a threshold number of Sertoli cells is present, they can recruit all other cells in the gonad (XX or XY) to the testis pathway (Burgoyne and Palmer, 1993). Thus, logical targets of Sry include intracellular factors that influence the development of Sertoli cells, and secreted signaling factors that exert a paracrine influence on the surrounding cells. However, most studies of signaling molecules in the testis have focused on juvenile development and adult functions of the testis, such as spermatogenesis and the regulation of hormone production (reviewed by Lamb, 1993; Yan et al., 1998; Chamindrani Mendis-Handagama and Siril Ariyaratne, 2001). Very little information is available regarding the expression and function of growth factors within the gonad during stages of early development and sex determination.

Recently, members of three signaling pathways have been identified that play a role in early testis formation: platelet-derived growth factor receptor alpha (Pdgfra) (Brennan et al., 2003), Desert hedgehog (Dhh) (Yao et al., 2002) and the insulin growth factor receptors (Nef et al., 2003). XY mice homozygous for deletions of fibroblast growth factor 9 (Fgf9) (Yao et al., 2002) and the Desert hedgehog (Dhh) (Yao et al., 2002) and the insulin growth factor receptors (Nef et al., 2003). XY mice homozygous for deletions of Pdgfra or Dhh exhibit abnormalities in the structural development of the testis and in the differentiation of the steroid cell lineage (Leydig cells), but they do not show primary defects in Sertoli cell differentiation, and they are not sex reversed. Thus, although Pdgfra and Dhh are necessary for aspects of testis development, they are not crucial for Sertoli cell differentiation and the primary commitment to the testis fate. Triple mutants of three insulin growth factor receptors (Ir, Igf1r, Irr), show male to female sex reversal. Sry levels are reduced and male development is not established; however, the primary role of insulin growth factor signaling in testis development is not yet clear.

We have previously shown that >80% of XY mice with homozygous deletions of fibroblast growth factor 9 (Fgf9) do not express Sertoli cell markers, such as Sox9 and Amh and fail to develop testis cord structures. As a consequence, most Fgf9–/– XY mice develop as sex-reversed females (Colvin et al., 2001a). However, although Fgf9 was known to be essential for testis development, the function of this growth factor, the cell types responsive to it, and the stages at which it was acting

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were not known. In this study, we show that Fgf9 acts downstream of Sry and the initiation of male development, and is essential for two events in early testis development: (1) the upregulation of proliferation in a population that contains Sertoli cell precursors, and (2) the localization of an FGFR receptor (FGFR2) in the nucleus of differentiating Sertoli cells. These events coincide with the earliest stages of sex determination, between 11.0 and 11.2 dpc, suggesting that they play a crucial role in this process. In fact, the nuclear localization of FGFR2 overlaps with Sry expression in Sertoli cells and colocalizes with the first known marker of male development downstream of Sry, SOX9. Although growth factor receptors have been seen in the nuclei of cultured cells (reviewed by Goldfarb, 2001; Wells and Marti, 2002), this is the first biologically relevant instance where the nuclear localization of these receptors is associated with the specification/differentiation of a specific cell type in a developing organ.

Materials and methods

Staging and genotyping prenatal gonads

Genotypes were determined by PCR as previously described (Colvin et al., 2001a). Mice carrying the Fgf9 deletion were received on a mixed background of 129 and C57BL/6 in which the contribution of each strain was unknown. To produce a more uniform phenotype, the Fgf9 deletion was backcrossed onto the C57BL/6 background. By the third backcross onto this background, testis morphology was no longer observed in Fgf9+/− XY gonads. Subsequently intercross and backcross generations were alternated. To produce staged embryos, female Fgf9+/− mice were housed with males of the same genotype overnight. Mating was assumed to take place at midnight. For accurate staging of the embryo, the number of tail somites (ts) between the hind limb and the tip of the tail were counted at the time of dissection. Using this method, 10.5 dpc corresponds to 8 ts, 11.5 dpc to 18 ts and 12.5 dpc to 30 ts (Hacker et al., 1995). To determine the sex of each fetus, the amniotic sac was stained to identify chromatin bodies in XX cells (Palmer and Burgoyne, 1991).

Immunofluorescence

Gonads were dissected from staged embryos and fixed in 4% paraformaldehyde. We could detect no difference between Fgf9+/− and Fgf9+/+ gonads. Therefore, these samples were pooled and all littermate controls were labeled Fgf9+/−, but may be either. Immunofluorescent staining used protocols previously described (Schmahl et al., 2000). The basal lamina surrounding testis cords and beneath the coelomic epithelium was detected using a 1:250 dilution of a rabbit polyclonal antibody against laminin, kindly provided by Harold Erickson. Cell types in the gonad were detected using 1:250 dilutions of a rat IgG antibody against PECAM (Pharmingen 01951D), and rabbit polyclonal antibodies against SF1 (kindly provided by Ken-ichiuro Morohashi) or WT1 (Santa Cruz Biotechnology C-19). FGFR1, FGFR2, FGFR3 and FGFR4 were detected using rabbit polyclonal antibodies from Santa Cruz Biotechnology (sc-121 C-15, sc-122 C-17, sc-123 C-15 and sc-7590 M20, respectively). These antibodies were raised to peptides mapping at the C terminus of these receptors (residues 808-822 of human FGFR1, 805-821 of human FGFR2, 792-806 of human FGFR3) and have been tested by immunoprecipitation and western blotting by Santa Cruz. We have also tested the specificity of the antibody against FGFR2 (see Fig. S1 at http://dev.biologists.org-supplemental). Appropriate secondary antibodies were obtained from Jackson Immunologicals. Most antibodies were used on whole gonads, except those against FGFR1, FGFR3 and FGFR4, which did not completely permeate whole tissue and worked best on cryosections. In these samples, gonads were fixed as above, then embedded in 3:1 OCT:20% sucrose and sectioned at 10 μm. Samples were mounted for confocal imaging as described by Karl and Capel (Karl and Capel, 1998). Images were collected using a Zeiss LSM 410 confocal microscope and processed using Adobe Photoshop.

SOX9 was detected using a 1:200 dilution of a rabbit polyclonal antibody (kindly provided by Peter Koopman). Both SOX9 and FGFR2 were raised in rabbits. Therefore, SOX9 and FGFR2 double labeling was detected using the double labeling protocol described by Albrecht and Eicher (Albrecht and Eicher, 2001). Controls samples with either secondary antibody omitted showed no crossreactivity with the inappropriate secondary antibody. Additionally, XX gonads (which do not express Sox9 at 12.5 dpc), showed only FGFR localization using this protocol, indicating that the overlap of SOX9 and FGFR2 labeling seen in XY samples is due to the colocalization of the proteins, not to crossreactivity of the two secondary antibodies.

To detect Sry expression, transgenic mice containing a EGFP reporter driven by a 7762 bp 5′ flanking region of the Sry gene were obtained from Eva Eicher (Albrecht and Eicher, 2001). Homozygous individuals were mated to ensure that the reporter was detectable by autofluorescence. Mice were typed by PCR as described previously Albrecht and Eicher (Albrecht and Eicher, 2001).

Detection of proliferating cells

BrdU was used to detect proliferating cells as previously described (Schmahl et al., 2000). To determine if FGF9 induced proliferation in culture, XX and XY gonads were dissected between 11.0 and 11.5 and cultured for 2 hours in culture media (10% FCS, 50 μg/ml ampicillin and 5% CO2 in DMEM) containing 10 μM BrdU, with or without 50 ng/ml FGF9 (15 nM, R&D systems #273-F9). After induction with FGF9, gonads were rinsed three times for 15 minutes in culture media, and cultured for 12 hours in shallow grooves on agar blocks as previously described (Martineau et al., 1997), then fixed in 4% paraformaldehyde at 4°C overnight.

In situ FGF9 binding assay

Embryonic gonads (11.5 dpc) were embedded in sucrose/OCI and cryosectioned at 12 μm. Sections were dried, then fixed in 4% paraformaldehyde for 30 minutes on ice. Before incubation with human FGF9, sections were blocked in 10% fetal calf serum in PBS for 1 hour. Sections were incubated in incubation solution (0.7 μg/ml human FGF9 and 1% fetal calf serum in PBS) for 1 hour at room temperature followed by the three washes for 30 minute each in PBS. For the heparinase assay, heparinase I and III (Sigma) were added at 0.1 U/ml to the sections, incubated for 1 hour at 37°C, and the enzyme-treated sections were tested for human FGF9 binding. Sections were then blocked using an M.O.M. kit (Vector Laboratories) and bound human FGF9 was detected with 1:50 mouse monoclonal anti-human FGF9 antibody (R&D systems) and FITC-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories). The anti-human FGF9 antibody did not detect endogenous mouse FGF9 in control sections.

In situ hybridization and FGF induction

To detect Fgf9 expression, gonads were fixed and processed for in situ hybridization as previously described (Henrique et al., 1995), using probes described in Colvin et al. (Colvin et al., 1999). At 11.5 dpc, Fgf9 could only be detected in gonads that had first been cryosectioned (see above protocol). To determine if FGF9 induces male-specific genes, gonads were cultured in culture media with or without 50 ng/ml FGF9 for 36 hours in shallow grooves on agar blocks as previously described (Martineau et al., 1997), fixed in 4% paraformaldehyde and processed for in situ hybridization or immunofluorescence. In situ probes used were Amh, Sox9, Scx and Dhh, kindly provided by Robin Lovell-Badge, Peter Koopman, Keith Parker and Andy McMahon, respectively.
Fig. 1. Fgf9+/– XY gonads on the C57BL/6 background do not form testis cords. Testis cords are visible in wild-type XY gonads by 12.5 dpc (A,C) and consist of groups of germ cells (red, detected with an antibody against PECAM) surrounded by a layer of Sertoli cells and enclosed by a basal lamina (green, detected with an antibody against laminin). PECAM (red) also labels cells in the vasculature. Fgf9+/– XY gonads on the C57BL/6 background did not form testis cords (B,D). In wild-type XY gonads, SF1 (green) is detected in the nuclei of Leydig precursors (ley) outside testis cords and, at a lower level, in the nuclei of Sertoli cells (s) within testis cords at 12.5 dpc (examples indicated in E). In Fgf9+/– XY gonads, a smaller number of SF1-positive cells were detected, located near the surface of the gonad (F). Laminin (green) and germ cell (red) localization in Fgf9+/– XX gonads at 12.5 dpc (H) were indistinguishable from Fgf9+/– XX gonads (G). Scale bars: 50 μm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from embryonic gonad tissues, which were dissected free of the mesonephros, using Trizol reagent as instructed by manufacturer (GIBCO BRL). The sample was amplified using SuperScript™ One-Step RT-PCR system (Invitrogen) using one cycle of 45°C for 15 minutes, followed by one cycle of 94°C for 2 minutes and 32 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 1 minute. The primer pairs for Fgf9 and Hprt are 5′AGGCAGCTGTACTGCAGGAC′ and 5′GTCAGG3′ and 5′GTCAGG3′ and 5′GTCAGG3′, respectively. Reaction products were resolved alongside a 100 bp DNA ladder on a 2% agarose gel.

Results

Fgf9 is essential for the development of the testis, but not the ovary

On a mixed background (129;C57BL/6), the phenotype of Fgf9+/– XY gonads is variable, ranging from the formation of small areas of disorganized testis structures and cell types in ~15% of samples, to the complete absence of testis-specific markers (Colvin et al., 2001a). The sample was a more uniform phenotype, the Fgf9 deletion was bred onto the C57BL/6 background. On this background, Fgf9+/– XY littermates formed normal testes by 12.5 dpc (Fig. 1A,C). However, in Fgf9+/– XY gonads testis structures did not form by 12.5-13.5 dpc, the basal lamina of testis cords was not detected and germ cells were dispersed throughout the gonad (Fig. 1B,D). Furthermore, cells expressing steroidogenic factor 1 (SF1; a marker of Sertoli and Leydig cells in the XY gonad), were reduced in number and concentrated at the surface of the gonad in a pattern consistent with the XX gonad at this stage (Fig. 1E,F). Subsequent studies were performed on this genetic background.

Although testis development was clearly affected in Fgf9+/– XY gonads, Fgf9+/– XX gonads developed normally. Early Fgf9+/– XX gonads had normal structures (Fig. 1G,H). The Fgf9 deletion is lethal at birth because of a defect in lung development (Colvin et al., 2001b), but Fgf9+/– XX fetuses developed normal female internal reproductive organs by the time of birth (Colvin et al., 2001a). Thus, Fgf9 is essential only for the early development of the XY gonad, not the XX, suggesting that Fgf9 acts downstream of Sry and the initiation of the male pathway, but upstream of the events of testis morphogenesis.

Proliferation is reduced in Fgf9+/– XY gonads before cord formation

Abnormalities in testis formation were observed in Fgf9+/– XY gonads by 12.5 dpc (Colvin et al., 2001a). However, the sexual fate of the gonad is determined earlier. Although XY and XX gonads are still morphologically identical at 11.5 dpc, Sry reaches its peak expression level and initiates the process of testis determination in the XY gonad by this stage (Hacker et al., 1995). The earliest known morphological change induced by Sry in the XY gonad is an increase in proliferation at the surface of the gonad (Schmahl et al., 2000). Previous experiments have shown that early stages of proliferation are necessary for cord formation and the expression of male-specific markers (Schmahl and Capel, 2003). As FGF9 is known to be mitogenic for a variety of cell types and is expressed near proliferating zones in many systems (Colvin et al., 1999), we examined proliferation in Fgf9+/– XY gonads at early stages of sex determination. For accurate staging during early periods of gonad development, tail somites (ts) were counted.

Using BrdU to label dividing cells, we found that proliferation was reduced in Fgf9+/– XY gonads as early as 11.2 dpc (14-15 ts; Fig. 2A,D). This stage occurs before any other known morphological differences between XY and XX gonads, during a period that proliferation blocking experiments have shown to be crucial for Sertoli cell differentiation (Schmahl and Capel, 2003). By 11.5 dpc (18 ts), a male-specific increase in proliferation is normally visible in cells at or near the surface of the XY gonad, in the coelomic epithelium
Between 11.5 dpc and 12.5 dpc, the XY gonad undergoes a rapid increase in width, such that the XY gonad is roughly twice the size of the XX by 12.5 dpc. This size increase is largely driven by the increase in proliferation in the XY gonad (Schmahl and Capel, 2003). Consistent with the loss of this rapid increase in width, such that the XY gonad is roughly twice the size of the XX by 12.5 dpc. This size increase is largely driven by the increase in proliferation in the XY gonad (Schmahl and Capel, 2003). Consistent with the loss of this

Thus, the disruption of internal structures, the smaller organ size and the reduced levels of proliferation seen in Fgf9+/- XY gonads are specific to testis development.

**Fgf9 and all four FGF receptors are expressed in both XX and XY gonads, but FGFR2 has a sexually dimorphic expression pattern**

The gonad phenotype of Fgf9+/- embryos is sex specific. However, using both in situ hybridization (Fig. 4A) and RT-PCR (Fig. 4B), Fgf9 transcripts were detected within both XY and XX gonads at 11.5 dpc, as well as within the mesonephric duct and tubules of the adjacent mesonephroi of both sexes, indicating that the sex-specific phenotype is not determined by sex-specific expression of Fgf9 at this stage. Later in testis development (12.5 dpc), Fgf9 expression is downregulated in the XX gonad and restricted to the testis cords of the XY gonad. To determine if the FGF receptors might mediate the early sex specific phenotype in Fgf9+/- gonads, we used receptor-specific antibodies against the four FGF receptors; FGFR1, FGFR2, FGFR3 and FGFR4 (Fig. 5). In these samples, germ cells were detected with an antibody against platelet endothelial cell adhesion molecule (PECAM). FGFR1, FGFR3 and FGFR4 showed widespread expression in germ cells, and, at a lower level, in most somatic cells throughout both XY and XX gonads at 11.5 dpc. However, the expression patterns of these receptors were not sex specific at this stage.

![Image](image_url)
Fig. 4. *Fgf9* expression. (A) Using in situ hybridization, *Fgf9* was expressed in both XY and XX gonads (g) during early stages of gonad development (11.5 dpc). By 12.5 dpc, *Fgf9* was specific to testis cords in XY gonads and not detected in XX gonads. In the neighboring mesonephros (m), *Fgf9* was expressed within the mesonephric duct and tubules (mt, arrows) of both sexes. (B) RT-PCR showed that FGF9 transcript was present in both XX and XY gonads at 11.5 dpc, but was specific to XY gonads by 12.5 dpc. Reactions without reverse transcriptase served as a control. *Hprt* and *Fgf9* were amplified together in each tube, with expected sizes of 325 bp and 400 bp, respectively.

Fig. 5. Location of FGF receptors. The FGF receptors 1, 3 and 4 (green) were found in many somatic cells, and, at a higher level, in germ cells (red) in both XX and XY gonads at 11.5 dpc. FGFR1 (A,B) and FGFR4 (E,F) were concentrated at the cell surface, while FGFR3 (C,D) was found within the cell. FGFR2 (G,H) was detected in somatic cells at and near the coelomic epithelium in both XY and XX gonads (arrows). FGFR2 was also detected in the nuclei of scattered cells within the XY gonad (G, arrowheads). Nuclear FGFR2 was not observed in XX gonads (H). The number of cells with nuclear FGFR2 increased in the interior of the XY gonad between 11.0 and 11.6 dpc (arrows in I-K). Scale bar: 50 μm.

FGFR2 was detected in the cytoplasm and outlining the plasma membrane of cells in and just under the coelomic epithelium at the surface of both XY and XX gonads. At 11.0 dpc, FGFR2 was detected weakly in coelomic epithelium, but by 11.2 dpc (14-15 ts), this receptor outlined most of the cells in these epithelial layers (Fig. 5J,K). FGFR2 was present at the surface of these cells in both XX and XY gonads. However, FGFR2 also showed a sexually dimorphic pattern. In the XY gonad at 11.5 dpc, FGFR2 was found in the nuclei of scattered cells within the XY gonad (G, arrowheads). FGFR2 was not observed in XX gonads (H). The number of cells with nuclear FGFR2 increased in the interior of the XY gonad examined (11.0-12.5 dpc; Fig. 5H, Fig. 8B, and data not shown).

Sex-specific binding of FGF9 in XY gonads

FGFR2 (and no other FGF receptor) was detected at the plasma membrane of cells in the coelomic epithelium, at the same time and place where an increase in cell proliferation is seen in the XY gonad, suggesting that FGF9 may be acting through this receptor to stimulate proliferation in the male. However, the proliferative increase in the coelomic epithelium is male specific, while neither FGF9 nor FGFR2 shows sexually dimorphic expression in these cells. Splicing isoforms of FGFR2 in the extracellular Ig-like domain III have been shown to have different affinity to FGF9 (Ornitz et al., 1996) and could account for sex-specific responses to FGF9. No antibodies specific for these isoforms are available, however, using RT-PCR, both IIIb and IIIc isoforms were detected in both XX and XY gonads at 11.5 dpc (data not shown). These data suggest that isoform differences do not explain the FGF9-dependent sexually dimorphic pattern of proliferation at the coelomic surface of the gonad.
Fig. 6. FGF binding and induction of proliferation. Gonadal sections were treated with exogenous human FGF9, which was detected with an antibody specific to the human protein (green). Human FGF9 bound to cells near the coelomic epithelium (arrowheads) in XY gonad sections (B), but not XX (A) or XY sections pretreated with heparatinase (C). Using BrdU labeling, many dividing cells (red) were observed within cultured XY gonads, concentrated in somatic cells (labeled with WT1, green) at and near the coelomic surface (D). Fewer dividing cells were observed in XX gonads (E). Addition of 50 ng/ml FGF9 in the culture media induced greater numbers of dividing cells in XX gonads. These dividing cells were concentrated at the surface of the gonad (arrows). Germ cells and vasculature are blue.

Components of the extracellular matrix (ECM), specifically heparin sulfate proteoglycans (HSPG), act as low-affinity receptors for FGFs and are required to efficiently activate FGFRs (Rapraeger et al., 1991; Yayon et al., 1991; Lin et al., 1999). Patterns of HSPG expression and modification have been shown to lead to cell- and tissue-specific effects of FGFs during development (Chang et al., 2000; Allen et al., 2001; Friedl et al., 2001; Ford-Perriss et al., 2002; Jenniskens et al., 2002; Habuchi et al., 2003). To determine if sex-specific differences in the HSPGs are responsible for the sex-specific requirement of FGF9 for proliferation in the XY gonad, we performed in situ binding assays (Rapraeger, 2002) on gonad sections. In these assays, purified human FGF9 was added to sections of XX and XY gonads, and bound human FGF9 was detected with an antibody specific to the human protein. In the XY gonad, human FGF9 accumulated on both somatic and germ cells, particularly on cells near the coelomic surface of the gonad (Fig. 6B). In the XX gonad, human FGF9 showed reduced binding to cells throughout the section (Fig. 6A), and bound at levels only slightly higher than the untreated controls. Pre-treatment of XY gonad sections with heparatinase abolished the XY-specific binding of human FGF9 (Fig. 6C), indicating that male-specific heparan sulfates at the cell surface and/or within the ECM lead to differential binding of human FGF9 in XY and XX gonads. Interestingly, induction of cultured gonads with 50 ng/ml FGF9 in the presence of BrdU revealed that exogenous FGF9 induces proliferation in XX gonads (Fig. 6F, arrows), suggesting that the ECM mediated sensitivity can be overcome with increased concentrations of FGF9.

Nuclear FGFR2 overlaps with both Sry and Sox9 expression in the nuclei of Sertoli cell precursors

The observation of FGFR2 in the nuclei of XY cells beginning at 11.0 dpc is intriguing because it is observed within hours of the initiation of Sry expression (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001; Lovell-Badge et al., 2002). To determine if FGFR2 is directed to the nucleus in cells that express Sry (Sertoli cell precursors), we localized FGFR2 in a transgenic reporter line that expresses EGFP driven by the Sry promoter (Sry:EGFP). In this line, EGFP can be detected within the nucleus and cytoplasm of Sertoli cell precursors (Albrecht and Eicher, 2001). In these gonads, the majority of EGFP expressing cells in the 11.5 dpc gonad also showed nuclear FGFR2 (Fig. 7A). Owing to the autosomal insertion site of the transgene, Sry:EGFP is also expressed in XX gonads; however, as in other XX gonads, EGFP-expressing XX cells showed no nuclear localization of FGFR2 (Fig. 7B).

In the XY gonad, a small proportion of the EGFP-expressing cells did not have nuclear FGFR2 and many of the cells with nuclear FGFR2 did not have EGFP expression. This incomplete overlap between FGFR2 and Sry:EGFP could be due to the transient nature of Sry expression, as Sry is expressed for only a short period of time in individual Sertoli cell precursors (Lovell-Badge et al., 2002). To confirm that nuclear FGFR2 is specific to the Sertoli cell lineage, we used an antibody against SOX9. SOX9 is a DNA-binding protein that is specific to the nuclei of cells in the Sertoli cell lineage and, unlike Sry, is maintained in this cell type through cord formation (Kent et al., 1996; Morais da Silva et al., 1996). At 11.5 dpc, every cell that expressed SOX9 also showed nuclear FGFR2, and vice versa (Fig. 7C-G), indicating that nuclear FGFR2 is specific to cells in the Sertoli cell lineage. Nuclear FGFR2 remained specific to Sertoli cells through 12.5 dpc, when Sertoli cells also could be identified by their location in testis cords (Fig. 8A).

Nuclear localization of FGFR2 is dependant on both Fgf9 and components of the male pathway

To determine if the nuclear localization of FGFR2 is dependant on Fgf9, we examined the pattern of this receptor in Fgf9–/– XY gonads. Nuclear FGFR2 was not observed in Fgf9–/– XY gonads (Fig. 8C). Instead, this receptor showed cytoplasmic and membrane localization in somatic cells throughout the Fgf9–/– XY gonad, in a pattern resembling the XX gonad (Fig. 8B). We conclude that the nuclear localization of FGFR2 is dependent on Fgf9. However, even though both Fgf9 and FGFR2 are expressed in XX gonads, FGFR2 is not transported to the nucleus in XX cells, indicating that other components of the male pathway are essential for the nuclear localization of this receptor.

It is possible that levels of active FGF9 protein are not high enough in XX gonads to trigger nuclear localization of FGFR2. To determine if exogenous FGF9 could induce the nuclear
localization of FGFR2 in the XX gonad independent of Sry, we cultured early (11.2-11.5 dpc) gonads with 50 ng/ml FGF9 protein for 36 hours. Nuclear FGFR2 was observed in the testis cords of cultured XY gonads (Fig. 8E), recapitulating the in vivo pattern of this receptor. However, FGF9 did not induce the nuclear localization of FGFR2 in cultured XX gonads (Fig. 8G), nor did it induce a greater number of XY cells to localize FGFR2 to the nucleus (data not shown). Thus, Fgf9 is necessary but not sufficient to direct FGFR2 to the nucleus. Additional components of the male pathway, specific to Sertoli cell precursors, are required for the nuclear localization of FGFR2. Consistent with the inability to induce nuclear FGFR2, FGF9 also did not induce the expression of other markers of Sertoli differentiation (Sox9, Amh and Dhh; Fig. 8H). However, FGF9 did induce the expression of a male specific gene normally found in the interstitium of the testis, P450 side chain cleavage enzyme (Scc; Cyp11a1 – Mouse Genome Informatics). This gene is a marker of differentiating Leydig cells, the hormone-producing cells of the testis. Leydig cell development occurs after Sertoli cell differentiation, and signals from Sertoli cells have been proposed to control the number and differentiation of Leydig cells. Thus, FGF9 may have a role in this process later in testis organogenesis, after its expression becomes sex specific.

Discussion

Previously, it has been shown that loss of Fgf9 results in male-to-female sex reversal (Colvin et al., 2001a). However, it was not known whether the observed abnormalities were due to disruptions at a primary step in the commitment to the male fate, or in the processes that shape testis morphology after sex determination. In this study, we provide evidence that Fgf9 functions at two early steps downstream of Sry: in the induction of proliferation of a population of cells that give rise to Sertoli precursors, and in the establishment of Sertoli cell fate. These functions may be mediated independently by additional components of the FGF pathway, including male-specific components of the extracellular matrix and the nuclear localization of FGFR2.

Fgf9 is necessary for male-specific proliferation

A rapid increase in proliferation in the XY gonad is observed before 11.5 dpc, and is the earliest known morphological difference between XY and XX gonads downstream of Sry (Schmahl et al., 2000). This male-specific proliferation is concentrated at the surface of the gonad, within and just under the coelomic epithelium, in a population of cells that give rise to Sertoli progenitors (Karl and Capel, 1998). In this study, we show that proliferation at the coelomic epithelium of XY gonads is reduced very early in the development of Fgf9−/− mutants (by 11.2 dpc). In addition, we show that exogenous FGF9 induces proliferation in these cells in cultured gonads. Interestingly, both Fgf9−/− XX and XY gonads maintained normal levels of growth prior to 11.0 dpc, and were equal in size to wild-type XX gonads by 12.5 dpc. Thus, despite the fact that Fgf9 and all four FGF receptors are expressed in both XX and XY gonads, proliferation and gonad size are affected only in Fgf9−/− XY gonads. These results indicate that Fgf9 is not necessary for all cell division in the gonad; instead, it is necessary specifically for the male increase in growth and proliferation initiated by Sry.

It has been proposed that a threshold number of Sertoli cells is necessary to recruit the other cells in the gonad to the testis pathway (Burgoyne and Palmer, 1993). In fact, it has been shown that blocking proliferation at a specific period of early gonad development not only decreases the size of the gonad, but also decreases or eliminates the expression of male-specific genes, the differentiation of Sertoli cells and the formation of testis cords (Schmahl and Capel, 2003). Thus, proliferation in the early testis is crucial for the normal patterning and, ultimately, the developmental fate of the XY gonad. In Fgf9−/− XY gonads, the reduction in proliferation is observed within the period that blocking experiments defined as crucial in testis development, and occurs at the time and place that pre-Sertoli cells are dividing. Therefore, the failure of cord formation and other aspects of testis development in Fgf9−/− XY gonads may be at least partially due to a reduction in the numbers of pre-Sertoli cells normally produced by early proliferation.

However, despite the importance of proliferation in testis development, it should be noted that the induction of proliferation at the coelomic epithelium in cultured XX gonads by FGF9 was not sufficient for Sertoli differentiation and cord
formation. Therefore, although proliferation has an important role in testis development, other elements downstream of \( Sry \) are necessary for Sertoli differentiation and normal testis development.

**Sex-specific components of the ECM may determine the sex-specific action of FGF9**

FGF9 is known to bind and activate four receptors (FGFR1, FGFR2, FGFR3 and FGFR4) (Ornitz et al., 1996). FGFR1, FGFR3 and FGFR4 were found in somatic and germ cells throughout both XY and XX gonads at 11.5 dpc, indicating that they could have a role in gonad formation. However, mice with null mutations of \( Fgfr3 \) and \( Fgfr4 \) are fertile (Colvin et al., 1996; Deng et al., 1996; Weinstein et al., 1998), indicating that these receptors are not required (or are redundant) for the development of a functional testis. Null mutations in \( Fgfr1 \) are lethal between 6.5 and 9.5 dpc, before gonad formation (Deng et al., 1994). However, studies with chimeric mice indicate that the testis develops normally even with up to 90% \( Fgfr1^{-/-} \) cells (Deng et al., 1997). Therefore, at least in many cells of the testis, FGFR1 is not essential for testis development. FGFR2 is necessary for many processes in development, including bone growth and the induction of the limb (Xu et al., 1998; Yu et al., 2003). However, because homozygous null mutations for \( Fgfr2 \) result in embryonic lethality at 10.5 dpc (Arman et al., 1999), the reproductive function of this receptor has not been assessed.

Despite the sex-specific proliferation phenotype in \( Fgf9^{-/-} \) gonads, the expression patterns of \( Fgf9 \) and all four known FGF receptors are not sex specific during the early stages of sex determination (11.5 dpc). FGFR2 is localized to the plasma membrane of proliferating cells; however, its expression in these cells is similar in XX and XY gonads. One explanation for the dimorphic effect of the \( Fgf9 \) mutation is that sex-specific components of the ECM, such as HSPGs, mediate the activity of FGF9 in XY gonads. HSPGs in the extracellular matrix form complexes with FGFs and FGFRs, and are required to efficiently activate FGFRs (Rapraeger et al., 1991; Yayon et al., 1991; Lin et al., 1999). HSPGs also stabilize secreted FGFs, limit their diffusion and maintain them in active or inactive states, thus generating sites of increased local activity and morphogenetic boundaries. Tissue-specific expression and modification of HSPGs have been shown to be responsible for the tissue specific action of FGFs in other systems (Chang et al., 2000; Pye et al., 2000; Allen et al., 2001; Friedl et al., 2001; Ford-Perriss et al., 2002; Jenniskens et al., 2002; Habuchi et al., 2003). In gonad sections, enhanced binding of FGF9 occurred specifically at the coelomic epithelium, the region where male proliferation increases occur. This enhanced binding of FGF9 was abolished by heparinases, indicating that the enhanced binding of FGF9 to the male gonad is dependant on sex-specific differences in the ECM, not differences in FGF9 or FGFR expression. These findings support a role for XY-specific components of the ECM in regulating the sex-specific activity of FGF9.

**FGFR2 translocates to the nucleus during the early differentiation of Sertoli cells**

FGFR2 showed a striking sex-specific expression pattern within the nuclei of a scattered population of cells located in the interior of the XY gonad. This dimorphic pattern in the nucleus of XY cells was detected by 11.0 dpc and is coincident with the appearance of nuclear SOX9, a marker of Sertoli cell differentiation and the earliest known difference between XY and XX gonads previously known to occur after the initiation of \( Sry \) expression. FGFR2 was not observed in the nuclei of \( Fgf9^{-/-} \) XY gonads, indicating that the nuclear localization of this receptor is dependent on \( Fgf9 \). However, addition of exogenous FGF9 did not induce the nuclear translocation of FGFR2 or other markers of Sertoli differentiation in XX gonads, indicating that nuclear localization of this growth factor receptor is also mediated by factors specific to the XY gonad.
Nuclear localization of FGFR2 does not occur in proliferating cells at the epithelial surface, but in cells that are initiating Sertoli cell differentiation within the gonad. Direct lineage tracing experiments indicate that Sertoli precursors originate from the coelomic epithelium (Karl and Capel, 1998), where FGFR2 is found at the plasma membrane. Once cells leave the coelomic epithelium, they initiate Sertoli cell differentiation within the interior of the gonad (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001), where the expression of Sry, the upregulation of Sox9, and the nuclear location of FGFR2 are observed.

It has been known for some time that many cell-surface growth factor receptors can accumulate within the nucleus. However, the biological relevance of this event is not known (reviewed by Goldfarb, 2001; Wells and Marti, 2002). It has been speculated that nuclear growth factor receptors may act as weak transcription factors, topoisomerases and/or nuclear kinases. Nuclear FGFR receptors have been observed in spliceosomes (Penderson, 1998; Peng et al., 2002). This is particularly interesting, as several components of the sex-determination pathway (including SRY, SOX9 and the +KTS isoform of WT) have been shown to associate with splicing factors, and have been demonstrated to have splicing activity (Hastie, 2001; Ohe et al., 2002). Another function of nuclear FGFR receptors may be to phosphorylate nuclear substrates, as the forced nuclear translocation of FGFR receptors leads to an increase in the phosphorylation of nuclear proteins, and some activities of the nuclear receptor are abolished by deactivation of the kinase domain (Maher, 1996; Reilly and Maher, 2001; Peng et al., 2002).

A potential role of FGFR signaling in the gonad is suggested by studies in chondrocytes. In this cell type, the activation of FGFR receptors in vitro can upregulate expression of Sox9 which is involved in chondrocyte differentiation in bone growth plates (de Crombrugghe et al., 2000; Murakami et al., 2000). Sox9 is known to be essential for male sex determination (Foster et al., 1994). In fact, because the induction of Sox9 expression can initiate the male pathway in the absence of Sry, this gene is hypothesized to lie immediately downstream of Sry in the sex-determination cascade (Bishop et al., 2000; Vidal et al., 2001). In early gonad development, Sox9 is found at low levels in the cytoplasm of cells in both XX and XY gonads. However, like FGFR2, SOX9 undergoes a change in subcellular localization and is found in the nucleus of cells in the XY gonad just after Sry is expressed (Morais da Silva et al., 1996; de Santa Barbara et al., 2000). Nuclear FGFR2 colocalizes with SOX9 in the gonad, suggesting that this receptor could be involved in the induction or maintenance of the nuclear localization of SOX9.

The discovery of the sex-specific subcellular localization of FGFR2 in the nuclei of Sertoli cell precursors provides a well-characterized biological context in which to study the function of nuclear growth factor receptors. In this context, the transition of FGFR2 from the cell membrane to the nucleus suggests that the nuclear localization of cell-surface receptors is linked to the initiation of cell differentiation. It is not yet clear how proliferation of Sertoli cell precursors in the coelomic epithelium and subsequent commitment to the Sertoli fate are interwoven; however, these findings suggest that FGF signaling may be involved in bridging these two processes essential to testis development.

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References


