Developmental Biology 370 (2012) 24–32

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Testis development requires the repression of *Wnt4* by Fgf signaling

Samantha A. Jameson, Yi-Tzu Lin, Blanche Capel *

Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA

ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form
5 June 2012
Accepted 7 June 2012
Available online 15 June 2012

Keywords:
Sex determination
Bipotential
Testis
Ovary
Fgf9
Wnt4

ABSTRACT

The bipotential gonad expresses genes associated with both the male and female pathways. Adoption of the male testicular fate is associated with the repression of many female genes including *Wnt4*. However, the importance of repression of *Wnt4* to the establishment of male development was not previously determined. Deletion of either *Fgf9* or *Fgfr2* in an XY gonad resulted in up-regulation of *Wnt4* and male-to-female sex reversal. We investigated whether the deletion if *Wnt4* could rescue sex reversal in *Fgf9* and *Fgfr2* mutants. XY *Fgf9*/*Wnt4* and *Fgfr2*/*Wnt4* double mutants developed testes with male somatic and germ cells present, suggesting that the primary role of Fgf signaling is the repression of female-promoting genes. Thus, the decision to adopt the male fate is based not only on whether male genes, such as *Sox9*, are expressed, but also on the active repression of female genes, such as *Wnt4*. Because loss of *Wnt4* results in the up-regulation of *Fgf9*, we also tested the possibility that derepression of *Fgf9* was responsible for the aspects of male development observed in XX *Wnt4* mutants. However, we found that the relationship between these two signaling factors is not symmetric: loss of *Fgf*9 in XX *Wnt4*$^{-/-}$ gonads does not rescue their partial female-to-male sex-reversal.

Introduction

The mammalian gonad forms initially as a bipotential primordium that is capable of developing into either a testis or ovary. The fate of a population of somatic cells called the supporting cells controls sex determination by either giving rise to Sertoli cells of the testis or a population of granulosa cells in the ovary. Prior to sex determination, supporting cells in XX and XY gonads have an identical expression profile. They express genes later associated with both the male and female pathways, although they show a bias toward the female pathway at the undifferentiated stage (Jameson et al., 2012).

In mammals, the adoption of the male fate is determined by the expression of the sex-determining gene from the Y-chromosome, *Sry* (sex determining region of Chr Y) in supporting cell precursors. Expression of *Sry* up-regulates *Sox9* (*Sry*-box containing gene 9), and initiates Sertoli cell differentiation and testis development (Gubbay et al., 1990; Koopman et al., 1991; Sekido et al., 2004; Sekido and Lovell-Badge, 2008). *Sox9* is critical for testis development (Bishop et al., 2000; Chaboissier et al., 2004; Huang et al., 1999; Vidal et al., 2001; Wagner et al., 1994), but other genes are also required, such as *Fgf9* and its receptor *Fgfr2*. Deletion of *Fgf9* (fibroblast growth factor 9) or *Fgfr2* (fibroblast growth factor receptor 2) results in male-to-female sex reversal (Bagheri-Fam et al., 2008; Colvin et al., 2001; Kim et al., 2007).

A robust transcriptional repression program characterizes the commitment of the supporting cells to the Sertoli fate (Jameson et al., 2012). Many female-associated genes (including *Wnt4*, wingless-related MMTV integration site 4) are initially expressed in the sexually undifferentiated supporting cells, and are repressed during male development (Jameson et al., 2012; Nef et al., 2005; Vainio et al., 1999). Exogenous FGF9 can repress *Wnt4* expression in cultured gonads (Kim et al., 2006), suggesting it is a mediator of female gene repression. Consistent with this result, in the absence of *Fgf9*, (*Fgf9*$^{-/-}$), *Wnt4* was up-regulated in XY gonads by E12.5. However, *Fgf9*$^{-/-}$ gonads also showed loss of *Sox9* expression and full male-to-female sex reversal (Kim et al., 2006). Based on these findings, we proposed that *Fgf9* and *Sox9* act in a feed-forward loop to reinforce SOX9 expression and repress *Wnt4* (Kim et al., 2006). However, within this general model, the primary cause of sex reversal was not determined.

There is reciprocal evidence that *Wnt4* acts as an antagonist of the male pathway. Stabilization of the *Wnt4* downstream target β-catenin in XY gonads leads to silencing of SOX9 and the male pathway (Maatouk et al., 2008). XX gonads mutant for the Wnt signaling components *Wnt4, Rspo1* (R-spondin homolog), or *Ctnnb1* (β-catenin) show partial female-to-male sex reversal. *Fgf9* is expressed in XX *Wnt4* mutants, endothelial cells migrate into the XX gonad and form male-type vasculature, and XX cells express steroidogenic enzymes characteristic of the testis,

* Corresponding author. Fax: +1 919 684 5481.
E-mail address: b.capel@duke.edu (B. Capel).

0012-1606/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2012.06.009

suggesting that *Wnt4* is normally involved in blocking male development of the gonad (Chassot et al., 2008; Jeays-Ward et al., 2003; Kim et al., 2006; Liu et al., 2009; Manuylov et al., 2008; Tomizuka et al., 2008; Vainio et al., 1999). Although constitutively active β-catenin can block male development of XY gonads, the importance of repression of the normal female program to Sertoli cell differentiation and testis development was not clear.

One open possibility was that *Fgf9* signaling is primarily required to block *Wnt4* and the female pathway rather than to activate *Sox9* and male development. To test this possibility, we generated XY mice mutant for both *Wnt4* and *Fgf9* or its receptor *Fgfr2*. If the primary role of *Fgf9* is to activate the male pathway, double mutants should be female. However, if the primary role of *Fgf9* is to repress *Wnt4* and the female pathway, these mice should be male.

In both sets of double mutants, the additional deletion of *Wnt4* rescued the XY *Fgf9* and *Fgfr2* male-to-female sex reversal phenotypes in somatic and germ cells. This indicates that the repression of *Wnt4* is required for male development. On the other hand, we found that the phenotype of the XX *Wnt4* mutants was not rescued by deleting *Fgf9*, indicating that the partial male sex reversal in XX gonads does not depend on the up-regulation of *Fgf9*. Thus, *Fgf* signaling is required to repress female genes during testis development, but it is not required to maintain *Sox9*, regulate male germ cell development, or activate male vascular development and steroidogenesis.

Materials and methods

Mice

All animals were maintained and experiments were conducted according to DUMC-IACUC and NIH guidelines, based on existing protocols. $Fgf9^{+/-}$ (Colvin et al., 2001) and $Wnt4^{+/-}$ (Stark et al., 1994) were separately maintained as heterozygotes on the C57BL/6 strain. For qRT-PCR experiments, $Fgf9^{-/-}$ embryos and their control littermates were generated by intercrossing $Fgf9^{+/-}$ mice. For more precise staging, tail somites distal to the hindlimb were counted (Hacker et al., 1995). E11.5 embryos had 16–19 tail somites, E11.75 embryos had 20–23 tail somites, and E12.5 embryos had 27–28 tail somites. Gonads were dissected away from the mesonephros prior to RNA extraction. "Control" mice were $Fgf9^{+/-}$ or $Fgf9^{+/+}$.

$Fgf9^{+/-}$ and $Wnt4^{+/-}$ mice (on the C57BL/6 strain) were intercrossed to generate $Fgf9^{+/-}$; $Wnt4^{+/-}$ offspring, which were intercrossed to generate double mutant embryos. The genotypes and chromosomal sex of the embryos were determined as previously described (Colvin et al., 2001; Munger et al., 2009; Stark et al., 1994). Embryos were isolated between about E16.5 and E17.5. Brightfield images of the gonads were collected on a Leica MZ12 following the dissection. "Control" mice were heterozygous or homozygous wild type at the *Fgf9* and *Wnt4* loci.

A mixed background $Fgfr2^{flox/flox}$ line (Yu et al., 2003) was backcrossed two or more generations to C57BL/6, during the processes of crossing on the *Wnt4* null allele (Stark et al., 1994) and the *Sf1-Cre* allele (Bingham et al., 2006) that are maintained on C57BL/6. These mice were intercrossed to generate $Fgfr2^{flox/flox}$; $Wnt4^{+/-}$ mice, some of which also had *Sf1-Cre*, and the line was maintained by intercrossing to preserve homozygosity at the *Fgfr2* locus. Timed matings were established between fertile *Sf1-Cre*; $Fgfr2^{flox/flox}$; $Wnt4^{+/-}$ males (see Fig. 2 for explanation) and $Fgfr2^{flox/flox}$; $Wnt4^{+/-}$ females. Similar methods were also used to generate mice that carried *Flk1-Cre* (Motoike et al., 2003) and $Fgfr2^{flox/+}$. *Flk1-Cre;* $Fgfr2^{flox/+}$ males were crossed to $Fgfr2^{flox/flox}$ females. E0.5 was defined as noon on the day a mating plug was detected. Embryos were collected from pregnant females on the morning of day E13.5. The genotypes (Stark et al., 1994; Yu et al., 2003) and chromosomal sex (Munger et al., 2009) of the embryos were determined as previously described. We determined whether Cre was present using the primers 5′-CCAGGGCGCGAGTTGATAGC-3′ and 5′-CTGCCACGACCAAGTGACAGC-3′. "Control" mice were all $Fgfr2^{flox/flox}$, negative for *Sf1-Cre*, and either $Wnt4^{+/-}$ or $Wnt4^{+/+}$.

qRT-PCR

Isolated gonads were frozen at −80 °C. RNA was extracted as previously described (Munger et al., 2009), treated with DNaseI, and converted to cDNA using the iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA). Each cDNA sample was run in technical triplicate on a StepOnePlus Real-time PCR system (Applied Biosystems, Carlsbad, CA). The threshold and baseline were set manually. Generally, the average C_T value of the technical replicates was analyzed using the "normalized expression" method described previously (Simon, 2003). *Canx* was used for normalization (van den Bergen et al., 2009). Since all primer sets appeared to have greater than 95% efficiency (data not shown), we assumed all primers were perfectly efficient. If the difference in C_T values of technical triplicates exceeded 0.5, the outlier sample was excluded from the average. If no sample was an outlier, all replicates were retained so long as the difference in C_T values was less than 0.75. The list of primers is provided (Table S1), several of which have been previously published (Manuylov et al., 2008; Munger et al., 2009).

Generally, three individual gonad pairs were analyzed for each genotype at each stage (three biological replicates). The normalized expression values from the biological replicates were averaged to calculate mean normalized expression (MNE). A significant difference in normalized expression between genotypes was determined using a *T*-test. To reduce technical variability in expression at E11.5 and E11.75, we used qRT-PCR data only when the reactions for all of the biological replicates were set up at the same time. Four independent E11.75 XY $Fgf9^{-/-}$ samples were analyzed. For one sample, the *Foxl2* reading was eliminated because the C_T value was within 3 cycles of the negative control, but data from that sample for other genes was retained in the analysis.

Immunofluorescence

Gonads were immunostained as previously described (Cook et al., 2009). All E13.5 gonads were stained as whole mounts with the mesonephros attached. At/after E16.5, phenotypically female samples (including XY $Fgf9^{-/-}$ samples) were stained as whole mounts after removing mesonephric structures. The whole mount samples were either immunostained immediately, or processed through a methanol series and stored at −80 °C prior to rehydration and immunostaining (Barske and Capel, 2010). Phenotypically male samples from E16.5 and after were cryosectioned after removing the mesonephric structures. Since the male and female samples were processed separately at E16.5–E17.5, we included CD-1 (Charles River, Wilmington, MA) positive/negative controls in each batch of immunostained samples (i.e., E13.5 CD-1 male gonads were stained as whole mounts with the E16.5 female samples, and E16.5 CD-1 female gonads were cryosectioned and stained with the E16.5–E17.5 males) (data not shown). E12.5 CD-1 male gonads were also used as the positive control in Fig. S3 for the Ki67 immunostaining. For the DNMT3L immunostaining (Fig. 5F–J), cryosectioned XY $Fgf9^{-/-}$ samples and CD-1 female controls are shown because the antibody did not work in whole mount.