Correct dosage of *Fog2* and *Gata4* transcription factors is critical for fetal testis development in mice

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Edited by Brigid L. M. Hogan, Duke University Medical Center, Durham, NC, and approved August 7, 2007 (received for review February 22, 2007)

Previous reports suggested that humans and mice differ in their sensitivity to the genetic dosage of transcription factors that play a role in early testicular development. This difference implies that testis determination might be somewhat different in these two species. We report that the *Fog2* and *Gata4* transcription factors are haploinsufficient for testis determination in mice. Whether gonadal sex reversal occurs depends on genetic background (i.e., modifier genes). For example, C57BL/6J (B6) XY mice develop testes if they are heterozygous for a mutant *Fog2* (*Fog2*−) or *Gata4* (*Gata4*^{ki}) allele. However, if the B6 Y chromosome (Y^{B6}) is replaced by the AKR Y chromosome (Y^{AKR}), B6 *Fog2*−/+ XY^{AKR} mice develop ovaries, and B6 *Gata4*^{ki}/+ XY^{AKR} mice develop ovaries and ovotestes (gonads containing both ovarian and testicular tissue). Furthermore, DBA/2J (D2) *Fog2*−/+ XY^{AKR} mice and (B6 × D2)F1 hybrid *Gata4*^{ki}/+ XY^{AKR} mice develop testes. *Sry* is expressed in the mutant XY gonads, indicating that the lack of *Sry* expression is not the cause of ovarian tissue development in B6 *Fog2*−/+ or *Gata4*^{ki}/+ XY^{AKR} mice. However, up-regulation of *Sox9* expression, which is critical for normal testicular development, does not occur in mutant XY gonads that develop as ovaries. We conclude that under certain genetic conditions, *Sox9* up-regulation depends on the proper dosage of *Fog2* and *Gata4*. We propose that in humans the *FOG2* and/or *GATA4* genes might be haploinsufficient for normal testis determination and thus could be the cause of some previously unassigned cases of XY gonadal sex reversal.

gonadal sex reversal | haploinsufficiency | sex determination | *Sry* | *Sox9*

During human fetal development, differentiation of the bipotential gonads into ovaries or testes depends on the proper dosage of transcription factors. For example, the presence of only a single functional copy of the autosomal transcription factor-encoding genes *SF1*, *SOX9*, or *WT1* can result in the development of XY females, and duplication of a chromosomal region containing *SOX9* can lead to the development of XX males (1–5). In contrast, the proper dosage of the transcription factors *Sf1*, *Sox9*, and *Wt1* appears not to affect testicular development in mice because XY mice containing only a single copy of these genes develop testes (6–9). This apparent species difference suggested that the correct dosage of gonadal sex-determining transcription factors is necessary for normal testis development in humans, whereas mice are less sensitive to these gene dosage effects (10–12).

Studies in our laboratory indicate that genetic background plays an essential role in the process of gonadal sex determination in mice. Specifically, mice of the C57BL/6J (B6) inbred strain are exceptionally sensitive to disturbances in the early events of gonad development and thus provide a genetic "litmus test" for identifying genes that cause sex reversal. For example, the *Sry*^{POS} gene carried on the *Mus domesticus poschiavinus* Y chromosome causes ovarian tissue development when present in C57BL/6J (B6) mice but not in DBA/2J (D2) or (B6 × D2)F1 mice (13, 14), and a mutant allele of the X-linked transcription factor *Dax1* (*Nr0b1*) causes gonadal sex reversal in B6 but not D2 or (B6 × D2)F1 XY mice (15). This "B6 sensitivity" is even stronger if the AKR/J Y chromosome (Y^{AKR}) is present. For example, if B6 mice are heterozygous for the *T*^{Orl} mutation, they develop testes. If the Y^{AKR} chromosome is present, however, they develop ovaries (16, 17). These findings suggest that genetic background, not species differences, could explain apparent differences in transcription factor dosage sensitivity for gonad development in XY humans and mice. The fact that not all XY humans are sex-reversed if only a single copy of a normal *SF1* or *WT1* allele is present (18–20) supports this possibility, as does the fact that XY males carrying a mutant *SRY* gene can pass this allele on to XY daughters (for review, see ref. 21).

To test the hypothesis that testis determination in XY mice is sensitive to levels of transcription factor gene dosage in a genetic background-dependent manner, we conducted experiments to determine whether the presence of only a single normal copy of the autosomal *Fog2* (*Zfpm2*) or *Gata4* gene causes XY sex reversal on specific genetic backgrounds. FOG2 is a multitype zinc finger cofactor that binds to and regulates the transcriptional activity of GATA4, a member of the GATA family of transcription factors (22–24). In mice, *Fog2* and *Gata4* are coexpressed in the somatic cells of XX and XY genital ridges as early as embryonic day (E) 10.5 (25–28). Previous work had shown that homozygosity for mutant alleles of *Fog2* or *Gata4* leads to abnormal fetal gonadal development in XY mice from an early developmental stage (29). But an abnormal gonadal phenotype was not observed in heterozygous mice on the mixed B6/129 genetic background, and the XX and XY heterozygotes appeared to have normal fertility.

We report that B6 XY mice develop testes if they are heterozygous for a *Fog2* null (*Fog2*−) (30) or a *Gata4* mutant (*Gata4*^{ki}) (31) allele. *Gata4*^{ki} encodes a mutant GATA4 protein that is unable to interact with FOG2. However, if a Y^{AKR} chromosome is substituted for the B6 Y chromosome, B6 *Fog2*−/+ XY^{AKR} mice develop ovaries (i.e., are completely sex-reversed), and B6 *Gata4*^{ki}/+ XY^{AKR} mice develop ovaries and ovotestes. On the other hand, D2 *Fog2*−/+ XY^{AKR} mice and (B6 × D2)F1 *Gata4*^{ki}/+ XY^{AKR} mice develop testes. These results indicate that, as is the case in humans, testis development in mice requires a sufficient gene dosage of autosomal transcription factors, and they suggest that genetic background (i.e., modifier genes) plays a role in testicular development in humans.

Author contributions: E.M.E. designed research; G.J.B., L.L.W., and K.H.A. performed research; G.J.B., L.L.W., K.H.A., and E.M.E. analyzed data; and G.J.B., K.H.A., and E.M.E. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: E, embryonic day; AMH, anti-müllerian hormone; FGFR2, fibroblast growth factor receptor 2.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0701677104/DC1.

Table 1. Depending on genetic background, the presence of a single functional allele of *Fog2* or *Gata4* causes XY gonadal sex reversal

Fetal genotype	Gonadal phenotype					
	O-O	O-OT	OT-OT	OT-T	T-T	Total
B6 *Fog2*−/+ XY^{B6}	0	0	0	0	12*	12
B6 *Fog2*−/+ XY^{AKR}	16	0	0	0	0	16
(B6 × D2)F1 *Fog2*−/+ XY^{AKR}	0	0	0	1†	19‡	20
D2 *Fog2*−/+ XY^{AKR}	0	0	0	0	14*	14
B6 *Gata4*^{ki}/+ XY^{B6}	0	0	0	0	22*	22
B6 *Gata4*^{ki}/+ XY^{AKR}	8	5	2	0	0	15
(B6 × D2)F1 *Gata4*^{ki}/+ XY^{AKR}	0	0	0	0	18	18

Numbers indicate the no. of mutant XY gonad pairs examined at E14.5–16. O, ovary; T, testis; OT, ovotestis.
*Both testes contained shorter testicular cords.
†One abnormal testis and one ovotestis.
‡One fetus with two normal testes, and 18 fetuses with two testes showing attenuated cord growth.

Results

Genetic Background Determines Whether the Presence of a Single Functional Copy of *Fog2* and *Gata4* Causes XY Gonadal Sex Reversal. To explore the role of *Fog2* and *Gata4* transcription factor gene dosage in gonadal development and to determine whether genetic background affects their role in this process, we examined fetal gonad development in B6 and (B6 × D2)F1 *Fog2*−/+ and *Gata4*^{ki}/+ mice. Testicular growth was abnormal in the gonads of B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY fetal mice (Table 1 and Fig. 1); the developing testicular cords in 12 B6 *Fog2*−/+ and 22 B6 *Gata4*^{ki}/+ XY fetuses were shorter compared with those in control B6 XY normal (+/+) siblings, giving the testes a flat appearance (Fig. 1). This abnormal development, however, does not affect fertility in these mice. The fetal ovaries observed in B6 *Fog2*−/+ or *Gata4*^{ki}/+ XX mice appeared normal (Fig. 1).

We also analyzed gonads from E14.5–16 B6 and (B6 × D2)F1 fetal mice carrying the Y^{AKR} chromosome. As shown in Fig. 1 and Table 1, the presence of only a single functional copy of *Fog2* or *Gata4* in B6 XY^{AKR} mice had a major effect on testis development. The gonads of the 16 B6 *Fog2*−/+ XY^{AKR} fetuses analyzed were ovaries (i.e., no testicular cords were observed), whereas the gonads of the 15 B6 *Gata4*^{ki}/+ XY^{AKR} fetuses were ovaries or ovotestes: eight fetuses contained bilateral ovaries, five contained one ovary and an ovotestis, and two contained ovotestes. The gonads of the 20 (B6 × D2)F1 *Fog2*−/+ XY^{AKR} fetuses analyzed were testes or ovotestes: one fetus contained bilateral normal testes, 18 contained bilateral testes with short cords, and one contained an ovotestis accompanied by a testis with short cords (Table 1). The gonads of the 18 (B6 × D2)F1 *Gata4*^{ki}/+ XY^{AKR} fetuses analyzed were normal testes. Because one (B6 × D2)F1 *Fog2*−/+ XY^{AKR} fetus contained an ovotestis and a testis, we also analyzed gonadal development in 14 D2 *Fog2*−/+ XY^{AKR} mice. No ovarian tissue was observed, but the testicular cords were shorter than normal [see supporting information (SI) Fig. 5]. We conclude that the presence of only one functional copy of a normal *Fog2* or *Gata4* allele can disrupt gonadal development in XY^{AKR} mice. Whether this disruption occurs, however, depends on genetic background.

B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{AKR} Mice Develop Ovarian Tissue. To conduct a more thorough morphological analysis of B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{AKR} gonads, the spatial localization of selected protein markers was determined by using whole-mount immunohistochemical analysis of E13.5 gonad–mesonephros complexes (Fig. 2). The antibodies used specifically recognized anti-müllerian hormone (AMH) (Sertoli cell marker), CD31 (germ cell and vascular endothelial cell marker), fibroblast growth factor receptor 2 (FGFR2) (somatic cell marker, localized to the nucleus in Sertoli cells), and FOXL2 (female somatic cell marker). For 11 of the 13 B6 *Fog2*−/+ XY^{AKR} and 9 of the 14 *Gata4*^{ki}/+ XY^{AKR} gonads analyzed, the expression and localization pattern of these four markers as well as the gonadal morphology resembled that observed in control XX ovaries. Because FOXL2 expression is ovary-specific and because it has been suggested to play a role in initiating the fetal ovarian pathway (32), these results indicate that the ovarian pathway is activated.

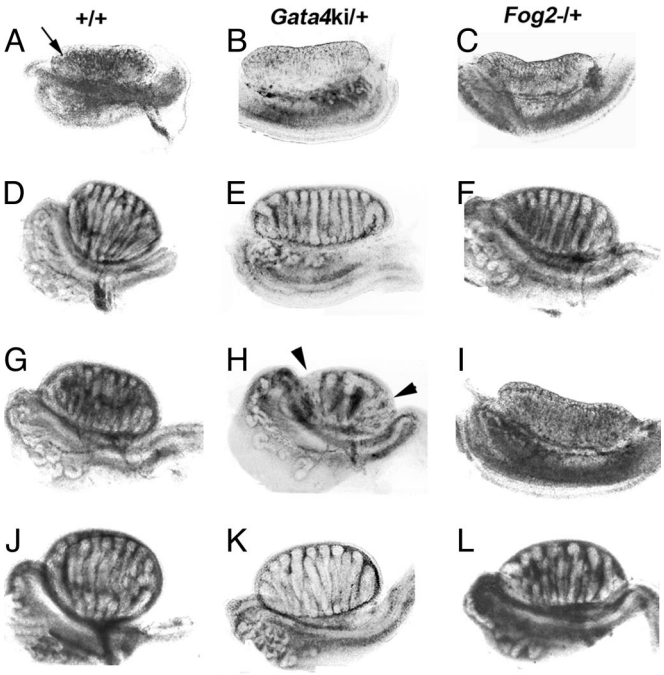

Fig. 1. Embryonic day (*E*) 14.5–15 gonad–mesonephros complexes from normal (+/+) (*A*, *D*, *G*, and *J*), and heterozygous mutant *Gata4*^{ki} (*Gata4*^{ki}/+) (*B*, *E*, *H*, and *K*), and *Fog2*− (*Fog2*−/+) (*C*, *F*, *I*, and *L*) fetuses. (*A*) B6+/+ XX ovary. (*B*) B6 *Gata4*^{ki}/+ XX ovary. (*C*) B6 *Fog2*−/+ XX ovary. (*D*) B6+/+ XY^{B6} testis. (*E*) B6 *Gata4*^{ki}/+ XY^{B6} testis. (*F*) B6 *Fog2*−/+ XY^{B6} testis. (*G*) B6+/+ XY^{AKR} testis. (*H*) B6 *Gata4*^{ki}/+ XY^{AKR} ovotestis. (*I*) B6 *Fog2*−/+ XY^{AKR} ovary. (*J*) (B6 × D2)F1+/+ XY^{AKR} testis. (*K*) (B6 × D2)F1 *Gata4*^{ki}/+ XY^{AKR} testis. (*L*) (B6 × D2)F1 *Fog2*−/+ XY^{AKR} testis. In each case, the gonad (arrow in *A*) lies above the mesonephros. (*E*) Testis with attenuated cord growth. (*H*) Ovotestis. The arrowheads in *H* point to ovarian tissue.

Interestingly, 2 of the 13 B6 *Fog2*−/+ XY^{AKR} and 3 of the 14 B6 *Gata4*^{ki}/+ XY^{AKR} gonads morphologically resembled ovaries but exhibited partial activation of the male pathway. In these gonads, a small number of centrally located cells expressed AMH and had nuclear localization of FGFR2, indicating that these cells had initiated differentiation as Sertoli cells (Fig. 2*D*). However, no testis cords were observed in these 5 gonads. On the other hand, 2 of the 14 B6 *Gata4*^{ki}/+ XY^{AKR} gonads were clearly ovotestes, with regions containing testis cords and expressing AMH and/or nuclear FGFR2. In these ovotestes, the ovarian and testicular regions were discrete, with the ovarian regions expressing FOXL2 or cell surface-localized FGFR2. None of the 13 B6 *Fog2*−/+ XY^{AKR} gonads was ovotestes (i.e., contained testis cords).

***Sry* Is Expressed in B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{AKR} Gonads.** Real-time RT-PCR results indicated that absence of *Sry* expression is not responsible for the failure to induce normal testicular development in B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{AKR} gonads. The level of *Sry* expression in E12 B6 *Fog2*−/+ XY^{AKR} and B6 *Gata4*^{ki}/+ XY^{AKR} gonads was similar to that observed in control E12 B6 XY^{AKR} gonads (Fig. 3). Contrary to what is observed in E14 B6

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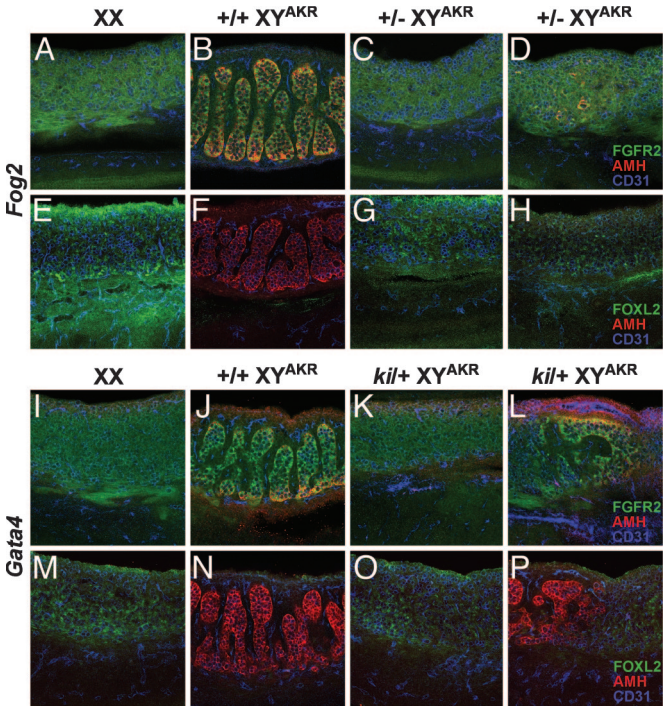

Fig. 2. Whole-mount immunohistochemical analysis of marker gene expression in E13.5 B6 *Fog2*−/+ XY^{AKR} (top two rows) and B6 *Gata4*^{ki}/+ XY^{AKR} (bottom two rows) gonads, and B6 XX and B6 XY^{AKR} control gonads. First and third rows illustrate expression of FGFR2 (green), AMH (red), and CD31 (PECAM; blue); the second and fourth rows illustrate expression of FOXL2 (green), AMH (red), and CD31 (PECAM; blue). The gonads in the first column (*A*, *E*, *I*, and *M*) are normal XX ovaries, and gonads in the second column (*B*, *F*, *J*, and *N*) are normal B6 XY^{AKR} testes. Gonads in the third column are B6 *Fog2*−/+ XY^{AKR} (*C* and *G*) and B6 *Gata4*^{ki}/+ XY^{AKR} (*K* and *O*) ovaries that appear similar to the control B6 XX ovaries (first column). Gonads in the fourth column are B6 *Fog2*−/+ XY^{AKR} ovaries (*D* and *H*) and B6 *Gata4*^{ki}/+ XY^{AKR} ovotestes (*L* and *P*). In each image, the gonad lies above the mesonephros.

XY^{B6} gonads, *Sry* expression was still detected in E14 B6 XY^{AKR} gonads. These results are consistent with a previous report that *M. domesticus Sry* alleles can exhibit prolonged expression in B6 gonads (33). However, at E14, *Sry* expression was significantly higher ($P < 0.05$) in B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{AKR} ovaries compared with control B6 XY^{AKR} testes (Fig. 3). These results are similar to the higher *Sry* expression observed in E14 B6 *Dax1*−/Y ovaries compared with control testes (15).

The Ovarian Genetic Pathway Is Activated in B6 *Fog2*−/+ and B6 *Gata4*^{ki}/+ XY^{AKR} Gonads. A multigene real-time RT-PCR assay was conducted to determine whether the ovarian, or testicular, or both developmental pathways were initiated in B6 *Fog2*−/+ XY^{AKR} and B6 *Gata4*^{ki}/+ XY^{AKR} gonads. The gene expression results indicate that the ovarian genetic pathway is activated despite persistent *Sry* expression (Fig. 4 and SI Table 2). For example, the ovarian-specific genes (i.e., genes expressed at significantly higher levels in ovaries than testes) *Fst*, *Wnt4*, *Bmp2*, and *Adamts19* were expressed at significantly ($P < 0.05$) higher levels, whereas the testicular-specific genes (i.e., genes expressed at significantly higher levels in testes than ovaries) *Amh*, *Dhh*, *Sox9*, and *Fgf9*, were expressed at significantly ($P < 0.05$) lower levels in E14 B6 *Fog2*−/+ XY^{AKR} ovaries and B6 *Gata4*^{ki}/+ XY^{AKR} ovaries compared with control B6 XY^{AKR} testes (Fig. 4 and SI Table 2). In addition, despite normal *Sry* expression at E12, *Sox9* expression was significantly lower ($P < 0.05$) in E12 B6 *Fog2*−/+ XY^{AKR} and B6 *Gata4*^{ki}/+ XY^{AKR} gonads compared with control gonads (2.0- and 2.3-fold, respectively) and *Fst* expression was significantly higher ($P < 0.05$) in E12 B6 *Fog2*−/+ XY^{AKR} and *Gata4*^{ki}/+ XY^{AKR} gonads compared with control gonads (3.5- and 4.6-fold, respectively) (SI Table 2).

We conclude that *Sox9* up-regulation fails even though the *Sry* gene is activated in B6 *Fog2*−/+ XY^{AKR} and B6 *Gata4*^{ki}/+ XY^{AKR} gonads. The result is that the ovarian pathway is initiated, and ovarian development proceeds.

Discussion

We report that *Fog2* and *Gata4* are haploinsufficient for normal fetal testicular development in certain XY mice. The severity of the gonadal phenotype, i.e., the extent of ovarian tissue development, depends on genetic background: gonadal sex reversal is complete in B6 *Fog2*−/+ XY^{AKR} mice, partial in B6 *Gata4*^{ki}/+ XY^{AKR} mice, and does not occur in D2 *Fog2*−/+ XY^{AKR} and (B6 × D2)F1 *Gata4*^{ki}/+ XY^{AKR} mice. These results provide insight into the genetic control of mammalian gonadal development and have implications for human syndromes involving abnormal gonadal sex determination.

Haploinsufficiency for specific transcription factors resulting in gonadal sex reversal has previously been reported in humans but not mice. For example, human XY females have been identified who are heterozygous for a mutant *SF1* or *WT1* allele (1, 4), whereas XY mice that are heterozygous for a mutant *Sf1* or *Wt1* allele were reported to develop as normal males (6, 7). These observations led to the idea that humans and mice differ in their transcription factor dosage requirement for fetal gonad development (11). Results presented here demonstrate that mouse fetal gonadal development is sensitive to transcription factor dosage. Moreover, similar to *Fog2* and *Gata4*, the presence of only a single functional copy of *Sf1* or *Wt1* also can cause gonadal sex reversal in XY mice (unpublished data), further underscoring the concept that testicular development in XY mice is sensitive to transcription factor dosage. When these findings are combined with the results involving B6 Y^{POS} and B6 DAX1 sex reversal in mice (14, 15), it is clear that genetic background and transcription factor dosage are

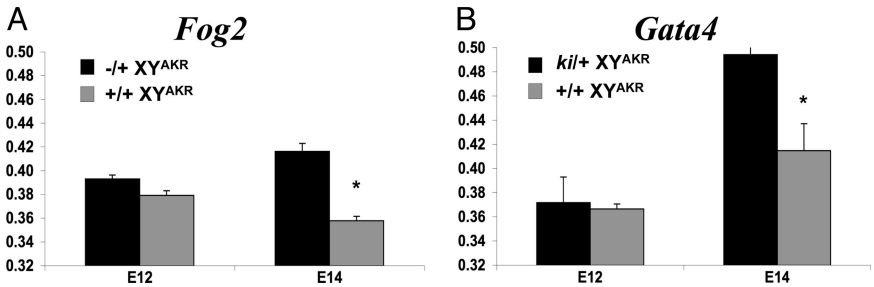

Fig. 3. Relative *Sry* expression in E12 (*A*) and E14 (*B*) *Fog2* and *Gata4* B6 XY^{AKR} homozygous normal gonads and heterozygous mutant gonads. At E14, only heterozygous mutant gonads classified as ovaries were used (i.e., no testicular cords observed). Expression levels are relative to 18S rRNA expression. The mean values represent the average values of a minimum of three cDNA samples (one cDNA sample represents both gonads from one fetus). *, Significant; $P < 0.05$ lower expression.

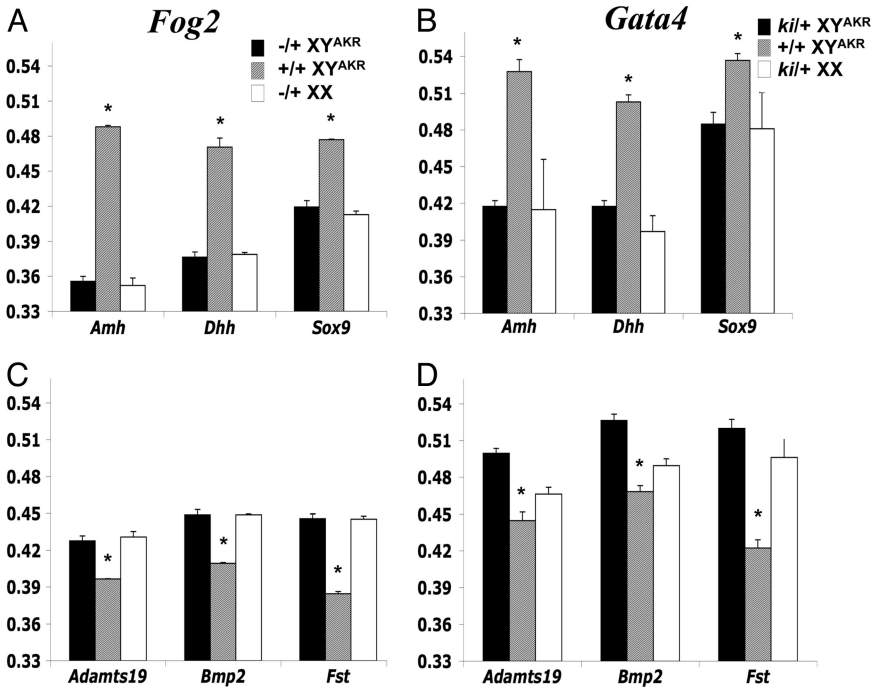

Fig. 4. Expression pattern of fetal testicular-specific (*A* and *B*) and fetal ovarian-specific (*C* and *D*) genes in B6 *Fog2* (*A* and *C*) and B6 *Gata4* (*B* and *D*) homozygous normal and heterozygous mutant E14 gonads. Expression levels are relative to 18S rRNA expression. The mean values represent the average values of a minimum of three cDNA samples (one cDNA sample represents both gonads from one fetus). *, Significant ($P < 0.05$) higher (*A* and *B*) or lower (*C* and *D*) expression levels.

key players in mammalian gonad sex determination and point to the possibility that a single copy of a mutant *FOG2* or *GATA4* gene can cause XY sex reversal in humans and other mammals.

Similar to our observation in B6 *Dax1*−/Y gonads (15), the failure to initiate complete testicular cord formation in B6 *Gata4*^{ki}/+ XY^{AKR} and B6 *Fog2*−/+ XY^{AKR} is not caused by a lack of *Sry* expression but is due to the failure to up-regulate *Sox9* expression in the somatic support cells of the gonad, thereby preventing these cells from differentiating as Sertoli cells. Furthermore, it is likely that the extended *Sry* expression observed in E14 mutant XY ovaries is caused by the lack of *Sox9* up-regulation. Results from Chaboissier *et al*. (9) indicate that *Sox9* up-regulation is responsible for *Sry* down-regulation in pre-Sertoli cells.

Previously, Tevosian *et al.* (29) reported that at E11.5, homozygosity for the *Fog2*− allele lowered *Sry* expression and prevented testicular cord formation. It is possible that before E12, *Sry* expression is lower in B6 *Fog2*−/+ XY^{AKR} gonads compared with B6 *Fog2*+/+ XY^{AKR} gonads. If this possibility is correct, our data suggest that *Sry* levels increase rapidly such that at E12, *Sry* levels are comparable between B6 *Fog2*−/+ and control B6 XY^{AKR} gonads. A single normal *Fog2* allele then is sufficient to establish normal *Sry* levels at E12, a time when the *Sry*^{4KR} allele is normally expressed at peak levels in B6 XY^{AKR} gonads [in B6 mice, the *Sry*^{4KR} allele peaks from 16 tail somites (E11.5) until 22 tail somites (E12) (34, 35)]. Future studies are needed to determine whether *Sry* expression is affected at earlier stages or whether FOG2 and GATA4 can physically interact with the *Sry* gene and regulate its expression.

All B6 *Fog2*−/+ XY^{AKR} gonads were ovaries, whereas B6 *Gata4*^{ki}/+ XY^{AKR} gonads were ovaries or ovotestes. The more severe phenotype observed when only one functional copy of *Fog2* is present compared with only one functional copy of *Gata4* may reflect the nature of the mutant alleles. The *Fog2*− allele is a null allele, whereas the *Gata4*^{ki} allele encodes a mutant GATA4 protein that fails to interact with FOG2. Our data indicate that the interaction between GATA4 and FOG2 is required for efficient *Sox9* up-regulation. However, it is possible that the GATA4^{ki} protein retains some function independent of its interaction with FOG2.

B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY mice develop testes and are fertile males. However, at E15, the testicular cords in these gonads are shorter compared with normal XY gonads, thus giving the testis a flat appearance (e.g., see Fig. 1 *D* and *E*). Fetal testicular cord development and growth are dependent on *Sry*-induced cell migration from the mesonephros into the gonad and cell proliferation (36–38). We propose that the presence of only a single functional *Fog2* or *Gata4* allele interferes with, but does not abolish, *Sry*-mediated signaling pathways involved in cell migration and proliferation, leading to attenuated testicular cord growth in B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{B6} gonads.

Sry is recognized as the mammalian testis-determining gene, although it alone is not sufficient to initiate testicular differentiation. Studies with mice have shown that in the absence of *Sry*, transgenic mice expressing *Sox9* can initiate the testicular developmental pathway (39, 40). *Sox9* up-regulation appears to be a complicated process involving interactions between *Dax1* and *Tda1* (15), and *Fog2* and *Gata4* (this work). The intricate regulation of *Sox9* transcription in pre-Sertoli cells is not surprising considering the number of upstream and downstream sequences involved in *Sox9* transcriptional regulation. Sequences ≈1 Mb upstream of *Sox9* can regulate *Sox9* expression in the gonad (41), and there are at least eight evolutionarily conserved regulatory elements located at the 5′ and 3′ of the *Sox9* gene involved in tissue-specific *Sox9* expression (42). How *Dax1*, *Gata4*, *Fog2*, and *Tda1* control signaling pathways and which signaling pathways are involved in up-regulating *Sox9* expression remain to be determined. Interestingly, Manuylov *et al*. (43) recently reported that the GATA4–FOG2 transcription complex regulates *Sox9* expression in XX transgenic mice that are sex-reversed males, adding further support to the idea that GATA4–FOG2 is involved in regulating *Sox9* expression.

In summary, the presence of only a single functional allele of the transcription factor *Fog2* or *Gata4* causes gonadal sex reversal in B6 XY^{AKR} mice. Gonadal sex reversal is not observed

in B6 *Fog2*−/+ and *Gata4*^{ki}/+ XY^{B6}, (B6 × D2)F1 *Gata4*^{ki}/+ XY^{AKR}, and D2 *Fog2*−/+ XY^{AKR} mice. Ovarian development in B6 *Gata4*^{ki}/+ and *Fog2*−/+ XY^{AKR} mice results from a failure of *Sox9* up-regulation. These results demonstrate that the process of gonadal sex determination in mice is sensitive to transcription factor gene dosage, and they strengthen the concept that genetic background plays an important role in mammalian gonadal development. We hypothesize that the presence of only a single functional *FOG2* or *GATA4* gene can cause gonadal sex reversal in some human XY individuals.

Materials and Methods

Mice. The C57BL/6J (B6) and DBA/2J (D2) *Fog2*− and *Gata4*^{ki} congenic strains were produced by transferring the *Fog2*− and *Gata4*^{ki} alleles (30, 31) to the B6 and D2 mouse strains with successive backcrossing. The B6 Y^{AKR} (16, 17) and D2 Y^{AKR} consomic strains were generated by mating AKR/J males to B6 and D2 females, respectively, followed by successive backcrosses of XY^{AKR} males to B6 or D2 females, respectively. All experiments were conducted by using mice that were at backcross generation N10 or greater. The Jackson Laboratory is American Association for Laboratory Animal Science-accredited, and The Jackson Laboratory Animal Care and Use Committee approved all animal procedures.

Embryo Staging and Genotyping. Timed matings were performed, and noon of the day that a vaginal plug was observed was considered E0.5. Precise fetal age was assessed for fetuses younger than E13 by counting tail somites distal to the hind limbs (44). Fetuses at E13–14.5 were staged according to limb morphology, and at E15 development was confirmed by limb morphology and the extent of formation of the cranial blood vessel (45).

The presence of the Y chromosome was determined by using a multiplex genotyping PCR assay on tissue lysate as described previously (37). The presence of the *Fog2*− allele was identified by using a multiplex PCR assay with a primer pair that amplified a fragment of the myogenin gene as a positive control (5′-TTACGTCCATCGTGGACAGCAT-3′ and 5′-TGGGCTGGGTGTTAGTCTTAT-3′) and a primer pair that amplified a fragment of the neomycin cassette (5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′). The PCR cycle conditions were 95°C for 2 min followed by 40 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s), and 1 cycle at 72°C for 5 min. The presence of the *Gata4*^{ki} and *Gata4*+ alleles was identified by using a primer pair that amplified both alleles (5′-TGCGGAAGGAGGGGATTCAAAC-3′ and 5′-TCTGAGAGAACTGAGGGGGTTAGC-3′) and the following PCR cycle conditions: 95°C for 3 min, followed by 39 cycles of 94°C (30 s), 55°C (30 s), 72°C (1 min), and 1 cycle at 72°C for 5 min. The amplified *Gata4*^{ki} and *Gata4*+ products are ≈300 and 210 bp, respectively.

Whole-Mount Immunohistochemistry. Whole-mount immunohistochemistry was performed on E13.5 gonad–mesonephros complexes and conducted as described previously (46) except the blocking buffer consisted of 10% donkey serum (Jackson ImmunoResearch, West Grove, PA)/3% BSA/0.01% Triton X-100/0.02% sodium azide in PBS. The following antibodies were used: CD31 (1:250, clone MEC13.3; BD PharMingen, San Diego, CA), FOXL2 (1:500; gift from Marc Fellous, Cochin Institute, Paris, France), FGFR2 (1:500, C17, sc-122; Santa Cruz Biotechnology, Santa Cruz, CA), and AMH (1:200, C20, sc-6886; Santa Cruz Biotechnology). All antibodies used in this work have been validated and used in previous published reports (15, 46–49). Images were obtained by using a Zeiss (Thornwood, NY) LSM 510 confocal microscope.

RNA Isolation and Multigene Real-Time RT-PCR Analysis. Multigene real-time RT-PCR was conducted with gonadal tissue collected at E12 (the first time when several ovary- and testis-specific genes are differentially expressed; e.g., see ref. 15) and E14 (ovaries and testes can be distinguished morphologically). Only E14 XY^{AKR} mutant gonads lacking any testicular cords (classified as ovaries) were used for the real-time RT-PCR analysis. E12 gonad–mesonephros complexes and E14 gonads were collected and placed in lysis buffer containing β-mercaptoethanol (RNAeasy kit; Qiagen, Valencia, CA), homogenized, and stored at −80°C until further use. Total RNA was isolated by using the RNeasy mini kit (Qiagen) as described previously (28), except tissue lysate was not applied to a QIAshredder spin column (Qiagen).

Multigene real-time RT-PCR was conducted by using the 56 gene-specific primer pairs described previously (28), and primers specific for *Lhx1* (LIM homeobox protein 1 (5′-TCTCCCCTTTTGATTTGCTAGT-3′ and 5′-GGAGCGACAGGGCAATTAGAG-3′) and the *M. domesticus Sry* gene (5′-TGCCTCAACAAAACTGTACAACCT-3′ and 5′-GGGATATCGACAGGCAGCA-3′). Significant changes in gene expression levels and fold changes were determined by using both Global Pattern Recognition (GPR version 2.0) (50) and Student's *t* test, as described previously (15, 28). At each developmental time point, a minimum of three cDNA samples (one cDNA sample is generated from gonads obtained from one fetus), except at E14, when only two cDNA samples were available from control B6 *Gata4*+/+ XY^{AKR} gonads. Experiments were repeated at least once, and representative results are shown in Figs. 3 and 4. Because gene expression profiles did not differ between B6 XX heterozygous mutant and control ovaries (data not shown), gene expression profiles of B6 XX heterozygous mutant ovaries were used as controls.

We are grateful to Dr. Stuart Orkin (Harvard Stem Cell Institute, Boston, MA) for providing mice containing the *Gata4*^{ki} and *Fog2*− alleles and Dr. Sergei Tevosian (Dartmouth College, Hanover, NH) for the *Gata4*^{ki} and *Gata4*+ primer sequences used for genotyping. We thank Meredith Crane, Andrew Recknagel, and Lisa Somes for help with the maintenance of the mouse strains and conducting PCR assays for determining genotypes; Dr. Marc Fellous (Cochin Institute, Paris, France) for kindly providing the FOXL2 antibody; and Michelle Musson for help with the whole-mount immunohistochemistry. Finally, we thank Drs. Greg Cox, Mary Ann Handel, and George Seidel for helpful comments concerning an earlier version of this paper. This work was supported by National Institutes of Health Grants GM20919 (to E.M.E.), HD07065 (to G.J.B.), and HD042779 (to K.H.A.) and National Cancer Institute Core Grant CA34196 (to The Jackson Laboratory).

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