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DEVELOPMENT AND DISEASE

Gonadal differentiation, sex determination and normal *Sry* expression in mice require direct interaction between transcription partners GATA4 and FOG2

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SUMMARY

In mammals, *Sry* expression in the bipotential, undifferentiated gonad directs the support cell precursors to differentiate as Sertoli cells, thus initiating the testis differentiation pathway. In the absence of *Sry*, or if *Sry* is expressed at insufficient levels, the support cell precursors differentiate as granulosa cells, thus initiating the ovarian pathway. The molecular mechanisms upstream and downstream of *Sry* are not well understood. We demonstrate that the transcription factor GATA4 and its co-factor FOG2 are required for gonadal differentiation. Mouse fetuses homozygous for a null allele of *Fog2* or homozygous for a targeted mutation in *Gata4* (*Gata4^{ki}*) that abrogates the interaction of GATA4 with FOG co-factors exhibit abnormalities in gonadogenesis. We found that *Sry* transcript levels were significantly reduced in XY *Fog2$^{-/-}$* gonads at E11.5, which is the time when *Sry* expression normally reaches its peak. In addition, three genes crucial for normal Sertoli cell function (*Sox9, Mis* and *Dhh*) and three Leydig cell steroid biosynthetic enzymes (*p450scc, 3βHSD* and *p450c17*) were not expressed in XY *Fog2$^{-/-}$* and *Gata$^{ki/ki}$* gonads, whereas *Wnt4*, a gene required for normal ovarian development, was expressed ectopically. By contrast, *Wt1* and *Sf1*, which are expressed prior to *Sry* and necessary for gonad development in both sexes, were expressed normally in both types of mutant XY gonads. These results indicate that GATA4 and FOG2 and their physical interaction are required for normal gonadal development.

Key words: Testis, GATA4, FOG2, *Sry*, Sertoli cell, Sex reversal, Mouse

INTRODUCTION

Mammalian gonads arise in both sexes from a bilateral genital ridges that have the potential to develop as ovaries or testes (reviewed by Capel, 1998; Swain and Lovell Badge, 1999; Capel, 2000). In mice, the genital ridges are first evident at embryonic day (E) 9.5. At ~E10.5, a critical switch in gonadal development occurs in which XY gonads express the testis determining gene *Sry* (sex-determining region Y chromosome) (Koopman et al., 1991), initiating the differentiation of the supporting cell precursors as Sertoli rather than granulosa cells (Burgoyne et al., 1988; Albrecht and Eicher, 2001). Sertoli cell signaling is thought to control further development of the male gonad (Magre and Jost, 1984; Magre and Jost, 1991; Jost and Magre, 1988), including cell proliferation (Schmahl et al., 2000), migration of mesonephric cells into the gonad (Buehr et al., 1993; Martineau et al., 1997; Merchant-Larios and Moreno-Mendoza, 1998) and differentiation of other cell lineages (Jost et al., 1973; Byskov, 1986). This male-specific differentiation leads to the development of two distinct compartments: testis cords containing germ cells and Sertoli cells surrounded by peritubular myoid cells, and an interstitial region containing Leydig (steroidogenic) cell precursors. In the absence of *Sry* expression (i.e. in XX gonads) or if *Sry* is expressed at insufficient levels, the gonad develops as an ovary or ovotestis (Laval et al., 1995; Washburn et al., 2001; Nagamine et al., 1999; Hammes et al., 2001).

The GATA zinc-finger transcription factors (designated GATA1 to GATA6) recognize the consensus target sequence (T/A)GATA(A/G) and play critical roles in various developmental processes, including hematopoietic and T cell differentiation, cardiac and coronary vasculature development, and liver, lung and gut morphogenesis (reviewed by Orkin, 2000; Molkentin, 2000; Ho and Glimcher, 2002; Van Esch et al., 2000; Rossi et al., 2001; Jacobsen et al., 2002; Yang et al., 2002). Although the precise roles GATA proteins play in

Table 1. In situ probes

Gene	Description	Cell type identified	Reference
Sf1 (Steroidogenic factor 1; *Nr5a1* – Mouse Genome Informatics)	0.25 kb fragment of cDNA (mouse)	Gonadal somatic cells	Ikeda et al. (1994)
Mis	Full-length cDNA (rat)	Pre-Sertoli cells	Shen et al. (1994)
Sox9 (SRY-box containing gene 9)	1.5 kb cDNA (mouse)	Gonadal somatic cells	Lefebvre et al. (1997)
Dhh (desert hedgehog homolog)	1.2 kb cDNA (mouse)	Pre-Sertoli cells	Bitgood et al. (1996)
Wnt4 (wingless-related MMTV integration site 4)	1 kb cDNA (mouse)	Gonadal and mesonephric somatic cells	Vainio et al. (1999)

gonadal development are not fully explored, two Gata genes are known to be expressed in fetal mouse gonads. *Gata2* (GATA-binding protein 2) is expressed between E10.5-15.5 in XX gonads and XX and XY mesonephroi, but not in XY gonads. *Gata2* is not expressed in XX gonads that lack germ cells, although mesonephric expression is maintained. At E13.5, *Gata2* expression is restricted to germ cells in XX gonads (Siggers et al., 2002), suggesting that it plays a role in ovarian germ cell development.

By contrast, *Gata4* appears to be the sole GATA family member active in somatic (and not germ) cells of the genital ridge (Heikinheimo et al., 1997; Viger et al., 1998). At E11.5, *Gata4* is expressed in somatic cells of both XX and XY genital ridges (Heikinheimo et al., 1997; Viger et al., 1998; Ketola et al., 2000). At E13.5, *Gata4* expression becomes sexually dimorphic: in XY gonads expression is upregulated in Sertoli cells and downregulated in interstitial cells, whereas in XX gonads, expression is downregulated in all cells. *Gata4* expression persists in the somatic cells of postnatal testes and is re-activated in adult ovaries with predominant expression in granulosa cells (Heikinheimo et al., 1997; Viger et al., 1998). GATA4 has been shown to bind to a consensus site in the *Mis* (Müllerian inhibiting substance, also known as anti-Müllerian homone, *Amh*) promoter and activate expression of a *Mis* reporter construct in vitro (Viger et al., 1998). Because *Gata4*$^{-/-}$ mice die at ~E7.0-9.5 (Kuo et al., 1997; Molkentin et al., 1997) analysis of gonadal differentiation in the absence of GATA4 is not possible. Both *Gata5* and *Gata6* also are expressed throughout the mouse urogenital system, but only during the late fetal and postnatal stages (Morrisey et al., 1996). In addition, Sertoli cells in the postnatal testis express *Gata1*, which represents the only reported extra-hematopoietic site of *Gata1* expression (Yomogida et al., 1994).

The normal in vivo function of GATA factors in vertebrates and *Drosophila* requires physical interaction with multitype zinc-finger proteins of the FOG (Friend of GATA) family (FOG1, FOG2, xFOG and USH) (for reviews, see Cantor and Orkin, 2001; Fossett and Schulz, 2001). Previously, we and others reported expression of a FOG family member, *Fog2* (friend of GATA2; *Zfpm2* – Mouse Genome Informatics), in the developing mouse gonad as early as E11.5 (Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999). *Fog2* also is expressed in cardiac and nervous tissues and is strictly required for mouse cardiac development. Mouse fetuses homozygous for a null allele of *Fog2* (*Fog2*$^{-/-}$) die at mid-gestation from a cardiac defect characterized by a thin ventricular myocardium, common atrioventricular canal and the Tetralogy of Fallot malformation. Because *Fog2*$^{-/-}$ embryos survive until ~E14.5, analysis of early gonad development in the absence of FOG2 is possible. Partial rescue of cardiac function using a cardiac alpha myosin heavy chain (αMHC) driven *Fog2* transgene specifically expressed in the myocardium, extends viability of *Fog2*$^{-/-}$ fetuses to ~E17.5 (Tevosian et al., 2000), thus allowing examination of gonad development in the absence of *Fog2* as late as E17.5. As noted above, *Gata4*$^{-/-}$ embryos die at ~E7.0-9.5, which precludes analysis of their gonadal differentiation. However, this problem is overcome by using a *Gata4* knock-in allele (*Gata4*^{ki}, a V217G amino acid substitution) that abrogates the interaction between GATA4 and FOG2 (or FOG1) (Crispino et al., 2001). Homozygous *Gata4*^{ki} embryos survive to E13.5 but then they die from cardiac abnormalities similar to those noted in *Fog2*$^{-/-}$ embryos (Crispino et al., 2001). Thus, the *Gata4*^{ki} allele allows unique insight into the importance of the GATA4/FOG interaction in mammalian gonad development. We report that either abrogation of GATA4/FOG interaction or *Fog2* loss result in the equivalent defect in mouse gonadal differentiation.

MATERIALS AND METHODS

Mice and genotyping assays

Generation of *Fog2*- and *Gata4*-targeted and αMHC-*Fog2* transgenically rescued mice, together with the assays used for determining presence of the *Gata4*^{ki} and *Fog2* mutant alleles have been described (Tevosian et al., 2000; Crispino et al., 2001). Genotyping for the presence of the Y chromosome followed the method of Koopman et al. (Koopman et al., 1991). Fetuses were collected from timed matings with E0.5 representing noon on the day a mating plug was detected. Fetuses were further staged according to limb morphology (Theiler, 1989). Fetuses used for the determination of *Sry* transcript levels were carefully staged by counting the number of tail somites (ts) distal to the hindlimbs (E11.5 is ~18 ts) (Hacker et al., 1995).

Whole-mount RNA in situ hybridization

All recombinant DNA work was accomplished using standard techniques. Probes provided by others are summarized in Table 1. Additional probes were generated according to Greco and Payne (Greco and Payne, 1994) from total testis RNA using a Superscript II RT-PCR kit according to the manufacturer's instructions (Gibco). The genes together with primers used for RT-PCR are: *P450scc* (cholesterol side-chain cleavage) 5′-CTGAGTACTGGAAA-GGGAGCTG-3′ and 5′-TCACTGATGACCCCTGAGAAAT-3′; *3βHSD* (3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase) 5′-TACATGGCTCTGGGAGTTATAAGGTCC-3′ and 5′-GCTTCAG-AAAGCAATGGGATTTTACC-3′; and *P450c17* (17α-hydroxylase/C17-20 lyase) 5′-GCCTGACAGACATTCTGATACAAGCC-3′ and 5′-CCCTTCATTGCTGCCAAGTAGAAAAC-3′. PCRs were performed for 30 cycles (95°C for 1 minute, 55°C for 1 minute 30 seconds and 72°C for 3 minutes), and 1 cycle for 5 minutes at 72°C. PCR fragments were cloned using the TOPO-TA cloning kit (Invitrogen) and fully sequenced. Whole-mount in situ hybridization with riboprobes labeled with digoxigenin-UTP (Roche) was performed following standard procedures. Gonads were dissected

Table 2. Primary antibodies

Antibody	Type	Antigen	Cell type identified	Source	Dilution used
SF1	Rabbit polyclonal	Steroidogenic factor 1	Gonadal somatic cells	Morohashi et al. (1993)	1:1000
GATA4 (C-20)	Goat polyclonal	GATA-binding protein 4	Gonadal somatic cells	Santa Cruz Biotechnology	1:400
WT1 (6F-H2)	Mouse monoclonal	Wilm's tumor 1	Gonadal and mesonephric somatic cells	Dako	1:300
PECAM	Rat monoclonal	Platelet endothelial cell adhesion molecule	Germ and vascular endothelial cells	BD PharMingen	1:100
Laminin	Rabbit polyclonal	Laminin	Basal lamina	Sigma	1:3000

from embryos that were fixed with 4% paraformaldehyde in 1×PBS and analyzed post hybridization with a Nikon Optiphot dissection microscope. Images were processed and assembled using Photoshop 5.5 (Adobe) and CorelDraw (Corel) software.

Determination of *Sry* transcript levels

Sry transcript levels were determined on single urogenital ridges obtained from individual fetuses using a semi-quantitative RT-PCR (Washburn et al., 2001). A total of 10 *Fog2*$^{-/-}$, 10 *Fog2*$^{+/-}$ and four *Fog 2*$^{+/+}$ urogenital ridges were analyzed by comparing the expression of *Sry* to *Lhx1* (LIM homeobox protein 1) as a control. [*Lhx1* is expressed in the mesonephros but not the gonad (Barnes et al., 1994; Fujii et al., 1998)]. Briefly, RNA was extracted from single urogenital ridges isolated from E11.5 fetuses using the RNeasy total RNA miniprep kit (Qiagen) and eluted in 30 μl H_2O. RNA was treated with DNase using the DNA-free kit (Ambion). One-third of the purified, DNA-free, RNA was used for first strand cDNA synthesis in a 20 μl reaction using the manufacture's protocol (Applied Biosystems). As controls, each RNA template was incubated in the presence and absence of reverse transcriptase and a no template (H_2O) reaction was included for each experiment. Two microliters of the RT reaction was then used for multiplex PCR in the presence of [α-^{32}P]dCTP and primers specific for *Sry* (5′-TGGTGAGCATACACCATACC-3′ and 5′-TTGCTGTCTTTGTGCTAGCC-3′, 377 bp product) and *Lhx1* (5′-GGCGAGGAGCTCTACATCATAG-3′ and CTTGGGAATCCGGA-GATAAAC-3′, 139 bp product). Thermal cycling conditions were 94°C for 30 seconds; 57°C for 30 seconds; 72°C for 30 seconds for 29 cycles. The PCR reaction was analyzed on a 3% agarose gel and Southern blotted. Phosphor imaging plates and Image Gauge software (Fuji Medical Systems) were used to determine the amount of radioactivity in each band. Transcript levels were compared between same-aged embryos as determined by ts number.

Immunofluorescent histochemistry and confocal microscopy

Detailed methods for examining gonad morphology and marker protein expression have been described (Albrecht and Eicher, 2001). Briefly, tissue samples were fixed in 4% paraformaldehyde in PBS, rinsed twice in PBS and incubated for 24 hours in blocking buffer (1% BSA, 0.1% Saponin, 0.02% sodium azide in PBS). Samples were incubated in primary antibody diluted in fresh blocking buffer for 24 hours, washed extensively, incubated in fluorphore-conjugated secondary antibody diluted in fresh blocking buffer for 24 hours, and washed extensively. All samples were analyzed by three-color confocal microscopy (Leica TCS-NT) as whole-mounts in SlowFade-Light Antifade (Molecular Probes). Confocal images were assembled using MetaMorph (Universal Imaging) and Photoshop (Adobe). Pertinent information regarding the primary antibodies used is listed in Table 2. All secondary antibodies were used at 1:500 dilution and were from Jackson ImmunoResearch (Cy3 and Cy5 conjugated) or Molecular Probes (AlexaFluor 488).

RESULTS

Failure of testis differentiation in *Fog2*-deficient gonads

As described above, mice homozygous for a targeted null mutation in *Fog2* die at ~E14.5 from cardiac defects. Partial rescue of cardiac function using a cardiac α myosin heavy chain (αMHC)-driven *Fog2* transgene specifically expressed in the myocardium extends viability of *Fog2*$^{-/-}$ fetuses to ~E17.5 (Tevosian et al., 2000). This affords the opportunity to examine morphogenesis in the absence of *Fog2* as late as E17.5 in organs other than the heart. The initial gross examination of gonads in E17.5 XY *Fog2*$^{-/-}$ αMHC-*Fog2* transgenic fetuses indicated that testicular development was abnormal (Fig. 1A-

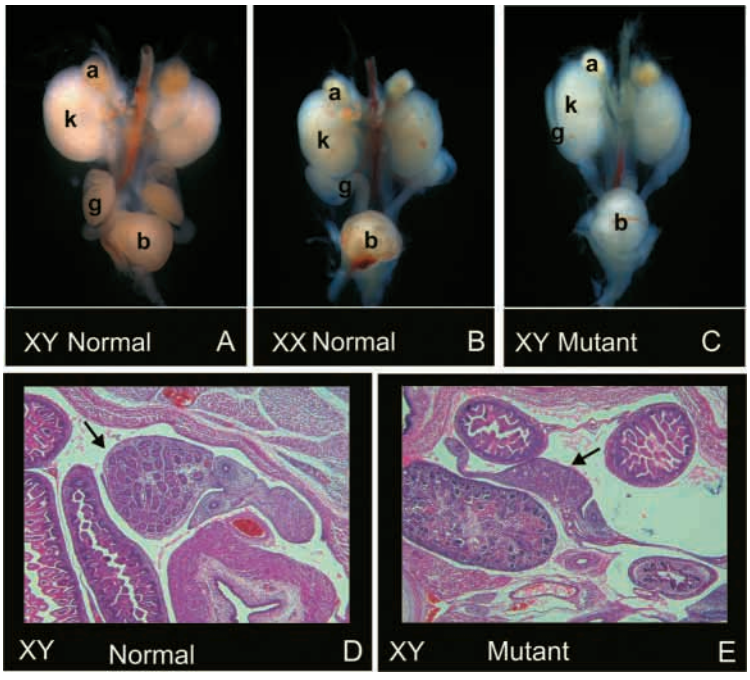

Fig. 1. E17.5 *Fog2*$^{-/-}$ αMHC-*Fog2* transgenic and normal mouse gonads. (A-C) Macroscopic view of the urogenital systems in male (A), female (B) and XY mutant (C) E17.5 fetuses. Mutant XY gonads are severely reduced in size and have not descended. a, adrenal, b, bladder, g, gonad, k, kidney. Only one paired organ is labeled. (D,E) Representative transverse sections through gonads at the same magnification of (D) normal testis and (E) *Fog2*$^{-/-}$ undifferentiated XY gonads (arrowhead). Notice the lack of testis cord development and greatly reduced size of mutant gonad.

C). In fact, mutant XY gonads (Fig. 1C) resembled normal XX gonads (Fig. 1B) more than normal XY gonads (Fig. 1A). Histological analysis confirmed that testis cords evident in the control (Fig. 1D) were absent in the mutant (Fig. 1E). Close examination revealed that both XY and XX mutant gonads of E17.5 *Fog2*$^{-/-}$ αMHC-*Fog2* transgenic fetuses looked alike and did not resemble the normal E17.5 XY or XX gonads (Fig. 1 and data not shown). Taken together, these results suggested that *Fog2* is needed for the normal development of ovaries and testes. (A detailed description of the defects found in the XX mutant ovaries will be reported elsewhere.) The striking nature of the defects in the XY *Fog2*$^{-/-}$ gonads prompted us to explore further the involvement of FOG2 and GATA4 in testis development.

SF1 and WT1 are expressed, but not upregulated in the mutant XY gonads

Examination of urogenital ridges in E11.5 XX and XY *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ embryos suggested that genital ridge differentiation was normal up to this stage of development. To confirm this, we analyzed the expression of *Sf1* and *Wt1* in both types of mutant XY genital ridges. Both *Sf1* and *Wt1* are required for gonadal and adrenal development; in addition, *Wt1* is essential for kidney and cardiac development (Kreidberg et al., 1993; Luo et al., 1994; Sadovsky et al., 1995; Moore et al., 1999; Hammes et al., 2001). In situ hybridization analysis revealed comparable levels of *Sf1* transcripts in control and mutant gonads and adrenals from E12.5 XX and XY E12.5 embryos (Fig. 2A-D, and data not shown). Confocal microscopy and immunofluorescent histochemistry of gonads from E13.25 XY *Fog2*$^{-/-}$ embryos confirmed that SF1 was expressed and established that WT1 protein was expressed as well (Fig. 2E-J). Expression levels of both proteins were grossly similar in wild-type and mutant gonads. However, at this developmental stage expression of SF1 and WT1 normally is upregulated in XY gonads in pre-Sertoli cells of the developing testis cords (Pelletier et al., 1991; Ikeda et al., 1994; Albrecht and Eicher, 2001). In contrast to control XY gonads, upregulation of neither protein was apparent in any cells within mutant XY gonads. In fact, the expression patterns closely resembled that in control XX gonads (compare Fig. 2H-J).

Testis cord development is absent in mutant XY gonads

To further evaluate if the structural organization of the testes is perturbed in *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ XY embryos, we examined GATA4, PECAM and laminin expression in both types of mutant gonads using confocal microscopy and immunofluorescent histochemistry. At E12.5, when testis cords are beginning to form in control XY gonads, XY mutant gonads displayed no evidence of cord formation but appeared more similar to XX control gonads, except that the mutant gonads were somewhat misshapen and less organized (Fig. 3A-C). At E13.5, testis cord development still was strikingly absent in XY mutant gonads, as confirmed by staining for laminin, which is expressed on the basement membrane surrounding the cords (Fig. 3D-F, green). Although both types of mutant XY gonads lacked testis cords, they did contain germ cells and vasculature, as demonstrated by PECAM staining (Fig, 3A-F, blue). Additionally, GATA4 expression was appropriately confined

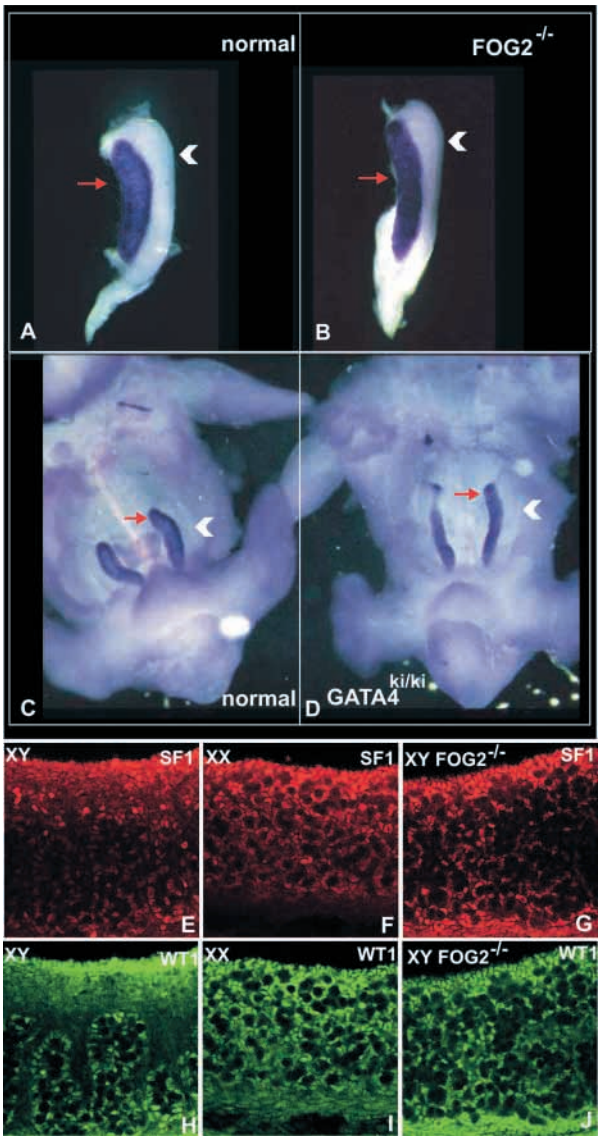

Fig. 2. Expression of molecular markers of early gonadal development. (A-D) In situ hybridization of E12.5 XY gonads with *Sf1* RNA probe. (A,C) Normal controls. (B) *Fog2*$^{-/-}$. (D) *Gata4*$^{ki/ki}$. Gonad (red arrow) and mesonephros (arrowhead) are indicated. (E-J) Analysis of SF1 (red) and WT1 (green) protein expression in E13.25 control XY gonads (E,H), control XX ovary (F,I) and XY *Fog2*$^{-/-}$ gonad (G,J) using immunofluorescent histochemistry and confocal microscopy. Notice that the XY *Fog2*$^{-/-}$ gonad lacks testis cords and resembles the control XX ovary at this stage of development.

to somatic cells (Fig. 3A-F, red). However, GATA4 expression was not upregulated in any cells of mutant XY gonads and expression was homogeneous and similar to control and mutant XX gonads.

Sry levels are significantly decreased in *Fog2*$^{-/-}$ XY gonads

To determine if impaired *Sry* expression might account for the absence of testicular cord development in *Fog2*$^{-/-}$ XY gonads, semi-quantitative RT-PCR was used to compare *Sry* expression

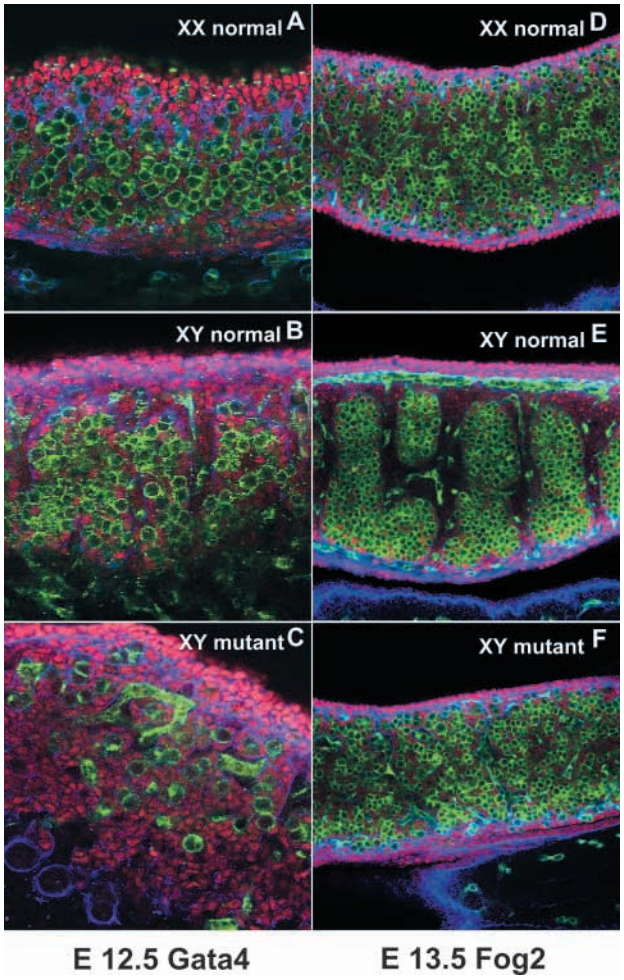

Fig. 3. Analysis of gonad differentiation using immunofluorescent histochemistry and confocal microscopy. (A-C) E12.5 *Gata4*$^{ki/ki}$ gonads at the time when testis cords are beginning to develop in normal XY gonads. (D-F) E13.5 XY *Fog2*$^{-/-}$ gonads at the time when testis cord are normally well developed and surrounded by a basal lamina in control XY gonads. (A,D) XX control gonads, (B,E) XY control gonads and (C,F) mutant XY gonads. Laminin, green; GATA4, red; PECAM, blue. In each image the gonad is above the mesonephros.

levels in mutant versus normal XY genital ridges (Fig. 4). *Sry* reaches its maximal expression at ~E11.5 in normal XY gonads (Hacker et al., 1995). We found a significant decrease in the *Sry* RNA transcript level in E11.5 *Fog2*$^{-/-}$ mutant gonads compared with *Fog2*$^{+/-}$ and *Fog2*$^{+/+}$ control XY gonads. In fact, the level of *Sry* expression in the mutant gonads was ~25% of the level in control gonads. This result suggests that initiation of the testis determination program, which is the primary function of *Sry*, is impaired in the absence of FOG2.

Sertoli cell differentiation is blocked in *Fog2*$^{-/-}$ and *Gata4*$^{ki/ki}$ XY gonads

Because *Sry* expression is dramatically reduced in *Fog2*$^{-/-}$ XY gonads and testis cord differentiation is absent in *Fog2*$^{-/-}$ XY and *Gata4*$^{ki/ki}$ XY gonads, we analyzed the expression of genes downstream of *Sry*. We first examined the expression of three Sertoli cell-specific genes that are central to sex determination

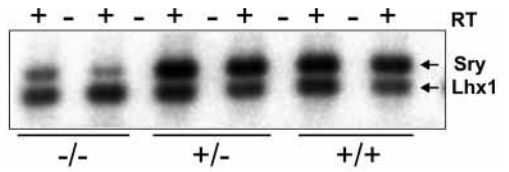

Fig. 4. *Sry* expression. Semi-quantitative RT-PCR analysis of *Sry* expression in E11.5 (15-20 tail somite stage) XY gonads. A representative analysis of *Sry* expression relative to *Lhx1* expression in the gonads of *Fog2*$^{-/-}$, *Fog2*$^{+/-}$ and *Fog2*$^{+/+}$ fetuses at the 18 tail somite stage is shown.

in mammals and possibly all vertebrates: *Sox9*, *Mis* and *Dhh*. Examination of *Sox9* was of special importance because up-regulation of *Sox9* expression in pre-Sertoli cells is one of the earliest markers of Sertoli cell differentiation (Kent et al., 1996; Morais da Silva et al., 1996), presence of *Sox9* as a transgene in XX mice causes testis development and female-to-male sex reversal (Bishop et al., 1999; Vidal et al., 2001), mutations in *SOX9* are associated with male-to-female sex reversal in humans (Foster et al., 1994; Wagner et al., 1994), and *Sox9* is required for activation of *Mis* expression in vivo (Arango et al., 1999). Expression of *Mis* and *Dhh*, which code for paracrine factors secreted by differentiating mouse Sertoli cells shortly after peak *Sry* expression at E11.5, was examined because both are essential for normal male development (Behringer et al., 1994; Bitgood et al., 1996). We found that expression of *Sox9*, *Mis*, and *Dhh* was absent in *Fog2*$^{-/-}$ XY and *Gata4*$^{ki/ki}$ XY gonads (Fig. 5 and data not shown). This finding indicates that Sertoli cell differentiation and the transcriptional program downstream of *Sry* are severely impaired in the absence of FOG2 or fully functional GATA4.

The steroidogenic program is not initiated in mutant XY gonads

To determine if cells other than Sertoli cells are abnormal in *Fog2*$^{-/-}$ XY and *Gata4*$^{ki/ki}$ XY gonads, we examined the expression of three genes that code for androgen biosynthetic enzymes in Leydig cells: *P450scc*, *3βHSD* and *P450c17* (Greco and Payne, 1994). Leydig cells are thought to originate from precursors that migrate into the genital ridge from the mesonephros before E11.5 (Buehr et al., 1993; Merchant-Larios and Moreno-Mendoza, 1998). Leydig cells differentiate later than Sertoli cells and their differentiation is probably controlled by Sertoli cell-secreted factors (for a review, see Habert et al., 2001). Because development of Sertoli cells is arrested at an early stage in *Gata4*$^{ki/ki}$ XY and *Fog2*$^{-/-}$ XY gonads, a block in steroidogenesis was anticipated and, in fact, this was the case. RNA transcripts for *P450scc*, *3βHSD* and *P450c17* were undetectable in *Fog2*$^{-/-}$ XY and *Gata*$^{ki/ki}$ XY gonads, indicating that steroidogenesis and maturation of Leydig cells are not initiated in the absence of *Fog2* (Fig. 6A-F and data not shown).

As the gonads emerge at E11.0, the signaling molecule *Wnt4* is expressed in the mesenchyme of the indifferent gonad in both sexes and in the mesonephos (Vainio et al., 1999). Activation of steroidogenesis in developing testes is thought to require downregulation of *Wnt4* expression and, correspondingly, ovary-specific expression of *Wnt4* suppresses Leydig cell development and activation of steroidogenic enzymes (Vainio et al., 1999). In normal E13.5 embryos, *Wnt4*

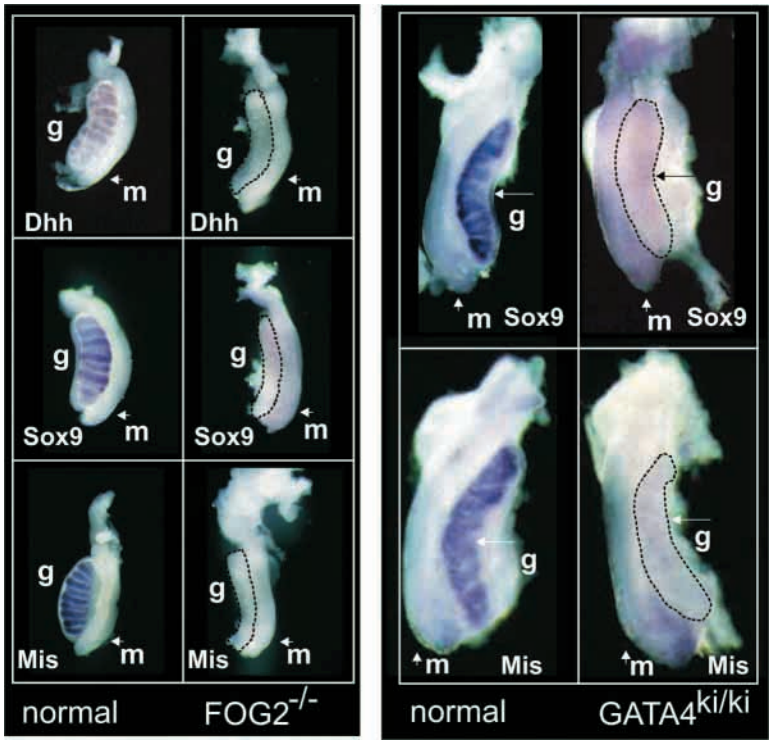

Fig. 5. Whole-mount in situ hybridization analysis of Sertoli cell marker gene expression in control and mutant E13.5 XY gonads. Left panels: *Dhh* (*top*), *Sox9* (*middle*) and *Mis* (*bottom*). Right panels: *Sox9* (top) and *Mis* (bottom). g, gonad; m, mesonephros. None of the Sertoli cell markers examined is expressed in mutant gonads.

is not expressed in XY gonads, whereas *Wnt4* is expressed robustly in XX gonads (Fig. 6G,H). We found that *Wnt4* expression persists in *Fog2*$^{-/-}$ XY gonads (Fig. 6I) similar to what is observed in normal ovaries. This result suggests that failure to downregulate *Wnt4* expression in the absence of FOG2 is relevant to suppression of steroidogenesis in mutant XY gonads.

DISCUSSION

GATA4/FOG2 are part of the sex determination cascade and regulate the expression of multiple gonadal genes in vivo

The results presented here clearly establish an essential role for FOG2 and GATA4 in gonadal differentiation and indicate that testis development is blocked by interfering with their direct physical interaction. Previous expression data suggested that GATA4 was involved in sex determination (Heikinheimo et al., 1997; Viger et al., 1998) and in vitro data suggested a role for GATA4 in the regulation of genes expressed in the gonads downstream of *Sry*, including *Mis*, inhibin α, and steroidogenic acute regulatory protein (reviewed by Hales, 2001; Hastie, 2001). Our results support the hypothesis that GATA4 is involved in sex determination. In addition, these results indicate that FOG2 also is involved in sex determination. Clearly GATA4 and FOG2 are major players in the cascade upstream of *Sry* and our results identify previously unsuspected downstream *Sry* genes that are dependent on GATA4/FOG2 physical interaction.

GATA4/FOG2 interaction is required for normal *Sry* expression

The differentiation of the genital ridge and induction of *Sf1* and *Wt1*expression appears to be grossly normal in the absence of FOG2 or GATA4. *Fog2*$^{-/-}$ XY gonads did initiate the male sex determination program by activating *Sry* expression, but *Sry* transcript levels were significantly reduced. Given that several inherited sex reversal conditions in mice are correlated with low *Sry* expression levels (Laval et al., 1995; Washburn et al., 2001; Nagamine et al., 1999; Hammes et al., 2001), it is likely that lack of testicular tissue development in *Fog2*$^{-/-}$ XY and *Gata4*$^{ki/ki}$ XY mice results from failure of Sertoli cell differentiation. The decrease in *Sry* transcript levels could be caused by a reduced number of *Sry*-expressing cells or a lower level of *Sry* expression per cell. Further experiments will distinguish

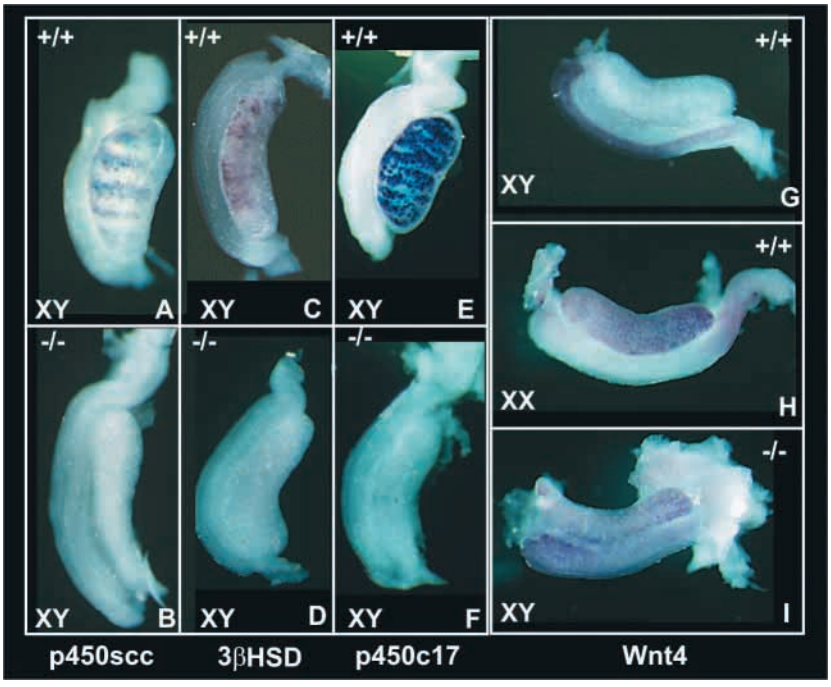

Fig. 6. Whole-mount in situ hybridization analysis of Leydig cell markers (encoding steroidogenic enzymes) and the *Wnt4* gene in E13.5-14.5 gonads. (A,C,E) Control gonads/mesonephroi; (B,D,F), *Fog2*$^{-/-}$ gonads/mesonephroi. Steroidogenic genes are not expressed in the absence of *Fog2* (B,D,F), indicating that Leydig cell differentiation is blocked. *Wnt4* expression is downregulated in XY normal gonads (G), but persists in XX control (H) and XY *Fog2*$^{-/-}$ gonads (I).

between these possibilities. Whichever the case, *Sry* is a target of GATA4/FOG*2* regulation, either directly or indirectly. It is possible that the GATA4/FOG2 complex interacts with the *Sry* promoter directly to activate its expression, or GATA4/FOG2 may participate in a parallel pathway. At E17.5 XY *Fog2*$^{-/-}$ gonads are not simply sex-reversed gonads because they do not resemble normal ovaries. Hence, *Sry* is unlikely to be the sole target of GATA4/FOG2 regulation and the data strongly indicates that GATA4/FOG2 regulation is needed for normal ovarian development as well. At this time the paucity of molecular markers expressed early in ovarian determination makes it more difficult to determine what affect the absence of FOG2 or GATA4 has on ovarian development. However, when early ovarian markers become available, the role of FOG2 and GATA4 in ovarian development can be explored more fully.

GATA4/FOG2 are likely to carry multiple functions during gonadal development

Whether GATA4 and FOG2 have in vivo roles in testis determination downstream of *Sry* cannot be readily assessed given that *Sry* expression was insufficient to initiate Sertoli cell differentiation, thus causing testis differentiation to be blocked early. For example, *Fog2*$^{-/-}$ XY gonads fail to properly downregulate *Wnt4* expression and activate steroidogenesis. However, because an early defect in the differentiation of pre-Sertoli cells prevents further testis differentiation, it is not yet possible to determine if FOG2 or GATA4 act cell-autonomously later in Leydig cells or if they participate in subsequent events in gonadal development and/or spermatogenesis. Also, given the decisive role FOG2 and GATA4 play in the transformation of migrating epicardial cells during cardiac development and formation of coronary vasculature (Tevosian et al., 2000; Crispino et al., 2001), it is possible that these proteins are involved in the migration of mesonephric cells into developing XY gonads. Conditional knockouts of *Fog2* and *Gata4* and other transgenic approaches will allow the dissection of the multiple roles these transcriptional regulators play in the development of various cell lineages in the gonads of both sexes.

In summary, our results provide a basis for incorporating the GATA/FOG paradigm into the genetic pathway of sex determination and sexual differentiation.

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