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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.

Keywords: Fog2; Gata4; Testis; Sox9; Sex reversal

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 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

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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′-350 kb and 3′-250 kb) 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 was 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 downregulation 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* is 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