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The Regulation of *Sox9* Gene Expression by the GATA4/FOG2 Transcriptional Complex in Dominant XX Sex Reversal Mouse Models.

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Abstract

We have previously established an *in vivo* requirement for GATA4 and FOG2 transcription factors in sexual differentiation. *Fog2* null mouse fetuses or fetuses homozygous for a targeted mutation in *Gata4* (*Gata4*^{ki}), which cripples the GATA4-FOG2 interaction, exhibit a profound and early block in testis differentiation in both sexes. Others have shown that XX mice with the *Ods* transgenic insertion or the *Wt1-Sox9* YAC transgene overexpress the testis differentiation gene, *Sox9*. Thus, these XX animals undergo dominant sex-reversal by developing into phenotypically normal, but sterile, males. Now we have determined that *Fog2* haploinsufficiency prevents (suppresses) this dominant sex-reversal and *Fog2*$^{+/-}$ *Wt1-Sox9* or *Ods* XX animals develop normally - as fertile females. The suppression of sex-reversal in *Fog2* heterozygous females results from approximately 50% downregulation of the expression from the transgene-associated allele of *Sox9*. The GATA4/FOG2-dependent sex reversal observed in the transgenic XX gonads has to rely on gene targets other than the Y chromosome-linked *Sry* gene. Importantly, *Fog2* null or *Gata4*$^{ki/ki}$ embryos (either XX or XY) fail to express detectable levels of *Sox9* despite carrying the *Ods* mutation or *Wt1-Sox9* transgene. *Fog2* haploinsufficiency leads to a decreased amount of SOX9-positive cells in XY gonads. We conclude that FOG2 is a limiting factor in the formation of a functional GATA4/FOG2 transcription complex that is required for *Sox9* expression during gonadogenesis.

INTRODUCTION

The basic principle of mammalian sexual determination is that genetic sex is already determined by the presence of the Y chromosome at fertilization. However, male and female embryos are morphologically indistinguishable during their early development; in both sexes the bipotential (indifferent) gonads arise from the urogenital ridges that appear on the surface of mesonephroi, a bilateral rudimentary nephric organ that lies parallel to the differentiating gonad. At a specific developmental stage the male and the female pathways diverge: the XY gonadal anlagen differentiate into testes and the XX anlagen form ovaries (Capel, 2000). This

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sex determination step in mammals is initiated by *Sry*, the Y chromosome-linked testis-determining gene. Triggered by SRY, formation of the testes rather than ovaries from a bipotential embryonic gonad is the decisive step for subsequent male sexual development. The molecular events that set *Sry* in motion and culminate in testis formation remain to be defined.

One of the major downstream targets of SRY in testis is thought to be *Sox9*. *Sox9* expression appears to be both necessary and sufficient for testis development; in mice, *Sox9* alone is sufficient to initiate testis differentiation, independent of *Sry* (Bishop et al., 1999;Qin and Bishop, 2005;Vidal et al., 2001). However, *Sox9* expression is not induced in the absence of *Sry*; thus one of the functions of *Sry* is to activate *Sox9* gene expression. Although the genetic relationship between *Sry* and *Sox9* has been established for quite some time, the mechanism of *Sox9* activation by SRY still remains enigmatic. In addition to a direct activation model of SRY acting through the *Sox9* cis-elements (reviewed in (Kanai et al., 2005;Koopman, 1999), it has been also hypothesized that SRY interferes either with the synthesis of a repressor of *Sox9* (yet unidentified) (McElreavey et al., 1993) or with the binding of this putative repressor to a *Sox9* enhancer (Bishop et al. 1999).

An interest in understanding the transcriptional regulation of *Sox9* expression has been also driven by the involvement of *SOX9* mutation in human disease as heterozygous defects in *SOX9* are associated with the skeletal malformation syndrome (campomelic dysplasia, CD) in humans. The observation that a large proportion of CD patients also experience XY sex reversal revealed a role for SOX9 in human sexual development. Importantly, in some patients chromosome rearrangements were found from 50 kb to 950 kb upstream of *SOX9* (Foster et al., 1994;Pfeifer et al., 1999;Wagner et al., 1994) thus implicating a long-range control for this gene. The involvement of a remote control element gained further support by the fact that mice transgenic for human *SOX9*-spanning YACs showed transgene expression patterns similar to those in endogenous *Sox9*, but only when the YAC transgene contained a 350-kb sequence upstream of *SOX9* (and approximately 250 kb of the 3'-flanking sequence) and not with a truncated YAC that contained only 75 kb of a 5'flanking sequence (Wunderle et al., 1998). Importantly even with these substantial (5'-350kb and 3'-250kb) flanking regions, gonadal expression from the YAC was not observed, thus leading one to propose that the *SOX9/Sox9* gonadal elements could reside even further upstream/downstream (Wunderle et al., 1998). These data have to be reconciled, however, with the reported observation that approximately 70 kb of the 5'- and 30 kb of the 3'-flanking sequence were sufficient for the testis-specific expression of *Sox9* (Lovell-Badge et al., 2002).

We have previously shown an *in vivo* requirement for GATA4 and its co-factor FOG (Friend of GATA)-2 transcription factors in testis differentiation (Tevosian et al., 2002). *Fog2* null (Tevosian et al., 2000) and *Gata4*$^{ki/ki}$ mutant (Crispino et al., 2001) XY gonads are able to initiate the expression of *Sry* (albeit at the substantially lower level compared to the wild-type controls), but not of *Sox9* (Tevosian et al., 2002). Hence, GATA4 /FOG2 function could be required for *Sox9* activation. Given the pivotal position of *Sox9* in gonad differentiation, we hypothesized that the absence of *Sox9* expression could be sufficient to cause the early and severe block in the development of *Gata4*$^{ki/ki}$ and *Fog2* null mutant testis.

It remained unclear, however, whether GATA4/FOG2 complex plays an essential (or any at all) role in testis differentiation subsequent to *Sry* activation. As mutations in GATA4/FOG2 lead to a significant decrease in the expression of the *Sry* gene (Tevosian et al., 2002), it was possible that concomitant loss of its primary target - *Sox9* expression - is indirect and results solely from the down-regulation of *Sry*. We sought to examine this possibility using the *Odd sex* (ocular degeneration with sex reversal, *Ods*) line of animals. In these mice, a fortuitous insertion of the tyrosinase transgene results in mis-regulation (a high level of expression) of the *Sox9* gene in the XX gonad; this high expression of *Sox9* in XX mice results in a dominant,

female-to-male, sex-reversal (Bishop et al., 1999). Since *Ods* allele of *Sox9* still retains all of the elements necessary to specifically activate the *Sox9* gene in the supporting cells of the gonad (Qin et al., 2003) we could derive the information about *Sox9* regulation by GATA4/FOG2. Importantly, the ability of the GATA4/FOG2 complex to regulate the *Sox9 (Ods)* in the XX gonads (in the absence of the Y chromosome) has to be independent from its ability to regulate the *Sry*, the Y chromosome-linked gene.

To further determine whether the loss of *Fog2* affects the steps in the testis differentiation program subsequent to *Sox9* induction, we used another line of transgenic mice, *Wt1-Sox9*. In these animals *Sox9* is expressed from the *Wt-1* regulatory elements within a yeast artificial chromosome (YAC), faithfully mimicking gonadal expression of the endogenous *Wt1* gene (Vidal et al., 2001). *Wt1* is expressed normally in the *Fog2* null gonads (Tevosian et al., 2002) and we anticipated that crossing the *Wt1-Sox9* animals into a $Fog2^{-/-}$ background should result in *Fog2*-independent *Sox9* expression. Hence, in the XY and XX *Wt1-Sox9* transgenic embryos one could potentially separate the SOX9-dependence and GATA4/FOG2-dependence for genes in the testis differentiation program. For example, it has been shown that the *Müllerian inhibitory substance* (*Mis*) gene is an *in vivo* target of SOX9 (Arango et al., 1999); there is also evidence that GATA sites (and GATA4 protein) are essential in the *Mis/MIS* promoter regulation (Viger et al., 1998;Watanabe et al., 2000). In the *Fog2* null or $GATA4^{ki/ki}$ XY embryos neither *Sox9* nor *Mis* are expressed; by restoring *Sox9* expression with *Wt1-Sox9* in *Gata4* or *Fog2* mutants we could determine whether SOX9 is still able to activate *Mis* in the absence of the functional GATA4/FOG2 complex.

Unexpectedly, we have now determined that *Fog2* haploinsufficiency prevents (suppresses) sex-reversal and $Fog2^{+/-}$ *Wt1-Sox9* or *Ods* XX animals develop normally - as fertile females. The suppression of sex-reversal in *Fog2* heterozygous females results from an approximately 50% downregulation of the expression from the transgene-associated allele of *Sox9*. The GATA4/FOG2-dependent sex reversal observed in the transgenic XX gonads has to rely on gene targets other than the Y chromosome-linked *Sry* gene. Importantly, *Fog2* null or $Gata4^{ki/ki}$ embryos (either XX or XY) fail to express detectable levels of *Sox9* despite carrying the *Ods* mutation or *Wt1-Sox9* transgene. We also show that *Fog2* haploinsufficiency results in a decreased amount of SOX9-positive cells in XY gonads. We conclude that FOG2 is a limiting factor in the formation of a functional GATA4/FOG2 transcription complex that is required for *Sox9* expression during gonadogenesis.

RESULTS

Induction of dominant XX sex reversal requires two functional *Fog2* alleles

Fog2 deletion is embryonic lethal in mice (Tevosian et al., 2000). To generate the *Ods*/+ or *Wt1-Sox9*/+ $Fog2^{-/-}$ embryos it was thus necessary to initially introduce these alleles into $Fog2^{+/-}$ background and then backcross the resulting *Wt1-Sox9*/+ $Fog2^{+/-}$ or *Ods*/+ $Fog2^{+/-}$ males with the $Fog2^{+/-}$ females. The *Ods* mutation, in addition to causing a female-to-male sex reversal, also results in the characteristic eye phenotype of micropthalmia with cataracts (Bishop et al., 1999;Qin et al., 2004). To our surprise, among the progeny of the first cross (XY *Ods*/+ x XX $Fog2^{+/-}$), we observed several phenotypic females with the eye phenotype. PCR genotyping of these females confirmed the presence of the *Ods* mutation (3 out of 3); however, in determining their *Fog2* status we also noticed that all of these animals are heterozygous for the *Fog2* mutation (Fig. 1A). In the *Wt1-Sox9* cross (XY *Wt1-Sox9*/+ x XX $Fog2^{+/-}$) all of the F_1 phenotypic female transgenics (XX *Wt1-Sox9*) were similarly positive for the *Fog2* null allele (5 out of 5). In summary, 100% (53/53) of the XX *Ods*/+$Fog2^{+/-}$ and 100% (62/62) of the XX *Wt1-Sox9*/+ $Fog2^{+/-}$ animals remained phenotypic females; all tested females were fertile (delivered offspring). To confirm the absence of sex reversal in these $Fog2^{+/-}$ females we have performed a qRT-PCR for *follistatin* and *Bmp2*

RNA in XX E12.5 gonads. We found no significant difference between these markers in the control (*Bmp2*: 100%, *Fst*: 100%), *OdsFog2*$^{+/-}$ (*Bmp2*: 93 ± 12%; *Fst*: 94±15%) and *Wt1-Sox9Fog2*$^{+/-}$ (*Bmp2* 108±12%; *Fst*:110±15%) females. We also isolated ovaries from the adult XX *Ods Fog2*$^{+/-}$ mice and found them grossly normal upon histological examination (Supplemental Figure 1).

We concluded that the induction of sex reversal in these two models requires a full complement of the functional *Fog2* gene.

XX *Fog2* heterozygous gonads have a decreased level of ectopic *Sox9*

Both the *Ods* mutation and the *Wt1-Sox9* transgene cause complete female-to-male sex reversal by inducing a male-specific expression pattern of *Sox9* in XX embryonic gonads (Bishop et al., 1999;Qin et al., 2003;Vidal et al., 2001). Remarkably, when crossed to the *Fog2*$^{+/-}$ females, the expression of the sex-reversed phenotype is inhibited and the F_1 XX Fog2$^{+/-}$*Ods*/+ or *Wt1-Sox9* mice develop as fully fertile, phenotypic females. To determine the mechanism of this suppression, we compared the *Sox9* expression in the *Ods* or *Wt1-Sox9*-positive E13.5 XX embryos that are either *Fog2*$^{+/+}$ or *Fog2*$^{+/-}$ by whole-mount *in situ* hybridization. We observed no drastic reduction in the *Sox9* staining in the *Fog2*$^{+/-}$ gonad compared to the *Fog2*$^{+/+}$ control (Fig. 1, B-G).

It has been shown recently that testis differentiation is extremely sensitive to *Sox9* levels and that a threshold of *Sox9* expression should be reached to set the testis differentiation program in motion (Barrionuevo et al., 2006;Chaboissier et al., 2004;Qin and Bishop, 2005). We reasoned that a subtle difference in the level of expression might escape detection by *in situ* hybridization. To better measure the level of *Sox9* expression we performed quantitative RT-PCRs. Real-time PCR analyses with RNA isolated from individual E12.5 urogenital ridges confirmed that the *Sox9* level in *Fog2*$^{+/-}$ heterozygous gonads is approximately 50% of the *Fog2*$^{+/+}$ controls (Fig. 1, H).

To evaluate the genetic consequences of the decrease in the *Sox9* expression level we analyzed the expression of the Müllerian-inhibiting substance (*Mis/Amh*) gene, which has been suggested to be a direct target of *Sox9* (Arango et al., 1999;Chaboissier et al., 2004;De Santa Barbara et al., 1998). As previously described, we observed a robust *Mis* expression in the E13.5 XX *Ods*/+ *Fog2*^{wt} and *Wt1-Sox9*/+*Fog2*^{wt} gonads (Figs. 2B and E); (Bishop et al., 1999;Vidal et al., 2001); however, in the transgenic XX *Fog2*$^{+/-}$ gonads *Mis* expression was undetectable (Figs. 2C and F), even when assessed by a sensitive RT-PCR assay (Fig. 2G).

If a decrease in the *Sox9* level is the sole reason for the suppression of sex reversal, merely increasing *Sox9* expression should be sufficient to cause sex-reversal in the *Fog2*$^{+/-}$ animals. Since both XX *Wt1-Sox9/Fog2*$^{+/-}$ and XX *OdsFog2*$^{+/-}$ are fertile females, it was possible to obtain the homozygous *Ods/Ods* or *Wt1-Sox9/Wt1-Sox9* animals by intercrossing them with the XY *OdsFog2*$^{+/-}$ or *Wt1-Sox9Fog2*$^{+/-}$ males, respectively. The double heterozygous, *Ods/Wt1-Sox9*, animals could similarly be obtained. We reasoned that having two copies of these genes should approximately double the ectopic *Sox9* expression in the *Fog2* heterozygous XX mice to the level sufficient for a sex-reversal (Fig. 1F). The analysis of the *Ods/Ods*, *Wt1-Sox9/Wt1-Sox9* or mixed *Ods/Wt1-Sox9* XX animals confirmed them to be the sex-reversed males irrespective of their *Fog2* status as we have predicted (data not shown). While the XX *Fog2*$^{+/-}$ embryos harboring the single *Ods* or *Wt1-Sox9* allele do not express *Mis* (Fig. 2C and F), *Mis* expression is robust in the gonads of the double-transgenic *Wt1-Sox9Wt1-Sox9 Fog2*$^{+/-}$ embryos (Fig. 2H). This experiment confirms that suppression of sex reversal in the *Fog2* heterozygous females results solely from ~50% down-regulation of the *Sox9* expression from the *Ods* or *Wt1-Sox9* allele.

To confirm that *Fog2*$^{+/-}$-mediated suppression of sex-reversal is not limited to *Mis* downregulation (which is a direct target of *Sox9*, (Arango et al., 1999) we examined the expression of other genes that are essential for male gonadal development. We could not detect the expression of *Dhh*, another marker of the Sertoli cell differentiation (Bitgood et al., 1996) as well as the Leydig cell-specific markers *P450Scc* and 3β-*Hsd* at any stage of development (Fig. 3 and data not shown). We conclude that the 50% reduction in the *Sox9* expression suppresses female-to-male sex-reversal and expression of the male-specific genes, *Mis*, *Dhh*, *P450Scc* and 3β-*Hsd*. This data provides a molecular basis for the normal (i.e., not sex-reversed) phenotype of the XX *Fog2*$^{+/-}$ *Ods* or the *Wt1-Sox9* XX *Fog2*$^{+/-}$ animals.

Ectopic Sox9 expression is lost in the absence of the functional GATA4/FOG2 complex

We and others have previously demonstrated that it is the GATA4/FOG2 partnership that is required to execute a number of critical decisions during organogenesis (Crispino et al., 2001;Tevosian et al., 2002). Specifically, fetuses homozygous for a targeted mutation (V205M) in *Gata4*, *Gata4*^{ki}, that cripples the GATA4-FOG2 interaction, exhibit a profound and early block in gonadogenesis that is similar to the block noted in *Fog2*-null fetuses. Hence the *Gata4*^{ki} allele allows a unique insight into the importance of the GATA4-FOG2 interaction for mammalian development (Crispino et al., 2001;Tevosian et al., 2002).

To determine whether the *Sox9* expression driven by the *Ods* mutation or *Wt1-Sox9* transgene requires the presence of the functional GATA4/FOG2 complex, we performed *Sox9 in situ* hybridization with the E12.0 *Ods Gata*$^{ki/ki}$ mutants (Fig. 4). We have shown previously that up-regulation of the endogenous *Sox9* expression in the XY fetal gonads requires GATA4-FOG2 interaction as *Sox9* expression is blocked in either the *Fog2* null or *Gata4*$^{ki/ki}$ embryos ((Tevosian et al., 2002); also Figs. 4B and F). The *Sox9* expression in the heterozygous XX *Ods Gata4*$^{ki/+}$ gonad (Fig. 4E) is comparable to that in the control *Gata4*$^{+/+}$ sample (Fig. 4D) and is functionally sufficient to induce *Mis* expression and sex-reversal (data not shown); however, no *Sox9* expression can be detected in either XY or XX *Ods Gata4*$^{ki/ki}$ homozygous mutants (Figs. 4C and G). Similarly, *Sox9* expression was absent in the *Wt1-Sox9 Gata4*$^{ki/ki}$, *Ods Fog2*-null, and *Wt1-Sox9 Fog2*-null E13.5 gonads in both sexes (Figs. 4H and I, and data not shown). We conclude that not only endogenous, but also ectopic *Sox9* expression requires the presence of a functional GATA4/FOG2 complex.

Fog2 is equally expressed in embryonic gonads of both sexes

XY *Fog2*$^{+/-}$ animals express the wild-type levels of the *Sry* gene (Tevosian et al., 2002) and sufficient *Sox9* (Fig. 1H; compare columns 1 and 2) to develop as normal fertile males; however, in the XX *Fog2*$^{+/-}$ mice the ectopic *Sox9* expression caused by either the *Ods* mutation or *Wt1-Sox9* transgene is suppressed below the threshold level (Fig. 1H; compare columns 2, 3 and 5 to columns 4 and 6). To exclude the possibility that this differential regulation is caused by the higher *Fog2* expression in the XY (male) vs. XX (female) gonad we examined the early expression of *Fog2* in mouse embryonic gonads. First, we evaluated *Fog2* expression by analyzing the β-galactosidase (*lacZ*) marker expression in the *Fog2-LacZ-ires-eGFP* line of mice. In these animals the *lacZ* gene is incorporated ('knocked-in') into the *Fog2* locus to allow *LacZ* expression as a fusion protein in-frame with the first 235 amino acids of the FOG2 protein. The *Fog2-lacZ* module is followed by an ires-eGFP cassette. This creates a null allele of the *Fog2* gene (Fig. 5A). Our analysis of the embryonic gonads demonstrated that *Fog2* is robustly expressed in both XY and XX as early as E11.5 (Fig. 5B-G). To further quantitate the *Fog2* expression level we isolated cDNA from the XY and XX E12.5 gonads and performed qRT-PCR. We did not observe any difference in the *Fog2* expression levels between the sexes at this stage; however, *Fog2* heterozygous males express approximately 50% of the wild-type RNA level (Fig. 5H). Our data is consistent with the previously performed

in situ hybridization analysis of *Fog2* expression in mouse embryonic gonads (Ketola et al., 2002).

Transgenically-derived SOX9 is not acting in the catalytic fashion

We observed that despite carrying the *Ods* mutation or *Sox9* over-expressing *Wt1-Sox9* transgene, *Fog2* null or GATA4$^{ki/ki}$ embryos (either XX or XY) fail to express detectable levels of *Sox9* (Fig. 5 and data not shown). Although this result could be envisaged for the *Ods* mutation (see Introduction), this was clearly unexpected for the *Wt1-Sox9*. In the *Wt1-Sox9* transgenics *Sox9* expression is driven by the regulatory elements of the *Wt1* locus encoded by a 620-kilobase mouse YAC (Vidal et al., 2001), the same elements that drive expression of the *Wt1* gene itself. *Wt1* expression is not affected by the *Fog2* deficiency either in male or in female gonads (Tevosian et al., 2002; Fig. 6).).

It was also important to eliminate the possibility that the transgenic *Sox9* in *Wt1-Sox9* could act in a catalytic manner (analogous to *Sry* in the XY gonad) to activate the endogenous *Sox9* in the pre-Sertoli cells of the XX gonad. In this case there could be a difference in the amount of *Wt1*-expressing vs. *Sox9*-expressing cells. To address this question we performed a qRT-PCR. The *Wt1*-driven *Sox9* RNA could be distinguished from the endogenous *Sox9* message by the presence of the SV40 polyadenylation sequence (Vidal et al., 2001). We detected no difference between the ectopic (SV40-containing) and the total *Sox9* level in the XX *Wt1-Sox9* E12.5 gonad (data not shown) confirming that *Sox9* expression in the transgenic gonad is derived solely from the *Wt1* transgene.

Fog2 haploinsufficiency leads to a decreased amount of *Sox9*-positive cells in the XY E12.5 gonads

Either *Ods* or the *Wt1-Sox9* transgene is not sufficient to activate *Sox9* expression in the absence of GATA4/FOG2 interaction. This suggested to us that GATA4/FOG2 interaction is specifically required either for the emergence of the Sertoli precursor cells or, more likely, for their subsequent survival. As *Fog2* heterozygous animals express 50% of the control *Sox9* level (Fig. 1) this should result in a corresponding decrease in the amount of SOX9-positive cells. To access the status of the SOX9-positive cells we performed a whole-mount staining of the E12.5 XY gonads with the SOX9 antibody. The amount of SOX9-positive cells in the E12.5 of the *Fog2* heterozygous males decreases approximately two-fold (Fig. 7, A–B and E); however, overall cellular proliferation (mitotic index) does not appear to be affected (Fig. 7, C–D). To confirm that the reduction in SOX9-positive cells on E12.5 is not due to the earlier decrease in total cell numbers, we performed an analysis of processes that could affect the cell quantity, proliferation and apoptosis, on E11.5 No difference in phospho-H3 staining could be detected between the control and *Fog2* heterozygous E11.5 samples (Fig. 8, A-B). Similarly, no changes in the amount of TUNEL-positive cells were observed between the control and *Fog2* heterozygous samples, although apoptotic cells could be easily detected in the developing mesonephros (Fig. 8, C–D). We conclude that FOG2/GATA4 function is required to maintain precise numbers of Sertoli cells in the developing gonads.

Discussion

SOX9/Sox9 is a key regulatory gene of the testis that is both necessary and sufficient for mammalian male sex differentiation. The regulation of *Sox9* gonadal expression ensures precise (sex, organ and time) expression of this crucial sex-determining gene and remains the subject of considerable interest (Kanai and Koopman, 1999;Koopman et al., 2001). Better understanding of *Sox9* regulation is essential to arrive at the genetic basis for mammalian testicular differentiation and function.

Examination of the *Sox9* regulation in an *Ods* line of mice initially seemed to pin down the location for the elusive *Sox9* gonadal cis-regulatory sequences. In *Ods* animals a tyrosinase minigene driven by the dopachrome tautomerase (*Dct*) promoter was randomly inserted ~1 Mb upstream of *Sox9*, additionally causing a 134-kb deletion of the adjacent sequence. *Ods* mice show female-to-male sex reversal, as well as microphthalmia with cataracts. The XX sex-reversal phenotype is accompanied by misexpression of *Sox9* in the XX gonad, where *Sox9* is usually repressed. It was originally proposed that the serendipitous deletion of the 134 kb region had removed the gonad-specific regulatory element(s) that would normally mediate the female-specific repression of *Sox9*, thus resulting in up-regulation of *Sox9* and the consequent male development (Bishop et al. 2000). However, subsequent experiments have shown that the 134-kb deletion alone is insufficient to cause the sex reversal (Qin et al., 2004). A follow-up experiment recreated both the *Ods* deletion and the tyrosinase insertion (this time driven by its own promoter instead of the *Dct*); however, the *Ods* sex-reversal phenotype still was not observed (Qin et al., 2004).

In this study we did not focus on the analysis of the phenotype in wild-type XX *Ods*/+ animals as *Ods*-mediated sex reversal has been analyzed previously (Bishop et al., 1999;Qin et al., 2003). Specifically, it was reported that the animals on the pure FVB develop as 100% *Ods*/+ males. The contribution of genetic background on sex reversal was also examined; specifically, FVB/N XY *Ods*/+ carrier males have been crossed with several inbred strains; again, with the notable exception of the A/J strain, it was reported that F1 XX *Ods*/+ mice derived from these crosses develop as typical sex-reversed males (Qin et al., 2003). These crosses included the C57BL/6 and 129/Sv strains used in this (our) study. Even when crossed to the ICR (outbred) strain of mice 90% of the XX *Ods*/+ animals developed as males; the rest remained females (Bishop et al., 1999).

Our results are in agreement with the results reported by the Bishop's group. For example, in this study external genitalia in 66 (95%) of the wild-type XX *Ods*/+ animals appeared male; only 4 mice (~6%) developed as females with microphtalmia. In stark contrast, all 53 (100%) of the XX *Ods*/+ *Fog2* heterozygous animals developed as females (judged by their external genitalia).

We have not observed any animals with ambiguous genitalia or who are clear hermaphrodites; although sexual differentiation in the XX *Ods*/+ animals could be somewhat delayed, they develop testes and are sex reversed males. We also dissected gonads from ten of the adult XX *Ods*/+ animals obtained by crossing of the *Ods*/+ males with wild-type females (all animals were on the FVB/C57Bl/6/129 mixed background) and we performed a histological examination of the gonads; all ten of them had two small well-descended testes devoid of germ cells (Supplemental Figure 2). We have previously established an *in vivo* requirement for GATA4 and FOG2 in sexual differentiation. *Fog2* null mouse fetuses or fetuses homozygous for a targeted mutation in *Gata4* (*Gata4*^{ki}) that cripples the GATA4-FOG2 interaction, exhibit a profound and early block in gonadogenesis in both sexes (Tevosian et al., 2002). We have now determined that *Ods* sex reversal (Bishop et al., 1999) as well as another previously described *Sox9*-dependent XX dominant sex reversal model, *Wt1-Sox9* (Vidal et al., 2001), require a full complement of *Fog2*. On the contrary, a single fully functional allele of *Gata4* provides sufficient amounts of the active GATA4/FOG2 complex to allow for ectopic XX *Sox9* expression and a sex-reversal in these models. We conclude that FOG2 is a limiting factor for the formation of a functional GATA4/FOG2 complex in gonads. We also propose that *Fog2* and/or *Gata4* should be considered as potential modifiers for the *Ods* in addition to the one described previously (Qin et al., 2003).

Sox9 expression in the *Ods* mutant XX gonads of the *Gata4*$^{ki/+}$ animals (Fig. 4E) (or in the XX *Gata4*^{ki} *Wt1-Sox9* transgenic, data not shown) appears unperturbed and is sufficient to

cause sex-reversal; however, the complete absence of a wild type GATA4 protein in the homozygous *GATA4*$^{ki/ki}$ embryos (both XX or XY) blocks *Sox9* expression (Fig. 4C and 4G) and the sex-reversal phenotype is suppressed even with the full complement of *Fog2* being present. Hence, an interaction between GATA4 and FOG2 is required for the expression of the trangene-driven (*Wt1-Sox9*) or the mutant (*Ods*) *Sox9*.

GATA4$^{ki/ki}$ *and FOG2*$^{-/-}$ XY gonads initiate the male sex determination program by activating *Sry* expression, but *Sry* levels in *FOG2*$^{-/-}$ gonads are insufficient to induce the differentiation of Sertoli cells. Given that *Sry* expression is not properly activated and therefore testis differentiation is blocked at the earliest stage, it was previously impossible to determine whether GATA4/FOG2 complex have an *in vivo* role in testis determination independent of *Sry* regulation. The suppression of sex reversal that we now observe in the XX gonads has to rely on gene targets other than the Y chromosome-linked *Sry* gene. This observation is in accordance with several previous reports that propose the role for GATA/FOG factors in the regulation of multiple testis-specific genes (Martin et al., 2005;Robert et al., 2002;Tremblay and Viger, 2001). At this point we are unable to determine whether other genes (besides *Sox9*) require GATA4/FOG2 complex for their expression.

The expression from the wild-type (endogenous) *Sox9* allele is shut down in the *Gata*$^{ki/ki}$ or *Fog2* mutant gonad (Tevosian et al., 2002), but we could not *a priori* predict whether the expression from the *Ods* allele of *Sox9* will be lost as well. As we have now determined, *Ods* is not expressed in the *Gata4/Fog2* mutant background; in fact, deletion of even a single functional *Fog2* allele already interferes with the *Ods* expression. We conclude that inactivation of the GATA4/FOG2 complex equally affects the wild-type *Sox9* and its *Ods* "version". In other words, while the *Ods* mutation releases *Sox9* from the negative control in XX gonads, it does not render *Sox9* insensitive to GATA4/FOG2 regulation. This implies that GATA4/FOG2 similarly regulates both *Sox9* and *Ods* and that this regulation is *Sry*–independent, although not necessarily direct (Fig. 9).

The 50% downregulation of expression from the *Wt1-Sox9* transgene in the XX Fog2$^{+/-}$ mutant background and the complete loss of its expression in the GATA4$^{ki/ki}$ or FOG2 null gonads were quite unexpected. In the *Wt1-Sox9* transgenics *Sox9* is expressed from the *Wt-1* regulatory element within a yeast artificial chromosome (YAC), faithfully mimicking gonadal expression of the endogenous *Wt1* gene (Vidal et al., 2001). As our previous data indicated that *Wt1* is expressed normally in the *Fog2* null gonad (Tevosian et al., 2002), we expected the *Wt1*-driven *Sox9* expression to be *Fog2*-independent. We cannot formally rule out the possibility that, in contrast to the endogenous *Wt1* gene, regulatory elements present in the YAC require the full dosage of *Fog2*; however, that seems unlikely. Instead, we show that a reduction in the dosage of *Fog2* gene expression leads to the concomitant decrease in the amount of SOX9 positive cells (Fig. 7, A–B) while the general proliferation and apoptosis in the gonad are not affected. Hence we propose that GATA4/FOG2 function is required to generate or maintain Sertoli cell precursors in the gonad. Although a direct, cell-autonomous activation of *Sox9* gene transcription by GATA4 or GATA4/FOG2 complex cannot be excluded, we currently favor a model where GATA4/FOG2-induced signaling (e.g. through FGF9) ensures the survival of the Sertoli cell progenitors in the developing testis.

Experimental Procedures

Animals

The *Odd sex* (*Ods*) FVB animals were a kind gift of Paul Overbeek (Bishop et al., 1999); *Wt1-Sox9* CBAxC57BL/6J transgenics were kindly provided by Andreas Schedl (Vidal et al., 2001). Transgenic lines were obtained by crossing these mice with *Fog2* heterozygous and *Gata4*^{ki} heterozygous animals (both mixed 129xC56BL/6 background); the generation and

genotyping of *Fog2*- and *Gata4*-targeted animals have been previously described (Crispino et al., 2001;Tevosian et al., 2000). The *Ods* genotype was scored as *Ods*/+ or +/+ by examination of the eyes and by PCR analysis using primers specific for the transgenic construct 5'-CTGTCCAGTGCACCATCTGGACC -3' and 5'-GATTACGTAATAGTGGTCCCTCAG-3'(Bishop et al., 1999). *Wt-Sox9* transgenic animals were genotyped using PCR primers sWTp 5'-CATCCGAGCCGCACCTCATG-3' and SS2 5'-GCTGGAGCCGTTGACGCG-3' as previously described (Vidal et al., 2001). Mice were genotyped for the presence of the Y chromosome by *Sry* or *Zfy* PCR as described previously (Tevosian et al., 2002) and also by the Southern blotting analysis using various fragments of the genomic Sry locus (p741, (Gubbay et al., 1990); generously provided by Kenn Albrecht and Eva Eicher) as probes. The mice were recorded as male or female after external and internal examination of the genitalia. The embryos were isolated and staged as previously described (Tevosian et al., 2002).

Generation of the reporter knock-in into the *Fog2* locus

All genomic DNA corresponding to various regions of the *Fog2* gene was obtained from the pBSK KSII (Stratagene) subclones of the λFixII 129 mouse genomic library (Stratagene). The NcoI-XbaI β-galactosidase gene from pSDKlacZpA (a kind gift of Janet Rossant) was subcloned into pBSK KSII (this cloning strategy does not retain the polyadenylation signal of the *LacZpA* cassette). A 2.6kb SalI-NcoI fragment containing a 5' homology region of the *Fog2* gene was cloned in-frame with the *LacZ* gene. Next, the *ires-EGFP-SV40pA* fragment from pIRES2-EGFP (Clontech) was cloned in the XbaI site of this vector. The resulting *Fog2-β-gal-ires-EGFP* cassette was transferred into the pTKLNCL targeting vector (Tevosian et al., 2000) to generate pTKLNCL-5'. The NotI fragment containing the 5.1kb of the *Fog2* 3' region was introduced into the NotI site of the pTKLNCL-5' to generate the targeting vector. The resulting plasmid was isolated by CsCl centrifugation, linearized with KspI and electroporated into the CJ7 ES cells. The neo/tk-selected ES clones have been genotyped by Southern blotting analysis. The correctly targeted ES cells were injected into the C57BL/6 blastocysts. The *Fog2-LacZ-ires-eGFP* strain was maintained on the mixed, C57BL/6x129 background.

Whole mount in situ hybridization

Embryos of various stages were dissected from the uterus and their internal organs were removed to expose the gonads. Embryos were fixed with 4% paraformaldehyde (PFA) in 1xPBS at 4^0C overnight. Further processing of embryos and in situ hybridization analysis were carried out essentially as described (Wilkinson, 1992). *Sox9*, *Mis*, *Scc*, *Dhh* and 3β*Hsd* dig-labeled RNA probes have been previously described (Tevosian et al., 2002). Gonads were photographed and images were processed and assembled as previously described (Adameyko et al., 2005).

Quantitative RT-PCR analysis

Individual gonad/mesonephros complexes were dissected in PBS from E11.5-E13.5 embryos and transferred in RNAlater solution (Ambion). RNA was isolated with RNeasy Mini kit (Qiagen) in 30μl of RNAse-free TE buffer. RNA samples were treated with RQ1 DNase (Promega) for 30 min and RNA was extracted with phenol:chloroform and ethanol precipitated according to standard procedures. Each sample was divided into two aliquots, one of which was reverse transcribed using the SuperScript First Strand Synthesis System (Invitrogen), following the manufacturer's instructions. The second aliquot was used as a control (RT-PCR without reverse transcription) to identify samples with DNA contamination. All real time PCR assays were carried out using TaqMan Universal PCR Master Mix (Applied Biosystems). The PCR reactions contained a) 25 ng of cDNA, b) the gene specific primers at a final concentration of 1 μM each and c) the Taqman probe at a final concentration of 0.25 μM. The assays were

run under standard Taqman conditions on the ABI 7500 instrument. A standard curve for each gene was generated using serial dilutions of cDNA. Relative expression levels of each sample were determined in the same run and were expressed as the ratio of the RNA amount (of interest) to the amount of a control RNA (β-actin). TaqMan reactions were performed in duplicates and the experiments were repeated independently at least three times (for at least three samples). Gene-specific primers and probes were designed using the Primer Express software (Perkin Elmer Life Sciences), namely qRT-PCR primers: β-actin; 5'-ACGGCCAGGTCATCACTATTG-3' and 5'-CAAGAAGGAAGGCTGGAAAAGA-3', with hybridization probe 5'FAM- CAACGAGCGGTTCCGATGCCC-BHQ3' *Fog2;* 5'-GCAAGTCCTGTGGCATCTG-3' and 5'-CTCGTTGCCTCCCACTACAGTA-3', hybridization probe 5'FAM- AGCGGAACCTGCAAGCCCATTTG-BHQ3' *Wt1;* 5'-AGGACACGACTGTGGATCTACATC -3' and 5'-TTCCGGCAAACCTGATAGGA -3', hybridization probe 5'FAM- TCCAAGACAGCACACCTGATTGACTGC-BHQ3' *Sox9;* 5'-CAAGCGGAGGCCGAAGA -3' and 5'-CAGCTTGCACGTCGGTTT -3', hybridization probe 5'FAM-CCACCCACCACTCCCAAAACCGAC-BHQ3' RT-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 RT-PCR primers for semi-quantitative analysis of the *Mis* gene expression were 5'-GCAGTTGCTAGTCCTACATCTGGCT -3' and 5'-TGGAGGCTCTTGGAACTTCAGCAA -3'. SV40 primers were 5'-TGAGTTTGGACAAACCACAAC -3' and 5'-CCCCCTGAACCTGAAACATA-3'. For an analysis of the *Fst* and *Bmp2* gene expression, quantitative Real Time RT-PCR was performed using a PCR SYBR Green I Kit (Applied Biosystems) according to the manufacturer's instructions in a 7500 Fast RT-PCR system machine. A standard amplification protocol was established for both genes. Calibration curves were generated using dilutions of a corresponding gel-purified RT-PCR product; calibration points for serial dilutions were from 10^6 to 10^2 copies. The value for each gene was normalized to the *Gapdh* gene value and these ratios were compared between XX controls and *Ods/Wt1-Sox9 Fog2* heterozygous samples. The gene copy number was calculated with ABI SDS Software Version 1.3.1. Values from five independent experiments were compared; standard deviation and a Student's t-test were calculated using Excel (Microsoft). Gene-specific primers were designed using the Primer3 software on the Web (Rozen and Skaletsky, 2000); *Fst* primers were 5'-AGAGGTCGCTGCTCTCTCTG-3' and 5'-AGCTTCCTTCATGGCACACT-3'; *Bmp2* primers were 5'-CGACGGAACATTCTCCAAAT-3' and 5'-ATTACGGGATTCTCGGAGGT-3'.

β-Galactosidase assay

Embryos were fixed and stained using X-gal essentially as previously described (Adameyko et al., 2005). The staining was continuously monitored until a satisfactory color development was achieved (2 to 5 hr). Embryos were then fixed overnight in 4% paraformaldehyde in PBS and photographed as previously described (Adameyko et al., 2005).

Whole-mount immunostaining and TUNEL assay

Whole-mount immunohistochemical analysis was performed as described previously (Albrecht and Eicher, 2001;Kim et al., 2006). Briefly, E11.5-E12.5 gonad-mesonephros complexes were fixed overnight at 4°C in 4% paraformaldehyde, followed by a 24 hour incubation in a blocking buffer (1% BSA, 0.1% saponin, 0.02% sodium azide in PBS) at 4°C. Samples were incubated with primary antibodies for 24 hours, washed and incubated for another 24 hours with secondary antibodies. Primary antibodies were rabbit anti-SOX9 (1:1000), rat anti-PECAM1 (BD Biosciences, 1:500) and rabbit anti-phosphorylated histone H3 (Cell Signaling; 1:250). Secondary antibodies used for visualization were anti-rabbit and anti-rat Alexa Fluor-488 and -555 conjugated (Invitrogen; 1:750). Fluorescently labeled samples were mounted in Slowfade (Invitrogen). Images were obtained using a Leica TCS-

NT laser-scanning confocal microscope, and assembled using Adobe Photoshop (Adobe). Apoptotic cells were detected in frozen sections of the E11.5-E12.5 gonads. Samples were frozen in the OCTcompound (VWR) and sectioned on the cryostat (Leica) to obtain 10μ sections. Apoptotic cells were detected by terminal deoxynucleotide UTP nick-end labeling (TUNEL) using an *In Situ* Cell Death Detection kit (Roche Biosciences) according to the manufacturer's instructions. Nuclei were counterstained with Vectashield with DAPI (Vector).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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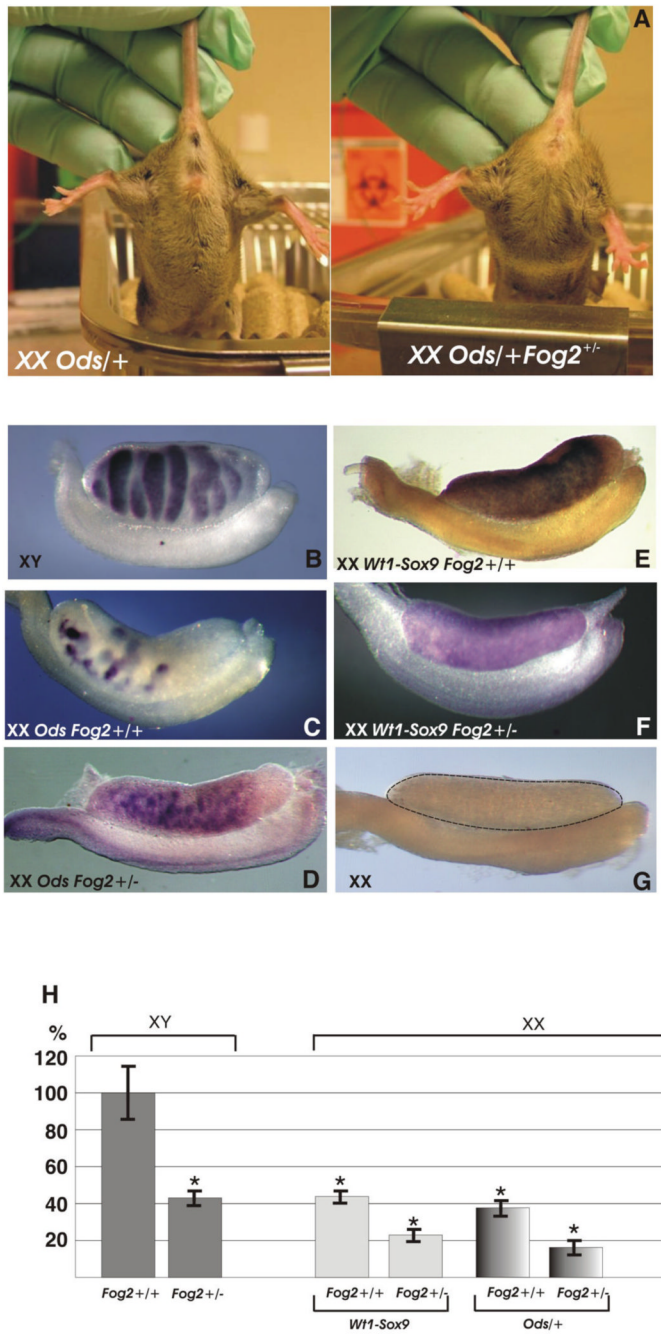

Figure 1.

(A) An XX *Ods/+ Fog2*$^{+/+}$ and an XX *Ods/+ Fog2*$^{+/-}$ transgenic littermates are shown. The *Ods/+* mouse has male external genitalia (*left*), while the *OdsFog2*$^{+/-}$ heterozygous animal is a fertile female (*right*). (B-) Whole mount in situ hybridization analysis of the *Sox9* expression in the E13.5 embryonic male (B), XX sex-reversed (C and E) and XX *Fog2* heterozygous (D and F) gonads. *Sox9* expression is present in all the samples with the exception of the E13.5 female (G). (F) Real-time PCR analysis (TaqMan) of *Sox9* expression in the E12.5 embryonic gonads.

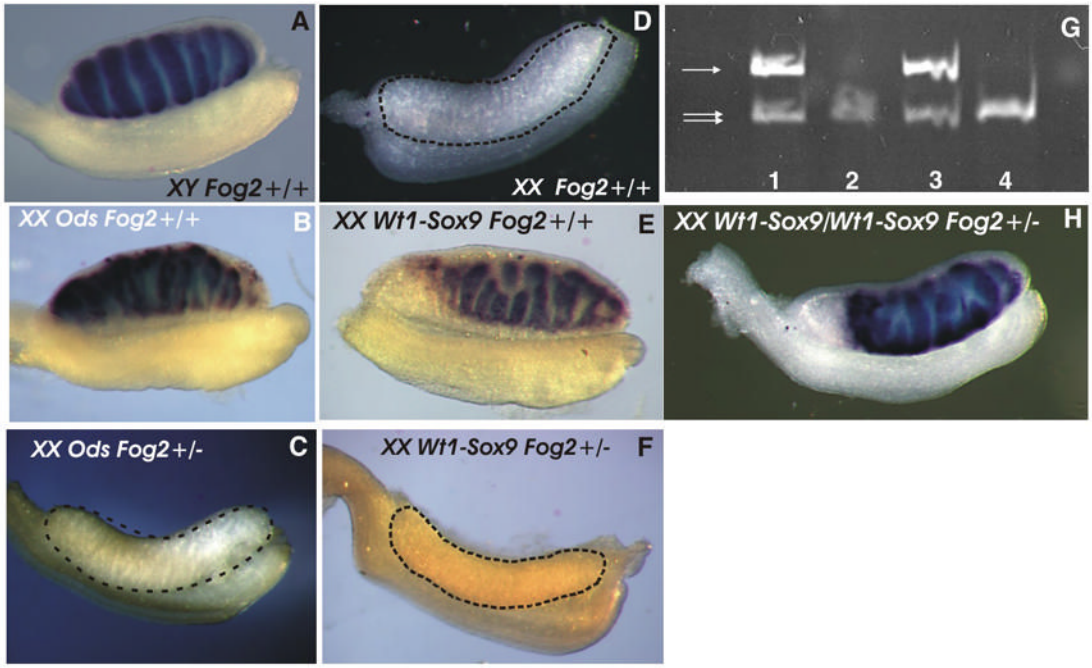

Figure 2.

(A-F) Whole mount in situ hybridization analysis of the *Mis* expression in the E13.5 embryonic male (A), XX sex-reversed (B and E), XX *Fog2* heterozygous (C and F) and XX (female) (D) gonads. *Mis* expression is absent from the control sample in (D); it is also not observed in the heterozygous *Fog2* transgenic (*Ods* or *Wt1-Sox9*) gonads (C, F). (G) Semi-quantitative RT-PCR analysis of *Mis* gene expression in the XY (1), XX (2), XX *Wt1-Sox9* (3) and XX *Wt1-Sox9 Fog2*$^{+/-}$ (4) E12.5 gonads. The positions of the bands corresponding to the *Mis* (arrow) and *Hprt* control (double arrow) PCR fragments are shown. (H) Whole mount in situ hybridization analysis of the *Mis* expression in the E14.0 double transgenic XX *Wt1-Sox9/Wt1-Sox9 Fog2*$^{+/-}$ gonad where robust *Mis* expression is apparent.

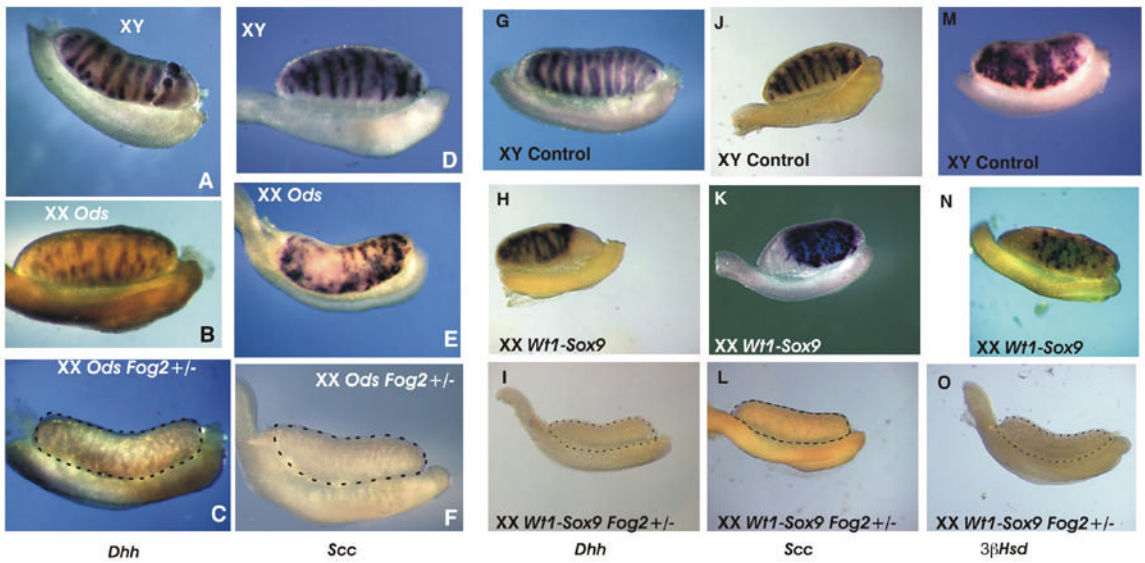

Figure 3.

Whole mount in situ hybridization analysis of the Sertoli cell (A-C; G-I) and Leydig cell (D-F; J-O) gene expression in the E13.5-E14.5 gonads. XY control gonads/mesonephroi (A, D, G, J, M), XX sex-reversed gonads/mesonephroi (*Ods*, B, E; *Wt1-Sox9*, H, K, N) and XX *Fog2* heterozygous gonads/mesonephroi (*Ods*, C, F; *Wt1-Sox9*, I, L, O) were hybridized with RNA probes to *Desert hedgehog Dhh* (A-C; G-I), *P450scc* (*Scc*)(D-F; J-L) and *3*β-*hydroxysteroid dehydrogenase/*Δ*[5]-* Δ *[4]-isomerase* (*3*β*Hsd*) (M-O). Marker gene expression is absent in the gonadal portion (encircled by a dotted line) of the XX *Fog2*$^{+/-}$ samples. All gonads within a set (A-C; D-F; G-I; J-L and M-O) are from the same experiment.

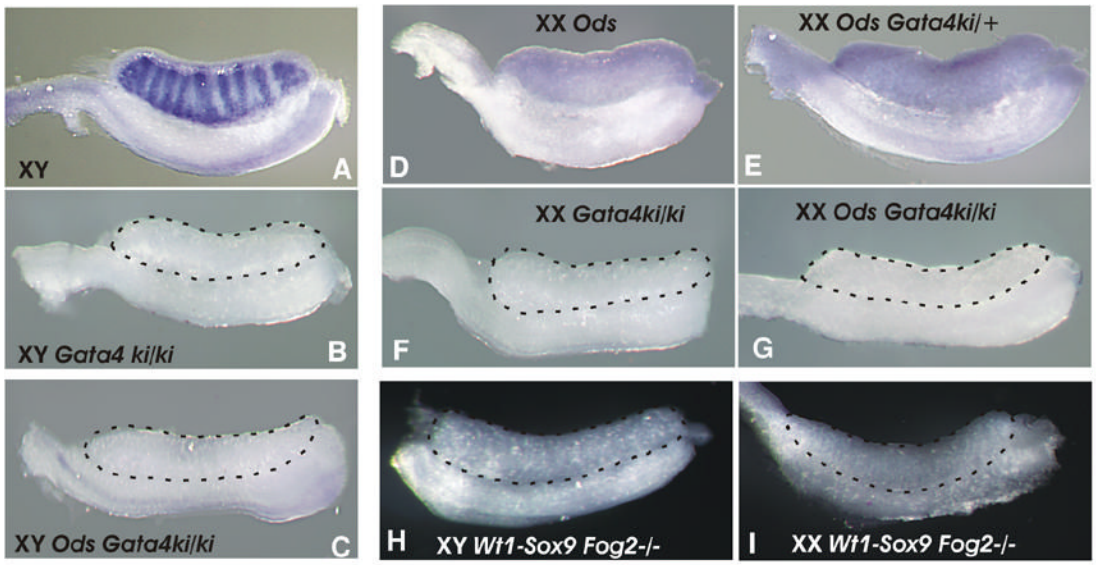

Figure 4.

(A-G) Whole mount in situ hybridization analysis of the *Sox9* expression in the XY (A-C) and XX (D-G) E12.0 gonads. *Sox9* expression is detectable in the male control and *Ods* sex-reversed gonads (A, D, E); *Sox9* expression is absent in the $GATA4^{ki/ki}$ homozygous gonads (B-C; F-G) even in the *Ods* mutants (XY, C; XX, G). (H-I) Whole mount in situ hybridization analysis of the *Sox9* expression in the E13.5 XY (H) and XX (I) *Wt1-Sox9* $Fog2^{-/-}$ gonads; *Sox9* expression is absent.

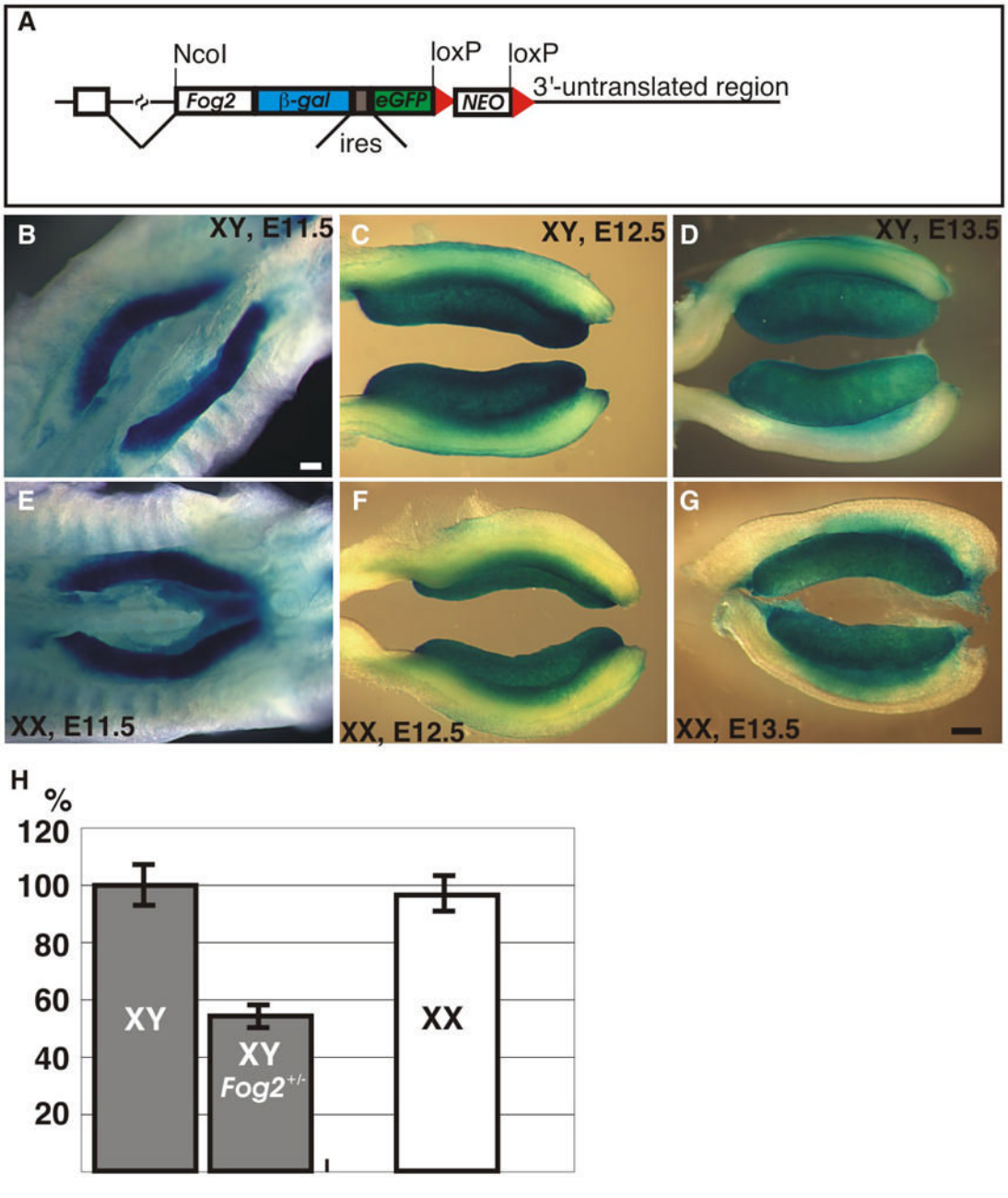

Figure 5.

(A) Schematic representation of the *Fog2-LacZ-eGFP* targeted allele. The positive selection marker (neo) is flanked by the loxP sites (red triangles). The positions of the ires and the NcoI site are shown. (B-G) Whole-mount β-galactosidase staining of the gonads in the XY (B-D) and XX (E-G) *Fog2-LacZ-eGFP* embryos at E11.5 (B, E), E12.5 (C, F) and E13.5 (D, G). The scale bar is 200μm. (H) Taqman qRT-PCR analysis of the *Fog2* gene expression in the XY (control and *Fog2* heterozygous) and XX E12.5 gonads.

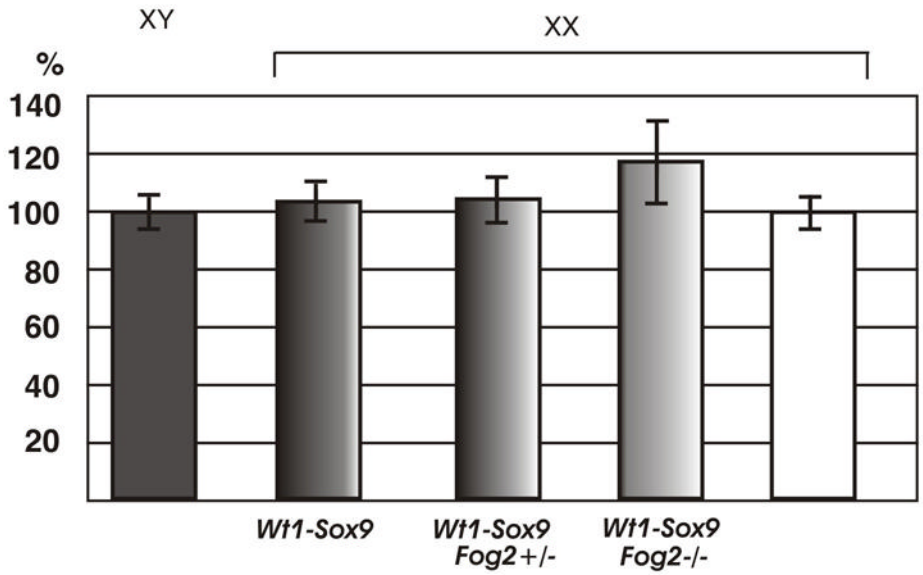

Figure 6.
Real-time PCR analysis (TaqMan) of *Wt1* gene expression in the E12.5 embryonic gonads. *Wt1* expression is not affected by the *Wt1-Sox9* transgene presence or *Fog2* deficiency.

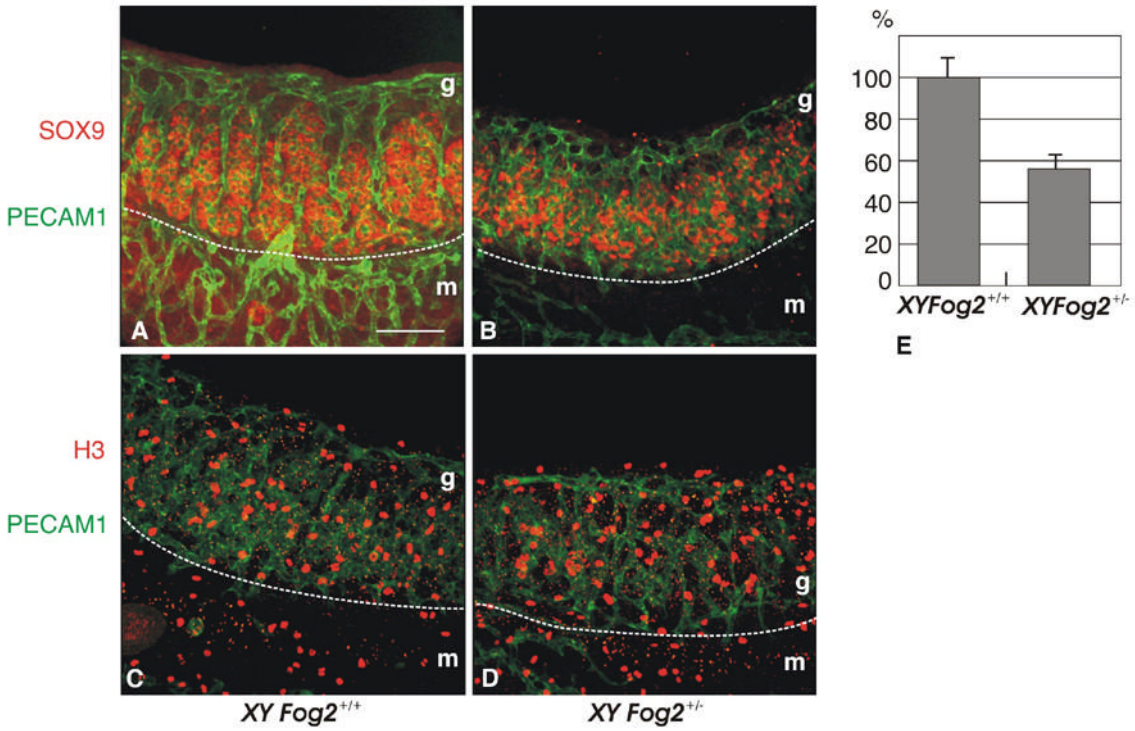

Figure 7.

Whole-mount immunostaining of E12.5 XY gonads isolated from control (A, C) and *Fog2* heterozygous (B, D) embryos. The gonads were stained with anti-PECAM1 (platelet/endothelial cell adhesion molecule 1) antibody that marks germ cells and vascular endothelial cells (green, A-D) and either anti-SOX9 antibody (red, A-B) or phosphorylated histone H3 (red, C-D). Fewer SOX9 positive cells were detected in the *Fog2* heterozygous sample (B) compared to the control (A), while proliferation did not appear to be affected (C-D). The white dotted line indicated a boundary between the mesonephros (m) and gonad (g). The scale bar is 100μM.

(E). SOX9 positive cells were counted in three independent pairs of gonads of both genotypes. The total number of cells was counted per sample (50 planes); the amount of cells in the control is normalized to be 100%.

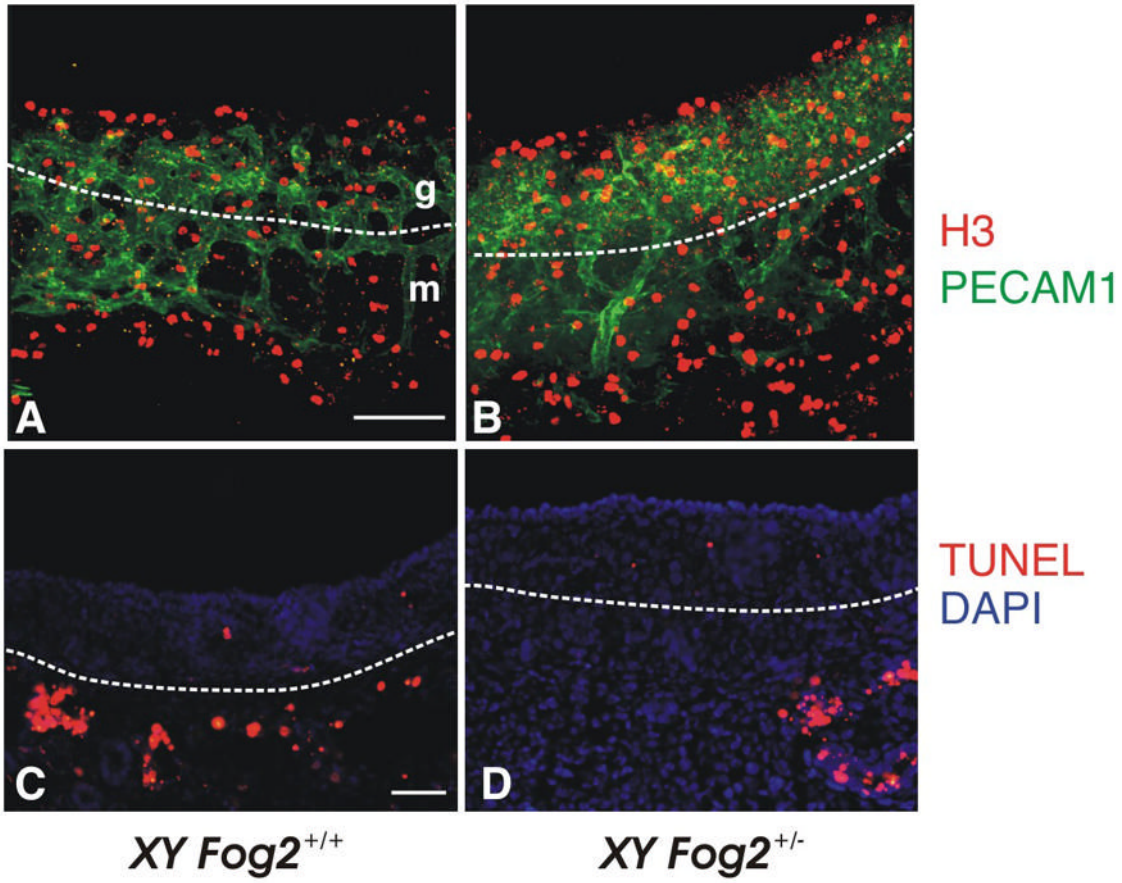

Figure 8.

Comparison of the proliferation and apoptosis in the E11.5 XY gonads isolated from control (A, C) and *Fog2* heterozygous (B, D) embryos. The gonads were stained with anti-PECAM1 antibody (green, A-B) and phosphorylated histone H3 (red, A-B). Frozen sections of gonad-mesonephros complex were stained to detect apoptotic cells/bodies (red; nick-end labeling, TUNEL) (C-D). No significant change in the proliferation (A-B) or apoptosis (C-D) are observed in the *Fog2*$^{+/-}$ samples compared to the control XY gonads. Apoptotic cells are easily detected around mesonephric tubules (arrows in D-F) as has been previously reported (e.g. (Allard et al., 2000;Kim et al., 2006). The white dotted line indicates the boundary between mesonephros (m) and gonad (g). The scale bar is 100 μM (A-B) and 50μm (C-D).

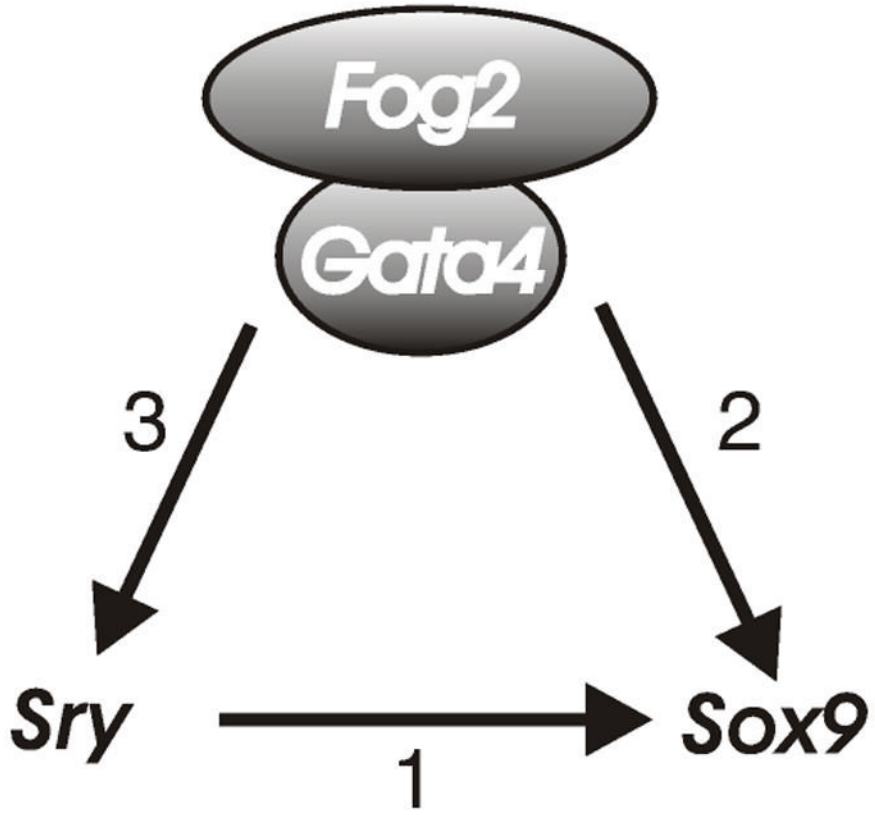

Figure 9.
A combined action of SRY (1) and GATA4/FOG2 (2) complex is necessary for the male-specific activation of *Sox9* gene expression. Normal *Sry* expression also requires GATA4/FOG2 complex (3) (Tevosian et al., 2002).