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XY *Sox9* embryonic loss-of-function mouse mutants show complete sex reversal and produce partially fertile XY oocytes

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ABSTRACT

Gonadal differentiation is the first step of mammalian sex determination. The expression of the Y chromosomal testis determining factor *Sry* leads to up-regulation of the transcription factor *Sox9* which promotes testis differentiation. Previous studies showed that *Sox9* deficiency induces expression of ovarian markers in XY mutant fetal gonads before they die. To better understand the genome-wide transcriptional profile underlying this process we compared samples from XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ mutant gonads in which *Sox9* is ablated in Sertoli-precursor cells during early stages of gonad development to XX *Sox9*$^{flox/flox}$ ovaries and XY *Sox9*$^{flox/flox}$ testes at E13.5. We found a complex mRNA signature that indicates wide-spread transcriptional de-regulation and revealed for XY mutants at E13.5 an intermediate transcript profile between male and female gonads. However, XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ mutant gonads develop as ovaries containing XY developing follicles at P0 but less frequently so than in XX control ovaries. Furthermore, we studied the extent to which developing XY mutant ovaries are able to mediate adult fertility and observed that XY oocytes from XY mutant ovaries are competent for fertilization; however, two thirds of them fail to develop beyond two-cell stage embryos. Taken together, we found that XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ females are capable of producing viable offspring albeit at a reduced level.

Introduction

In most mammalian species, sexual differentiation is determined by the paternal transmission of the *SRY* gene located on the Y chromosome (Gubbay et al., 1990; Sinclair et al., 1990). *SRY* expression promotes testis differentiation of the XY bipotential gonad whereas an XX gonad develops into an ovary. In mice, transgenic experiments have shown that *Sry* is necessary and sufficient for testis differentiation (Koopman et al., 1990; Lovell-Badge and Robertson, 1990) and the only function of *Sry* reported so far is to induce the transcription factor *Sox9* (Canning and Lovell-Badge, 2002; Qin and Bishop, 2005; Sekido and Lovell-Badge, 2008) for which we will employ the mouse nomenclature unless specified otherwise. *Sox9* is initially expressed at low levels in both XX and XY genital ridges prior to *Sry* expression in mouse and human embryos (Morais da Silva et al., 1996). Upon SRY expression, *Sox9* becomes strongly induced in pre-Sertoli cells and subsequently Sertoli nurse cells which are critical for XY gonad development (Sekido et al., 2004; Sekido and Lovell-Badge, 2008; Wilhelm et al., 2005). *Sox9* gain-of-function experiments promote testis development of XX gonads (Bishop et al., 2000; Vidal et al., 2001) and one case of duplication of the *SOX9* gene has been shown to be associated with XX sex reversal in a patient (Huang et al., 1999). Thus the gene is sufficient for testis differentiation and male sex determination.

Heterozygous mutations of *SOX9* are associated with campomelic dysplasia (CD), a skeletal malformation syndrome (Houston et al., 1983). Two thirds of XY patients exhibit male-to-female sex reversal implying that efficient levels of *SOX9* expression are required for testis differentiation and subsequent male development. In human and mice, the gene is required for various developmental processes such as chondrogenesis or neural development (Guth and Wegner, 2008). A null mutation of mouse *Sox9* is lethal during the neo-natal period due to respiratory failure which is a common condition described in CD patients (Bi et al., 2001). However, in contrast to what was observed in humans, heterozygous mutations of *Sox9* do not trigger XY sex reversal in mice.

The expression of CRE recombinase in germ cells (Chaboissier et al., 2004), in embryonic ectoderm, in embryonic mesoderm or in definitive endoderm (Barrionuevo et al., 2006) deletes *Sox9* from cells within the urogenital ridge. However, its expression in tissues other than gonads causes an embryonic lethal phenotype (Akiyama et al., 2004; Chaboissier et al., 2004) thereby preventing a thorough analysis

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of *Sox9* function during sex determination. This problem was circumvented by using either gonad cultures or the few surviving *Sox9* loss-of-function embryos to show that *Sox9* induces Sertoli cell differentiation which triggers testis formation (Barrionuevo et al., 2006; Chaboissier et al., 2004). The mouse *Fgf9*/*Fgfr2* and *Pdg2*/*Pdgs* signalling pathways also promote testicular development and maintenance of *Sox9* expression once *Sry* expression has ceased (Kim et al., 2006; Moniot et al., 2009). Indeed, *Sox9* ablation promotes the expression of ovarian markers in XY gonads implying male-to-female sex reversal of XY mutant gonads. However, their fertility was not assessed because the developing foetuses died early during development (Barrionuevo et al., 2006; Chaboissier et al., 2004).

Sex-reversed B6Y^{TIR} females bear a Y^{TIR} chromosome from the *Mus musculus domesticus* species captured in Tirano (TIR, Italy) in a C57BL/6J background. These females are infertile because of a defect in Meiosis II which is rescued by transfer of the karyoplast into an enucleated oocyte; this suggests that XY oocytes cannot undergo embryonic development (Obata et al., 2008). Mouse mutants bearing a partial deletion allele of *Sry* can, however, produce offspring which in most cases exhibits chimerism due to non disjunction between sex chromosomes in meiosis (Gubbay et al., 1990; Lovell-Badge and Robertson, 1990). In human patients characterized by a 46, XY karyotype presenting with disordered sex development (DSD), gonadal dysgenesis ranges from streak gonads to ovaries and one female with high levels of sex chromosome chimerism has given birth (Mendonca et al., 2009). These cases of sex reversal are associated with the Y chromosome and thus with genes required and expressed during testis differentiation. In contrast, *Sox9* expression is not restricted to the XY gonads but has also been detected in steroidogenic cells of the preantral/antral follicles in mice suggesting a role for this gene in follicle maturation (Notarnicola et al., 2006).

To gain further insight into *Sox9* function during sex determination and early sexual development, we crossed mice bearing the *Sf1:Cre* transgene (Bingham et al., 2006) and the *Sox9*$^{flox/flox}$ allele thereby removing exons 2 and 3 of the *Sox9* gene specifically in somatic cells of the developing gonad. We report male-to-female sex reversal at different stages as assessed by histology, histochemistry, and *in situ* hybridization and provide evidence via genome-wide expression profiling for gonads from XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ mutant mice at E13.5 to display an intermediate expression signature between ovaries and testes. Moreover, we find that XY follicles develop at P0 in XY mutant ovaries and XY mutant oocytes are fertilizable and are capable of mediating embryonic development. Taken together, our data suggest that, although XY follicle formation in XY mutant ovaries occurs at a reduced level, XY oocytes in XY *Sf1:Cre*$^{Tg/+}$; *Sox9*$^{flox/flox}$ mice are at least partially able to be fertilized and to undergo embryonic development, and that *Sox9* is not essential for fertility in female mice.

Materials and methods

Mouse strains and genotyping of embryos and mice

The experiments here described were carried out in compliance with the relevant institutional and French animal welfare laws, guidelines and policies. All mouse lines were kept on a mixed 129/C57BL6/J background. The generation of *Sox9*^{flox} allele (Akiyama et al., 2002) and the *Sf1:Cre* line (Bingham et al., 2006) were reported previously. The tail somite (Ts) number was determined by counting them from the middle of hind limb until the end of the tail. PCR was carried out using DNA extracted from tail tip of embryos. Wild-type and *Sox9*^{flox} alleles were identified using the primers 5′-GGGGCTTGTCTCCTTCAGAG-3′, or 5′-ACACAGCATAGGCTACCTG-3′ and 5′-TGGTAATGAGTCATACACAGTAC-3′, respectively. The *Sox9* knockout allele was identified using the primers: 5′-GTCAAGCGACCCATG-3′ and 5′-TGGTAATGAGTCATACACAGTAC-3′. Genotyping for the *Sf1:Cre* transgene was performed as described (Bingham et al., 2006). The presence of the *Sry* gene was determined using primers 5′ GTGACAATTGTCTAGAGAGC 3′ and 5′ ACTGCA-GAAGGTTGTACAGT 3′. *Pax6* primer set 5′ GCAACAGGAAGGAGGGG-GAGA 3′; 5′ CTTTCTCCAGAGCCTCAATCTG 3′ was included in each PCR reaction as an internal control.

Histological and immunological analyses

Embryonic samples from mated animals (day of vaginal plug = E0.5) were fixed with 4% paraformaldehyde (for immunodetection) or Bouin's solution (for histological analysis) over night at 4 °C and then embedded in paraffin. 8 μm or 5 μm sections were stained with Hematoxylin and Eosin Staining. Immunofluorescence analysis was performed after antigen retrieval in 10 mM Sodium citrate (pH 6) for 2 min in a pressure cooker. Sections were incubated for 45 min in blocking solution (3% BSA, 10% donkey serum, 0.1% Triton) at room temperature. The blocking solution was replaced by the primary antibodies diluted in 3% BSA, 3% donkey serum, 0.1% Triton at the following concentrations: SOX9 (provided by Michael Wegner, 1/1000), SDMG1 (provided by Ian Adams, 1/1000), FOXL2 (Abcam 1/300) and DDX4/MVH (Abcam 1/200) SPRRD2 (Enzo Life Sciences, 1/100). Cy3- or Fitch-conjugated anti-rabbit or anti-goat secondary antibodies were diluted at 1/150 (Jackson Laboratories). Slides were mounted using Vectashield and DAPI (Vector Labs). Fluorescent studies were performed with an AxioImager Z1 microscope (Zeiss), and pictures were taken with an AxioCam Z.I CCD camera (Zeiss).

Germ cell quantification

For each genotype four sections of three embryos each were processed for immunohistological experiments. The anti DDX4/MVH antibody was used to identify germ cells. The total number of germ cells was quantified within a defined area and for each genotype the mean and standard deviation was calculated and graphically displayed after statistical analysis. The results were analyzed using Graphpad.

Whole mount in situ hybridization analysis

Embryos were fixed with 4% paraformaldehyde in PBS overnight at 4 °C. Further processing of the embryos and *in situ* hybridization was carried out as previously described (Chassot et al., 2008). Riboprobes for *Rspo1* (Parma et al., 2006), *Stra8* (Menke et al., 2003), *Bmp2* (Furuta et al., 1997), *Emx2* (plasmid provided by Seppo Vainio), *Sox8* (Sock et al., 2001), *Sox10* (Britsch et al. 2001), *ErbB3* (Britsch et al. 2001), *Pdgs* (provided by P. Koopman) and *Fgf9* (Colvin et al., 2001) were synthesized as described previously. The *Phf19* (NM_028716) probe was provided by D. Badro.

RNA preparation

Individual gonads without mesonephros dissected from E13.5 embryos were snap frozen at −80 °C and the RNA was extracted using the RNeasy kit (Qiagen). The developmental stage and the phenotype of each embryo were recorded. Each RNA sample consisted of 12 gonads from six pooled mutants. Separate sets of mutant gonads and wild-type gonads were used for each of the microarray analyses.

RNA isolation and GeneChip hybridization

Three independent experiments were performed for XX and XY wild-type (*Sox9*$^{flox/flox}$) and XY Sox9 mutant (*Sf1:Cre*$^{Tg/+}$ *Sox9*$^{flox/flox}$) samples. RNA quality was assessed by measuring the optical density at 230 nm (A^{230}), 260 nm (A^{260}) and 280 nm (A^{280}) using a ND-1000 Spectrophotometer (Nanodrop, Wilmington, DE 19810, USA) as well as the electrophoretic mobility using the Agilent 2100 BioAnalyzer